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HPLC JAKO SOFISTIKOVANÝ NÁSTROJ PŘI STANOVENÍ TOXICKÝCH METABOLITŮ BAKTERIÍ A PLÍSNÍ V BIOLOGICKÝCH MATRICÍCH

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habilitační práce

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Na tomto místě bych chtěl také poděkovat manželce a všem, kteří mne v mém profesním i osobním životě provázeli a podporovali.

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Předmluva

V současné době patří kapalinová chromatografie mezi nejpoužívanější separační metody, kdy zejména díky technickému pokroku poskytuje velmi vysokou separační účinnost při relativně krátké době potřebné pro analýzu. Ve spojení s vhodnými typy detektorů je možné ovlivnit celou řadu analytických parametrů metody, jako jsou selektivita, specifita, citlivost či limit detekce. Hmotnostní detekce navíc přináší možnost identifikace analytu na základě jeho hmotnostního spektra.

Z tohoto důvodu jsou chromatografické metody, nejen v kombinaci s hmotnostní detekcí, rutinně užívány například při stanovení biologicky aktivních látek (biogenních aminů) či toxických kontaminantů (mykotoxinů) v potravinách a jiných velmi složitých biologických matricích.

Předkládaná habilitační práce je komentovaným souborem odborných publikací, zabývajících se využitím vysokoúčinné kapalinové chromatografie pro stanovení nejčastěji se vyskytujících toxických metabolitů plísní ve vzorcích rostlinného původu a také možností využití kvantifikace plísní na základě stanovení ergosterolu ve zkoumaném materiálu. Dalším okruhem zájmu jsou tzv. biogenní aminy, zpravidla fyziologicky aktivní produkty, často vzniklé mikrobiologickou dekarboxylací volných aminokyselin.

V pracech zabývajících se stanovením biogenních aminů je autorský podíl RNDr. Dohnala dán analytickou částí jednotlivých prací (cca 40-50 %), v přehledovém článku 50%, v publikaci o vývoji nové rychlé metody pro jejich stanovení v kultivačních médiích asi 90%.

U článků týkajících se mykotoxinů je v případě review jejich metabolismu autorský podíl RNDr. Dohnala asi 40%, u publikací prezentujících vývoj a validaci nových analytických metod asi 80-90% a u článků, kde jsou tyto metody pouze aplikovány pak 40-80% v závislosti na počtu dalších použitých metod.

Úvod

Tato habilitační práce je souhrnem části vědecké činnosti autora, publikované ve vědeckých a odborných časopisech a v knize v letech 2004 až 2011. Společným rysem všech uvedených prací je využití kapalinové chromatografie (HPLC) při separaci a stanovení obsahu vybraných toxických metabolitů bakterií a plísní v biologických materiálech.

První část prací je věnována vzniku a stanovení biogenních aminů (BA) a polyaminů (PA) ve fermentovaných potravinách živočišného původu, v sýrech a salámech, metodou vysoce účinné kapalinové chromatografie. Úvod je věnován přehledu používaných analytických postupů. Následující práce se věnují stanovení BA a PA ve vzorcích sýrů a salámů. Získané výsledky spolu s údaji z mikrobiologické analýzy jsou pak využity pro zhodnocení aspektů ovlivňujících tvorbu těchto látek. Poslední práce této kapitoly je věnována vývoji a aplikaci nové rychlé metody pro screening přítomnosti BA ve vzorcích mikrobiologických kultivačních médií s využitím high-throughput rapid resolution chromatografických kolon plněných velmi malými částicemi o velikosti 1,8 μm.

Další část souboru je věnována fugální kontaminaci potravin či krmiv. Je zde diskutována možnost využití stanovení ergosterolu pro kvantifikaci plísní a také stanovení vybraných mykotoxinů v různých matricích. Největší pozornost je věnována fuzáriovým mykotoxinům (deoxynivalenolu, a fumonisinům B_1 a B_2), jejichž producenti patří mezi nejrozšířenější. V úvodní části jsou zařazeny přehledové články, popisující současný stav problematiky biodegradace a metabolismu vybraných mykotoxinů. Následuje práce, zabývající se vývojem postupu pro stanovení deoxynivalenolu ve vzorcích ječmene a sladu metodou HPLC-MS. Osud tohoto mykotoxinu byl poté sledován i v průběhu kultivace různých druhů kvasinek. Fumonisiny B_1 a B_2 a steroidní látka ergosterol byly stanovovány ve vzorcích kukuřice. Výsledkem bylo nalezení korelace mezi obsahem ergosterolu a úrovní kontaminace rostlinného materiálu plísněmi. V neposlední řadě byla vyvinuta a aplikována nová metoda pro stanovení citrininu, mykotoxinu, který společně s ochratoxinem A je pravděpodobným původcem Balkánské endemické nefropatie. V současné době je právě citrinin v centru zájmu Evropské agentury pro bezpečnost potravin (European Food Safety Authority, EFSA).

Pro stanovení všech výše uvedených látek, počínaje počínaje biogenními aminy, přes ergosterol, až po mykotoxiny, byly vyvinuty nebo adaptovány, následně validovány a použity analytické postupy s charakteristikami odpovídajícími specifikům analyzovaným vzorkům. Limity

detekce těchto metod umožňují spolehlivou kvantifikaci mykotoxinů v množstvích, která jsou uvedena v evropských normách či nařízeních Evropské komise jako maximální přípustná pro daný typ vzorku.

Souhrn publikací je doplněn průvodním textem, který dává této práci celistvost a dává do souvislostí jednotlivé části tak, aby byl zřejmý vývoj, kterého bylo za uvedenou dobu dosaženo. V každé kapitole jsou v úvodu podány základní informace, týkající se dané problematiky, podkapitoly jsou pak věnovány podrobnějšímu rozboru řešených úkolů.

RNDr. Vlastimil Dohnal, Ph.D.

1. Cíle habilitační práce

Cílem této habilitační práce je:

- Vyvinout a aplikovat rychlou chromatografickou metodu pro screeningové stanovení produkce biogenních aminů jednotlivými kmeny mikroorganismů
- Vyvinout a zavést analytické metody pro stanovení mykotoxinů deoxynivalenolu, fumonisinů B_1, B_2 a B_3 a citrininu a aplikovat tyto metody při sledování jejich obsahu v reálných vzorcích rostlinného původu

2. Kapalinová chromatografie

Princip

Kapalinová respektive vysoce účinná kapalinová chromatografie (HPLC) je separační metoda, u nichž dochází k dělení analytů na základě rozdílných distribučních koeficientů mezi dvěmi fázemi - mobilní kapalnou fází (MF) a fází stacionární.

Instrumentace

Z hlediska technického provedení sestává kapalinový chromatograf ze zásobníku mobilní fáze, čerpadla, zařízení pro dávkování vzorku, chromatografické kolony a detektoru. Mezi zásobník mobilní fáze a čerpadlo může být zařazeno zařízení pro odstranění plynů rozpuštěných v mobilní fázi. Za kolonu nebo za nedestruktivní detektor (optické detektory) lze zařadit sběrač frakcí či destruktivní detektor, například hmotnostní.

Čerpadlo zabezpečuje definovaný průtok mobilní fáze systémem a v případě gradientové eluce i změnu jejího složení v průběhu analýzy v průběhu času. Dávkovací systém musí splňovat podmínku dávkování definovaného a reprodukovatelného množství vzorku. U jednodušších přístrojů je jím dávkovací kohout s kalibrovanou smyčkou, pro rutinní analýzy pak automatizovaný dávkovač vzorků, který pojme desítky až tisíce vzorků. Dávkovaný objem vzorku vytváří ostře ohraničenou zónu, která je unášena proudem MF do chromatografické kolony naplněné stacionární fází, zde dochází k vlastní separaci.

V současné době je nejvíce používanou stacionární fází hydrofobní, tzv. „reverzní fáze“, kde je na křemenném nosiči zakotven oktadecylový či jiný nepolární fragment. V takovém uspořádání je hlavním mechanismem dělení látek hydrofobní interakce molekuly analytu se stacionární fází. Tento princip je vhodný pro separaci nepolárních látek. V dalších případech však může být separace založena i na jiných principech, jako jsou například iontová výměna na iontoměničích, ion-párová chromatografie, dipól-dipól, dipól-indukovaný dipól, π-π interakce apod. Pro separaci polárních sloučenin bývá zpravidla volena polárnější stacionární fáze, nebo jsou tyto látky převedeny do méně polární formy například derivatizací a separovány na nepolárních kolonách.

V poslední době se klade velký důraz na zkrácení doby analýzy a s ní související finanční nákladností. Snížení velikosti částic náplně chromatografické kolony vede k vyšší účinnosti separace a tím i k možnosti zkrácení délky kolony při zachování obdobné účinnosti jako při použití

kolon „klasických“ rozměrů. Ekonomickou nákladnost je také možné snížit volbou kolony s menším vnitřním průměrem a tím i menším průtokem mobilní fáze při zachování stejné lineární průtokové rychlosti. Takové řešení však klade i větší požadavky na použitou instrumentaci, neboť menší částice kladou větší hydrodynamický odpor, což vede k nutnosti zvýšit tlak pro zajištění patřičného průtoku mobilní fáze. Také dávkovací systém musí být schopen reprodukovatelně dávkovat nižší objemy vzorku, který tyto užší kolony vyžadují. V neposlední řadě je nutné dbát na pečlivou přípravu vzorku k analýze, neboť kolony plněné malými částice mají větší tendenci se zanášet mechanickými nečistotami.

K detekci stanovovaných analytů se využívá celá řada fyzikálně-chemických metod. Díky své jednoduchosti a hlavně nízké pořizovací ceně jsou velmi oblíbené spektrofotometrické metody v ultrafialové či viditelné části spektra. Velká část organických látek absorbuje záření právě v této oblasti. Mnohem selektivnější a citlivější je fluorescenční detekce. U látek, které neobsahují v molekule fluorofor, je nutné provést derivatizaci a tím do ní zavést fluoreskující skupinu.

V posledních asi 15 letech dochází k velkému rozvoji spojení HPLC s hmotnostními detektory. Tyto detektory, v závislosti na konstrukci, umožňují selektivní a stopové stanovení analytů ve vzorcích s velmi složitou matricí, aniž by bylo nutné provést náročné oddělení matrice. Další jejich výhodou je i možnost konfirmace chemické struktury látek, zvláště ve spojení s dalšími metodami, jako je nukleární magnetická rezonance, infračervená spektroskopie apod.

3. Biogenní aminy a jejich stanovení

Pod pojmy biogenní aminy (BA) a polyaminy (BP) lze zařadit jednoduché organické dusíkaté báze, které převážně vznikají mikrobiologickou dekarboxylací aminokyselin. Z hlediska chemické struktury se dělí na heterocyklické (například histamin a tryptamin), aromatické (tyramin a 2-fenylethylamin) a alifatické (agmatin, putrescin, kadaverin, spermidin a spermin apod.) aminy. Kromě těchto látek náleží do skupiny BA také některé významné biologicky aktivní látky – dopamin, adrenalin, noradrenalin a serotonin. Tyto aminy působí v tělech savců jako hormony a neuropřenašeče, přítomnost v potravinách ve větším množství však může vést k projevům různých negativních účinků, jako jsou zvýšení krevního tlaku, bolesti hlavy apod. [1]. Navíc tyto látky slouží jako prekurzory pro tvorbu karcinogenních N-nitrosoaminů.

Biogenní aminy a polyaminy se běžně vyskytují v nízkých koncentracích v některých potravinách. K významnému zvýšení obsahu však dochází činností mikroorganismů během fermentace. Proto jsou mezi rizikovější potraviny řazeny například sýry [2], salámy [3], víno [4], pivo [5] apod.

Ke stanovení obsahu BA a BP je využívána celá řada analytických metod, nejčastěji však kapalinová chromatografie s UV či fluorescenční detekcí. Celá metodika začíná homogenizací vzorku. Zpravidla je pro jejich kvantifikaci aplikována metoda vnitřního standardu a ke vzorku je tedy přidán 1,7-diaminoheptan, který se v těchto potravinách běžně nevyskytuje. Dále pokračuje postup extrakcí aminů z matrice, přes čištění extraktu (extrakce kapalina-kapalina, solid-phase extrakce) až po vlastní stanovení. Jelikož molekuly těchto aminů neobsahují silně absorbující či fluoreskující skupiny, provádí se jejich derivatizace. Jen velmi málo publikovaných prací se věnuje stanovení BA a BP bez předchozí derivatizace. Problematika stanovení BA a BP v mléčných výrobcích je shrnuta v přehledovém článku **[I]**.

V práci **[II]** byla použita metoda HPLC s UV respektive fluorescenční detekcí pro stanovení osmi BA a PA (histamin, tyramin, tryptamin, fenylethylamin, kadaverin, putrescin, spermidin a spermin) v sýrech typu Eidam od dvou výrobců v průběhu jejich zrání. Dále byla sledována dekarboxylázová aktivita jednotlivých vyizolovaných mikroorganismů. Pro stanovení obsahu biogenních aminů a polyaminů ve vzorcích sýra byla použita metoda popsaná v práci [6]. Účelem práce bylo zhodnotit vliv faktorů, jako jsou producent, typ startovací kultury, místo odběru (okraj, střed) a tučnost, na produkci BA a BP.

K 10 g vzorku sýra bylo přidáno 0,5 ml roztoku vnitřního standardu a BA a BP extrahovány 3x roztokem 0,1 M HCl. Extrakty byly spojeny a doplněny na objem 50 ml. K 1 ml extraktu bylo přidáno 0,5 ml nasyceného roztoku uhličitanu sodného a 1 ml roztoku dansylchloridu v acetonu (5 mg/ml). Derivatizace byla prováděna při 40 °C po dobu 60 minut. Poté byl nezreagovaný dansylchlorid odstraněn přídavkem roztoku amoniaku. Derivatizované BA a BP byly 3x extrahovány do etheru. Spojené extrakty byly odpařeny pod proudem dusíku do sucha, rozpuštěny v acetonitrilu a stanoveny pomocí HPLC s UV detekcí.

Největší vliv na obsahy histaminu, tyraminu a putrescinu měla doba zrání, která vyjadřovala 67, 67 respektive 76 % variability. Také místo odběru hrálo roli, když v okrajové části rostl obsah BA a PA více jak třikrát rychleji než ve středové části. Z dalších rozborů též vyplynulo, že obsah histaminu a tyraminu negativně koreloval s počtem bakterií mléčného kvašení.

Stejný postup byl aplikován i v práci **[III]**, kde byl sledován biogenní amin tyramin. Zde byl použit stejný plán pokusů, jako v práci **[II]** a získány obdobné výsledky.

Kromě sýru eidamského typu byl sledován obsah BA a BP v sýrech plísňových. Jako modelový byl vybrán sýr s modrou plísní Niva **[IV]**. Zde opět byla nejvýznamnějším faktorem ovlivňujícím obsah sledovaných látek doba zrání sýra. Nejvíce zastoupen byl tyramin. Zatímco v povrchové části se jeho obsah lineárně zvyšoval, ve středové části zůstával konstantní. S výjimkou kadaverinu a tyraminu byl obsah ostatních BA a PA velmi nízký.

Kromě mléčných výrobků byly též analyzovány i fermentované masné výrobky, jako jsou salámy. Zde se příprava vzorku k analýze mírně lišila a to zejména v extrakčním médiu. Na rozdíl od vzorků sýrů, kde je standardně používán roztok kyseliny chlorovodíkové, v případě salámů se jednalo o kyselinu trichloroctovou.

V práci **[V]** byl sledován obsah jednotlivých BA a BP v závislosti na výrobci, startovacích kulturách, kořenících směsích, průměru salámu a době fermentace. Opět byly nejvíce zastoupeny tyramin a putrescin, jejichž obsah rostl v průběhu skladování. Salámy s menším průměrem, kořením obsahujícím papriku a startovací kulturu obsahující *Pediococcus pentosaceus* měly oproti jiným variantám nižší obsah BA a BP.

V případě analýzy mikrobiologických kultivačních médií na obsah BA a BP v pracech **[II - VI]** byl pro úpravu vzorku adaptován postup publikovaný v práci [7]. K 1 ml média byl nejprve přidán 1 ml 0,1 M HCl a po promíchání byl roztok centrifugován při 18 000 ot./min. Čirý supernatant byl poté derivatizován o-ftalaldialdehydem a vzniklé deriváty stanoveny na HPLC s fluorescenční detekcí. Avšak vzhledem k obrovskému množství vzorků, které byly kultivací

mikroorganismů a jejich postupným přeočkováváním získány, bylo nutné vyvinout novou metodu pro screening BA a BP. Také bylo nutné eliminovat možnost falešně pozitivních/negativních výsledků získávaných standardní metodou, založené na principu změny pH média při dekarboxylaci aminokyseliny.

V práci **[VII]** byla tedy pro stanovení BA a BP v mikrobiologických kultivačních médiích vyvinuta nová metoda, využívající HPLC s fluorescenční detekcí. K separaci je ale použita tzv. rapid resolution high throughput (RRHT) chromatografická kolona. Náplň kolony je tvořena částicemi o velikosti 1,8 μm, což vede k vyšší účinnosti separace a tím i možnosti zkrátit délku kolony a snížit tak čas potřebný pro separaci. Právě díky těmto vlastnostem jsou RRHT kolony předurčeny pro sériové analýzy velkého množství vzorků. Navíc, díky vysoké účinnosti separace dochází k menšímu rozmytí eluovaných píků a tím i vyšší citlivosti stanovení než s použitím standardních kolon a podstatnému snížení doby analýzy.

Závěr

V referovaných vlastních pracích i ve vlastních pracích souvisejících s řešenou problematikou byla aplikována metoda vysokoúčinné kapalinové chromatografie pro stanovení biogenních aminů v celé řadě složitých matric, jako jsou sýry, salámy respektive mikrobiologická kultivační média. Pro analýzu kultivačních médií byla vyvinuta a úspěšně používána velmi rychlá screeningová metoda, která umožnila i přesnější hodnocení vzorků testovaných na dekarboxylázovou aktivitu.

4. Stanovení vybraných mykotoxinů

Mykotoxiny jsou sekundárními metabolity plísní, ale i vyšších hub, které vyvolávají toxický efekt u zvířat i lidí. Role tvorby mykotoxinů není stále zcela objasněna. Nejpravděpodobnější příčinou jejich produkce je eliminace ostatních mikroorganismů, soutěžících o stejný substrát. Dále se podílejí na regulaci primárního metabolismu a také na usnadnění invaze houby do hostitelských tkání.

Ne všechny houby jsou však producenty mykotoxinů. Mezi nejrozšířenější producenty patří některé kmeny rodu *Aspergillus*, *Penicillium* a *Fusarium*. V této habilitační práci jsou obsaženy práce, týkající se stanovení mykotoxinů produkovaných plísněmi rodu *Fusarium* - fumonisinů B_1 a B_2 a deoxynivalenolu (DON) a *Aspergillus* respektive *Penicillium* - citrininu v různých matricích.

Produkce mykotoxinů plísněmi závisí na celé řadě biotických a abiotických faktorů a za určitých podmínek nemusí i toxikogenní kmen tyto látky tvořit. Velmi často se vyskytují v prostředí, které je vhodné pro růst plísní, tedy ve vlhku a teplotě kolem 25 – 30 °C. Odhaduje se, že 25 % světové produkce obilnin může být kontaminováno mykotoxiny [8]. Z důvodu mírnějších klimatických podmínek a správné zemědělské praxe v Evropě a Severní Americe je v těchto regionech kontaminace potravinových zdrojů nižší než například v rovníkových oblastech.

Toxické působení na živé organismy je známo zejména z veterinární praxe. Akutní otravy mykotoxiny je relativně málo četné. Během nich dochází k degeneraci jater, ledvin a centrálního nervového systému.

Jak již bylo zmíněno výše, k expozici mykotoxinům dochází převážně orální cestou, v některých provozech, jako jsou výroby krmiv, mlýny, pekárny také inhalačně a dermálně. S přihlédnutím k faktu, že nejčastější cestou expozice mykotoxinům je potrava, je z toxikologického hlediska mnohem významnější toxicita chronická než akutní. Účinek je způsoben dlouhodobým příjmem malých dávek toxinu. Nejčastěji se jedná o potlačení imunity, teratogenitu, mutagenitu a kancerogenitu. Během metabolizace toxinu dochází zpravidla k jeho deaktivaci, ale například aflatoxin B_1 je metabolizován na velmi reaktivní 8,9-epoxid, který snadno reaguje s DNA či bílkovinami a způsobuje tak často nevratné poškození organismu včetně možnosti vyvolání rakovinného bujení.

Pro některé velmi rozšířené mykotoxiny byly proto příslušnými organizacemi, jako jsou například Evropská komise či americká Food and Drug Administration, stanoveny limity v řádech ppb či ppt. Jiné mykotoxiny jsou stále sledovány a vztah mezi expozicí jim a toxickými účinky neustále vyhodnocován. Případné překročení limitů je v Evropské Unii hlášeno do systému rychlého varování RASFF (Rapid Alert System for Food and Feed) a poté jsou provedena příslušná opatření.

V poslední době se obrací pozornost i na sledování speciace mykotoxinů. Ty mohou být ve zkoumaném materiálu přítomny nejen ve volné podobě, ale například i ve formě glykosidů, z nichž se uvolní například při zpracování působením enzymů. Přesto je v současnosti drtivá většina metod zaměřena na stanovení volných mykotoxinů.

Výběr vhodné analytické metody pro stanovení mykotoxinů ve vzorcích je dán účelem jejího použití, koncentrací analytu či počtem analyzovaných vzorků [9]. Pro screening možné přítomnosti některých kontaminantů ve velkém počtu vzorků byly vyvinuty komerčně dostupné, citlivé a rychlé imunochemické metody.

V případě pozitivního nálezu mohou nastoupit chromatografické metody k potvrzení přítomnosti mykotoxinu a přesnému stanovení jeho množství. Výsledná hodnota obsahu je pak porovnána s limity danými pro danou komoditu.

Každé stanovení obsahu mykotoxinů v rostlinném materiálu začíná již odběrem vzorku. Právě jeho reprezentativnost je v mnoha případech klíčovým faktorem, nutným pro zajištění správnosti výsledku. Například u obilnin je obsah mykotoxinů výrazně vyšší na povrchu zrna či těsně pod ním, zatímco vnitřní část, endosperm, obsahuje velmi malá množství těchto látek.

Dalším krokem v přípravě vzorku je převedení analytu do roztoku, zpravidla extrakcí vhodným extrakčním činidlem. Poté následuje oddělení matrice. Zpravidla jsou využívány různé extrakční metody, kapalinová extrakce, extrakce na tuhé fázi (solid phase extraction, SPE), ale i imunoafinitní metody. Extrakce kapalinou probíhá buď na třepačce, nebo pomocí mixéru. Druhá varianta je intenzivnější a u některých mykotoxinů poskytuje kvantitativně vyšší výtěžky. Mívá však nižší reprodukovatelnost.

V takto připraveném extraktu je pak stanoven obsah příslušného toxinu. Pro screening jsou používány imunochemické metody. Pro kvantifikaci jsou nejvíce využívanými metodami vysokoúčinná kapalinová chromatografie, chromatografie na tenké vrstvě a pro těkavé deriváty mykotoxinů plynová chromatografie. K jejich detekci slouží prakticky všechny druhy detektorů,

jejichž výběr je dán chemickou strukturou analytu, požadovaným limitem detekce a v neposlední řadě i finančními možnostmi konkrétní laboratoře.

Každá vyvinutá metoda musí být validována a její analytické parametry, jako jsou přesnost, správnost, limit detekce, lineární rozsah, opakovatelnost aj. by měly odpovídat nárokům konkrétního případu.

Velká variabilita v chemické struktuře jednotlivých typů mykotoxinů a tím i jejich chemických vlastností vede k závěru, že není možné vyvinout jedinou metodu pro účinnou extrakci všech mykotoxinů jediným extrakčním činidlem. Proto jsou pro každou skupinu strukturně podobných látek vyvíjeny specifické postupy přípravy vzorku. Navíc zde může hrát významnou roli i složení matrice. Například burské oříšky obsahují více tuku, než jej obsahují pícniny. Pro každý mykotoxin jsou analytické postupy diskutovány v příslušné kapitole.

Obsah mykotoxinů v krmivech či potravinách je možné snížit kromě preventivních opatření (správná zemědělská a výrobní praxe) také využitím mikroorganismů, které mykotoxiny metabolizují zpravidla za vzniku méně toxických metabolitů. Již dnes jsou komerčně dostupná aditiva, která obsahují příslušné enzymy či extrakty z buněčných stěn. V přehledových článcích **[VIII, IX, X]** byly shrnuty různé cesty biodegradace mykotoxinů trichothecenů, T-2 toxinu a aflatoxinů. V publikaci **[XI]** pak byla testována schopnost různých druhů kvasinek *Saccharomyces* vázat deoxynivalenol.

Důležitým indikátorem přítomnosti plísní může být i množství ergosterolu ve vzorku. Tato steroidní látka je produkována téměř výhradně houbami a kvasinkami. V případě jeho detekce je velmi pravděpodobné, že daný vzorek byl napaden plísněmi. Ve specifických případech koreluje obsah ergosterolu s obsahem mykotoxinů [16]. Obsah ergosterolu byl též navržen jako jeden z parametrů jakosti rajčat [17].

4.1. Stanovení fumonisinů

Úvod

Fumonisiny jsou relativně novou skupinou fuzáriových mykotoxinů. Poprvé byly objeveny a izolovány roce 1988 v Jihoafrické republice ze vzorků kukuřice, napadené plísní *Fusarium moniliforme*. Jejich přítomnost v krmivech vyvolává mimo jiné leukoencefalomalacii u koní a otoky plic u prasat. Toxický účinek fumonisinu B_1 u člověka je způsoben hlavně ovlivněním syntézy sfingolipidů, kde tento mykotoxin inhibuje enzym ceramidsynthetázu katalyzující jejich syntézu. Proto také změny v obsahu sfingolipidů v krvi slouží jako marker otrav fumonisiny. V současnosti je známo přes 20 fumonisinů. Nejrozšířenější jsou fumonisiny řady B – B_1, B_2 a B_3 (FB_1, FB_2, FB_3), z nichž nejtoxičtějším a také nejvíce zastoupeným je fumonisin B_1. Fumonisiny jsou klasifikovány jako možné karcinogeny pro člověka (třída 2B) a jsou charakterizovány jako promotory karcinogenního procesu.

Z chemického hlediska jsou fumonisiny diestery různých 2-amino-12,16-dimethylpolyhydroxyeikosanů, které mají hydroxylovou skupinu na uhlíku C_{14} a C_{15} esterifikovanou propan-1,2,3-trikarboxylovou kyselinu. Jsou to tedy polární látky, rozpustné v polárních rozpouštědlech, např. voda, acetonitril, methanol či jejich směsi. V alkalickém prostředí částečně či úplně hydrolyzují, proto práce s nimi probíhá v neutrálním či mírně kyselém prostředí, které zvyšuje jejich ionizaci a tím i rozpustnost. Čištění extraktu probíhá na SPE kolonách plněných iontoměničem, reverzní fází či imunosorbentem.

Fumonisiny mají relativně vysokou molekulovou hmotnost a jsou polární. Jsou proto málo těkavé pro stanovení pomocí plynové chromatografie a k jejich separaci se používá především HPLC. Obdobně, jako je tomu u některých jiných mykotoxinů, nemají fumonisiny silný chromofor, proto je fotometrická detekce v UV či viditelné oblasti spektra velmi málo citlivá. Do jejich molekuly se proto zavádí například fluorofor a to reakcí vhodného činidla s aminoskupinou fumonisinu.

Mezi běžné metody stanovení fumonisinů ve vzorcích potravin či krmiv patří kapalinová chromatografie s fluorescenční detekcí. Separace bývá prováděna na kolonách s reverzní fází. Pro dosažení vyšší separační účinnosti je třeba snížit disociaci karboxylových skupin analytu. Proto je mobilní fáze okyselována nízkomolekulární organickou kyselinou.

V posledních letech je k detekci fumonisinů používán i hmotnostní detektor [10]. Díky amfoterní povaze analytu mohou být fumonisiny stanovovány ve formě kladně i záporně nabitých iontů.

Screening i kvantifikace je často prováděna tenkovrstvou chromatografie či imunologickými metodami (enzyme-linked immunosorbent assay, ELISA). Nevýhodou běžných ELISA testů je tzv. křížová reaktivita (cross-reactivity), kdy jsou neselektivně stanovovány fumonisiny B_1, B_2 i B_3, bez možnosti jejich samostatné kvantifikace. V prodeji jsou za vyšší cenu i selektivnější ELISA kity. Dalšími negativními vlastnostmi ELISA metod je jejich relativně nižší přesnost. Z tohoto důvodu bylo nutné vyvinout metodu, umožňující stanovení jednotlivých fumonisinů B_1 a B_2.

Vývoj a aplikace analytické metody

Pro extrakci a stanovení těchto mykotoxinů ve vzorcích kukuřice byla vyvinuta nová metoda. Příprava vzorku spočívala v extrakci mykotoxinu směsí acetonitril:methanol:voda (1:1:2, v/v/v), tak jak je uvedeno ve standardním postupu dle normy EN 114352:2004 "Foodstuffs – Determination of fumonisin B_1 and B_2 in maize based food – HPLC method with immunoaffinity column clean up". Oproti postupu v uvedené normě byl upraven způsob extrakce. Zatímco původně je k extrakci použito třepačky, v inovovaném postupu je nahrazena více invazivní metodou – ultraturraxem (mixérem). Výsledkem trojnásobného opakování extrakce v mixéru byla mnohem vyšší výtěžnost fumonisinů ze vzorku. U FB_1 došlo ke zvýšení výtěžnosti ze 75,6 na 94,7 %, pro fumonisin B_2 ze 72,0 na 93,9 %. Během dalších opakování extrakce již nedošlo k výraznějšímu nárůstu výtěžnosti.

Fumonisiny neobsahují silně absorbující chromofor v ultrafialové nebo viditelné oblasti. Pro jejich citlivou detekci je využívána fluorescence. Protože nemají ani vlastní fluorofor, musí být před detekcí derivatizovány, což stanovení komplikuje. Z tohoto důvodu bylo přikročeno k použití hmotnostní detekce, kde tento krok odpadá. V kyselém prostředí dochází k tvorbě pozitivně nabitých molekulových iontů a fragmentů, proto byl detektor nastaven na detekci kladně nabitých částic. Tvorba pozitivních iontů byla úspěšně podpořena přídavkem vhodné koncentrace amonných iontů. Výhodou použitého octanu amonného je těkavost iontů, ze kterých je složen. Nedochází tak k zanášení hmotnostního detektoru netěkavými solemi. Byla tedy vyvinuta a optimalizována i nová chromatografická metoda pro stanovení fumonisinů B_1 a B_2.

V souladu s nařízením Evropské komise 2002/657/EC byly k detekci na hmotnostním detektoru využity pro každý analyt 3 ionty. Během analýz byl sledován i poměr mezi těmito jednotlivými ionty, což je jedním z parametrů zvyšujících selektivitu detekce. Limity detekce 62,0

respektive 58,5 µg/kg pro FB_1 a FB_2 jsou výrazně pod úrovní vyžadovanou nařízením Evropské komise č. ES 1881/2006, ze dne 19. prosince 2006, kterým se stanoví maximální limity některých kontaminujících látek v potravinách. Zde je povoleno v případě nezpracované kukuřice 2000 µg FB_1+FB_2/kg.

Metoda byla validována s uměle kontaminovanými vzorky, do kterých bylo přidáno podlimitní, limitní a nadlimitní množství mykotoxinů. Závěrem byla správnost metody ověřena evropským mezilaboratorním testem organizovaným Central Science Laboratory (Ministry of Agriculture, Fisheries and Food, UK). Tato organizace pořádá největší a nejobsáhlejší schéma testování analytických laboratoří v potravinovém sektoru – FAPAS (food analysis performance assessment scheme). Naměřené výsledky byly v toleranci. Vyvinutá metoda byla následně použita pro rutinní stanovení fumonisinů v kukuřici (povolený limit 2 mg/kg). Bylo provedeno i interní porovnání vyvinuté metody s ELISA testy, kde byla pozorována velmi vysoká korelace obou metod. V reálných vzorcích byl detekován i fumonisin B_3. Vzhledem k nízké toxicitě i relativnímu nízkému zastoupení v přirozeně infikovaných vzorcích nebylo uvažováno o kvantifikaci fumonisinu B_3. Metoda byla následně použita pro stanovení fumonisinů ve vzorcích kukuřice **[XII, XIII]**.

Byl založen polní experiment a pěstováno 26 hybridů kukuřice včetně dvou geneticky modifikovaných (Bt-modifikace). V těchto hybridech bylo sledováno napadení plísněmi. Po sklizni byl stanoven obsah fusáriových mykotoxinů – deoxynivalenolu, fumonisinů, zearalenonu. Dále byl sledován obsah ergosterolu, který lze považovat za indikátor napadení substrátu plísněmi. Bohužel, klimatické podmínky v roce 2007 nebyly příliš vhodné pro růst plísní a proto i obsahy sledovaných látek byly velmi nízké. Pouze obsah ergosterolu byl v silné korelaci s mírou napadení kukuřice. Obsah mykotoxinů v geneticky modifikované kukuřici byl nižší než v ostatních vzorcích.

Závěr

Byla vyvinuta nová citlivá analytická metoda pro stanovení fumonisinů ve vzorcích surové nezpracované kukuřice. Změnou provedení extrakce z třepání na intenzivnější mixování došlo k výraznému zvýšení výtěžnosti extrakce. Kapalinová chromatografie s hmotnostní detekcí vedla k odstranění jinak nutného kroku derivatizace a tím i ke zjednodušení celého postupu stanovení. Metoda splňuje parametry kladené nařízeními Evropské komise. Vyvinutá metoda byla použita pro stanovení obsahu fumonisinů v 26 hybridech kukuřice, z toho ve dvou geneticky modifikovaných.

4.2. Stanovení deoxynivalenolu

Úvod

Deoxynivalenol (DON) neboli vomitoxin je mykotoxinem produkovaným zejména plísněmi *Fusarium culmorum* a *F. graminearum*. Patří mezi velmi rozšířené mykotoxiny, zejména v obilninách [11]. Řadí se do skupiny B trichothecenových mykotoxinů (s karbonylovou skupinou na uhlíku C_8). Trichotheceny mají tetracyklickou seskviterpenovou strukturu, včetně šestičlenného kruhu obsahujícího kyslík, epoxyskupinu mezi uhlíky 12 a 13 a dvojnou vazbu mezi uhlíky v poloze 9 a 10. DON je přítomen ve velké části vzorků kontaminovaných fuzáriovými mykotoxiny a je proto prakticky markerem možnosti jejich přítomnosti. Jeho toxicita pramení z přítomnosti epoxidové skupiny mezi uhlíky C_{12} a C_{13}. Deoxynivalenol je dáván do souvislosti s celou řadou otrav, například alimentární toxickou aleukií, akutní DON toxikózou, otravou červenou plísní (red-mold disease) apod.

Kromě možnosti alimentárních otrav vede přítomnost DON v obilninách k ovlivnění jejich technologických vlastností. Například v ječmeni infikovaném plísněmi se DON tvoří i během sladování. DON se také v průběhu sladování dostává z ječmene do sladu. Poté přechází do piva a pravděpodobně se podílí na tzv. „přepěňování“ piva. Jedná se o jev, kdy po otevření lahve dochází k nadměrnému pěnění a vytečení větší části jejího obsahu. Legislativně jsou maximální limity obsahu DON v příslušných potravinách a potravinářských surovinách zakotveny v příslušných vyhláškách a nařízeních Evropské komise.

Z fyzikálně-chemického hlediska je DON bílý krystalický prášek, rozpustný v běžných polárních organických rozpouštědlech, jako jsou acetonitril a methanol. Mírně rozpustný je i ve vodě.
Proto se k extrakci DON z matrice používají právě tato rozpouštědla či jejich směsi. Po extrakci následuje čištění extraktu a to zpravidla pomocí Mycosep® kolonek respektive SPE kolonek, naplněných aktivním uhlím, imunosorbentem apod. [9].

Mezi nejpoužívanější screeningové metody patří ELISA, ke kvantifikaci se využívají chromatografické metody, například plynová či kapalinová chromatografie. Pro analýzu na plynovém chromatografu je nutné provést derivatizaci DON k získání těkavého derivátu. HPLC separace je prováděna téměř výhradně na reverzní fázi. S přihlédnutím k nízké absorpci nativního deoxynivalenolu v UV oblasti spektra (maximum okolo 220 nm), bývá v případech potřeby nízkých detekčních limitů používána jiná, citlivější metoda, jakou je například hmotnostní detekce.

V práci **[XIV]** byla řešena právě problematika přechodu DON z ječmene do sladu a to včetně vývoje nové analytické metody pro stanovení DON. Vyvinutá metoda byla následně použita pro sledování korelace obsahu deoxynivalenolu a ergosterolu ve vzorcích ječmene a z něj vyrobeného sladu **[XV]**.

Další řešenou problematikou bylo studium obsahu deoxynivalenolu v tělech kvasinek **[XI]**. Ta jsou součástí výpalků vznikajících při výrobě bioethanolu, a které mohou být zkrmovány hospodářskými zvířaty. V těchto odpadních produktech, sestávajících z velké části z těl odumřelých kvasinek, dochází ke zkoncentrování mykotoxinů. Ty z důvodu nízké těkavosti zůstávají v destilačním zbytku po oddestilování etanolu a působit tak negativně na zvířata, jimiž jsou zkrmovány.

Cílem práce bylo vyvinout metodu pro stanovení deoxynivalenolu ve vzorcích ječmene a sladu a také ve vzorcích kultivačního média.

Vývoj metody

V **[XIV]** byly studovány možnosti využití extrakce na pevné fázi k přípravě vzorku ječmene a sladu pro stanovení DON.

Vzorek ječmene či sladu byl extrahován směsí acetonitril:voda (84:16) a tento extrakt byl dále čištěn pomocí SPE. Na základě experimentálních dat byly ze tří testovaných SPE kolonek zavedeny SPE kolonky se sorbentem na bázi aktivního uhlí. Pro použitý druh vzorků kolonky vykazovaly nejvyšší výtěžnost DON a také dostatečné oddělení matrice. Daný způsob přípravy vzorku vede ke stanovení pouze nevázaného DON. Metoda byla následně použita i v práci **[XV]**.

Další matricí, ve které bylo nutné sledovat obsah deoxynivalenolu, jsou mikrobiologická kultivační média. Ta obsahují jednak bakterie a živiny, nutné pro růst mikroorganismů, ale i celou řadu jejich rozpustných metabolitů, jako jsou nízkomolekulární organické kyseliny, alkoholy, aminy apod.

V prováděném experimentu **[XI]** se jednalo o vyzkoušení možnosti dekontaminace roztoku za využití různých kmenů kvasinek. Proto bylo nutné zvolit levnou, rychlou a snadno proveditelnou screeningovou metodu pro stanovení DON. Z důvodu toxického působení acetonitrilu, ve kterém byl rozpuštěn standard DON na kvasinky, bylo kultivační médium připravováno vždy tak, že do kultivační nádoby byl odpipetován daný objem roztoku standardu a acetonitril odpařen. Čistý DON byl pak rozpuštěn v médiu.

Deoxynivalenol se v takovém systému vyskytuje v různých formách – volné, vázané, eventuálně může být metabolizován. Za předpokladu, že DON není těmito kmeny kvasinek metabolizován, stačí stanovit obsah DON v živných médiích po uplynutí dané doby kultivace různých kmenů kvasinek. Rozdíl mezi počátečním množstvím DON, přidaným do roztoku a množstvím zjištěným pak udává mykotoxin sorbovaný (vázaný) kvasinkami. Buněčná stěna kvasinek disponuje celou řadou komponent, jako jsou ß-D-glukomanany, které jsou schopny vázat mykotoxiny. K separaci vázaného a volného DON byla využita filtrace přes membránový filtr s velmi malými póry. Kvasinky díky své velikosti neprocházejí filtrem a ve filtrátu se nachází pouze volný DON. Pro předčištění extraktu byla zvolena metoda, využívající jednorázové SPE kolonky MycoSep®. Bylo postupováno dle návodu dodavatele. Filtrát byl zředěn acetonitrilem v poměru 16:84 (v/v) a prolit SPE kolonkou. Poté byly 2 ml eluátu odpařeny pod dusíkem do sucha a odparek rozpuštěn ve 400 µl mobilní fáze. Takto připravené vzorky kultivačních médií byly následně analyzovány pomocí HPLC/MS.

K chromatografickému stanovení DON v předčištěných extraktech z ječmene, sladu či živného média byla vyvinuta a optimalizována metoda, využívající kapalinovou chromatografii s hmotnostní detekcí. Separace probíhala na koloně s reverzní fází. Pro snížení limitu detekce byla mobilní fáze okyselena 1 mM kyselinou mravenčí, což vedlo k podpoře vzniku kladně nabitých iontů v elektrospreji detektoru. Vyvinutá metoda je dostatečně citlivá pro daný účel a její limit detekce DON leží výrazně níže, než je povolený obsah DON v obilninách. Získané výsledky byly porovnány s komerčně používanými ELISA kity (enzyme-linked immunosorbent assay).

Naměřené výsledky jednoznačně neprokázaly, zda při procesu sladování vzrůstá či klesá množství deoxynivalenolu ve sladu. Obsah deoxynivalenolu ve všech analyzovaných vzorcích byl pod maximálním povoleným limitem daným legislativou.

V případě měření úbytku DON v živných médiích bylo zjištěno, že v průběhu kultivace kvasinek *Saccharomyces* dochází k úbytku tohoto mykotoxinu **[XI]**. Příčinou poklesu byla sorpce na buněčných stěnách kvasinek. Jednalo se o pilotní projekt a dále bude tato problematika studována.

Závěr

Byla vyvinuta a aplikována nová metoda pro stanovení deoxynivalenolu ve vzorcích ječmene, sladu a mikrobiologickém médiu. Byly vyzkoušeny různé SPE kolonky pro předčištění extraktu z ječmene a sladu obsahujícího deoxynivalenol. Nejvyšší výtěžnosti bylo dosaženo s kolonkami plněných grafitizovanými sazemi. Pro screening DON v kultivačních médiích byla vyvinuta jednoduchá metoda přípravy vzorku k analýze založená na použití MycoSep® kolonek. Pro stanovení DON byla vyvinuta a validována metoda HPLC s hmotnostní detekcí. Metodika byla použita na stanovení DON v ječmeni, sladu a vzorcích mikrobiologických kultivačních médií.

4.3. Stanovení citrininu

Citrinin (CIT) je toxickým metabolitem produkovaným různými druhy plísní, jako jsou například *Penicillium verrucosum*, *P. citrinum*, eventuelně *Monascus* sp., která je hojně používaná jako zdroj červeného barviva k barvení potravin. Spolu s dalším mykotoxinem ochratoxinem A citrinin kontaminuje potraviny. Typická koncentrace nacházená v cereáliích a potravinách z nich vyrobených se pohybuje v rozmezí 0,1 až 1130 mg/kg [12].

Citrinin spolu s ochratoxinem má negativní efekt převážně na ledviny a játra. Spolu jsou zodpovědní za onemocnění ledvin u lidí I zvířat, nádory a pravděpodobně jsou jedním z faktorů způsobujích Balkánskou endemickou nefropatii. Expozice ledvinových buněk citrininu vedla k inhibici proliferace a k projevům genotoxicity (DNA fragmentace a aberace chromozomů). Tyto toxické projevy citrininu jsou potencovány ochratoxinem A [13].

Zatím nejsou zavedeny žádné legislativní limity pro obsah citrininu v potravinách, obilovinách či krmivech. Vzrůstající pozornost vzhledem k obsahu citrininu v potravinách je zřejmý i z toho, že v roce 2010 Evropská komise požádala Evropskou autoritu pro bezpečnost potravin (European Food Safety Authority, EFSA) o vydání vědeckého stanoviska mimo jiných mykotoxinů právě i pro citrinin.

Pro účely monitoringu bylo nutné vyvinout novou citlivou a rychlou metodu pro stanovení citrininu v těchto matricích. V praxi jsou používané různé metody, zpravidla chromatografické, například tenkovrstevná chromatografie s fluorescenční detekcí pro detekci citrininu v sýrech či kultivačních médiích. Detekce je následně prováděna derivatizací či reakcí s p-anisaldehydem, pyridinem, acetanhydridem či chloride hlinitým. Detekční limit se u těchto metod pohybuje kolem 10-50 ng/g [14].

V současné době je velmi často pro stanovení citrininu ve složitých biologických matricích využívána vysokoúčinná kapalinová chromatografie s detekcí fluorescenční, UV či luminiscenční. Mnoho metod v kapalinové chromatografii používá kyselou mobilní fázi k dosažení nižších detekčních limitů. Nejvyšší intenzita fluorescence citrininu je dosažena při pH 2,5. Naneštěstí, většina stacionárních fází není pro toto pH vhodná. Někteří autoři řeší tento problém okyselením eluentu za kolonou 1M kyselinou chlorovodíkovou [15].

V práci **[XVI]** byla vyvinuta nova metoda pro stanovení citrininu, využívající chromatografickou kolonu s rozšířeným operačním rozsahem pH a fluorescenční detekcí. Metoda byla poté úspěšně

aplikována na reálné vzorky, kdy byly poprvé měřeny české vzorky a výsledky porovnány s jinou metodou na spolupracujícím pracovišti ENSAT v Toulouse ve Francii **[XVII]**.

Závěr

Byla vyvinuta nová, vysoce citlivá metoda pro stanovení citrininu ve vzorcích obilovin a následně porovnána s výsledky získanými u stených vzorků jinou metodou na zahraničním pracovišti. Výsledky stanovení jsou jedny z prvních v České republice a do budoucna mohou sloužit jako výchozí pro další projekty.

7. Splnění cílů habilitační práce

Kapalinová chromatografie s různými způsoby detekce byla využita pro stanovení významných látek, jako jsou biogenní aminy a polyaminy či mykotoxiny ve vzorcích potravin.

Byly vyvinuty nové analytické metody využívající HPLC pro stanovení nejčastěji se vyskytujících mykotoxinů. Pro stanovení fumonisinů B_1 a B_2 bylo modifikací extrakčního procesu dosaženo velmi vysoké výtěžnosti z uměle kontaminovaných vzorků kukuřice. K analýze extraktů byla vyvinuta a validována nová HPLC metoda s hmotnostní detekcí. Správnost metody byla ověřena kruhovým testem FAPAS.

Pro stanovení deoxynivalenolu byla vyvinuta nová metoda čištění extraktu pomocí SPE. K analýze byla vyvinuta HPLC metoda s hmotnostní detekcí v oblasti pozitivních iontů. Metoda byla využita pro stanovení DON v ječmeni a z něj vyrobeného sladu. Nebyla nalezena žádná souvislost mezi obsahem DON v těchto dvou komoditách. Dále byla tato metoda využita pro sledování možnosti detoxifikace materiálu kontaminovaného deoxynivalenolem pomocí různých druhů kvasinek.

Jako jedna z prvních v České republice byla vyvinuta a aplikována nová velmi citlivá metoda pro stanovení mykotoxinu citrininu ve vzorcích rostlinného původu. Následně byla porovnána se zavedenou metodou na zahraničním pracovišti. Výsledky získané touto metodou mohou do budoucna sloužit jako podklad při dalším plánování sledování výskytu mykotoxinů v České republice či Evropské unii, zvláště když se touto problematikou začala zabývat i Evropská komise.

V rámci stanovení vybraných mykotoxinů byl sledován i obsah ergosterolu, který je markerem přítomnosti plísní a může sloužit i pro jejich kvantifikaci. V případě infikované kukuřice byla nalezena významná korelace s vážností jejího onemocnění. Naopak, v případě sladování nebyly zaznamenány významné změny.

Vyvinuté analytické metody mohou pomoci ke zlepšení kvality a hygienické nezávadnosti potravin. Získané výsledky jsou využitelné nejen pro prevenci nebo minimalizaci rizika vzniku biogenních aminů ve fermentovaných potravinách a mykotoxinů na poli či v průběhu skladování, ale i pro sledování účinnosti dekontaminace vzorků kvasinkami *Saccharomyces*.

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Příloha

TEXTY PRACÍ I-XVII

Publikace I

Chapter 41

Amines

Tomáš Komprda and Vlastimil Dohnal

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41.1 Structure

Under the term "amines," biogenic amines (BA) and polyamines (PA) will be recognized in this chapter. They are relatively simple organic nitrogenous bases, formed predominantly by the decarboxylation of their particular precursor amino acids.

Based on a purely chemical structure, three main groups of quantitatively important amines, which can occur in dairy foods, can be recognized: heterocyclic (histamine and tryptamine), aromatic (tyramine and 2-phenylethylamine), and aliphatic (agmatine, putrescine, cadaverine, spermidine, and spermine) amines (Figure 41.1).

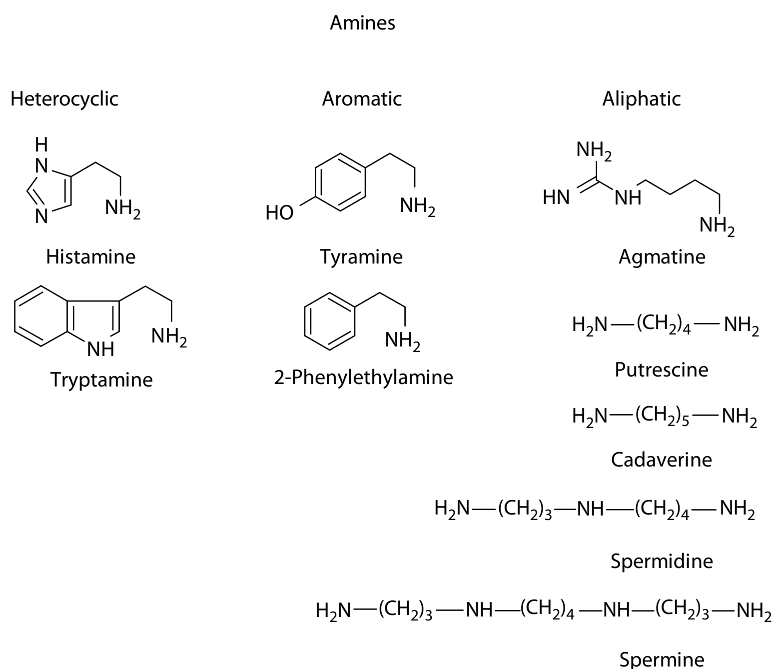

Figure 41.1 Chemical structure of BA and PA with likely occurrence in dairy foods.

Some physiologically important bioactive substances, such as dopamine, adrenalin (epinephrine), noradrenalin (norepinephrine), and serotonin (5-hydroxytryptamine), also belong to the group of BA. However, these amines act as hormones and neurotransmitters in mammals and their presence cannot be expected at physiologically relevant levels in dairy foods, and therefore, these amines will not be taken into account from the analytical viewpoint in this chapter.

The decarboxylating enzymes are mainly of microbial origin. Therefore, BA are formed in dairy foods predominantly as a consequence of the presence of microorganisms (either starter or contaminant bacteria). However, PA (putrescine, spermidine, and spermine) have recently been recognized as a distinct group owing to the biochemical (they are also formed by an alternative metabolic pathway both in bacterial and mammalian cell) and toxicological (see Section 41.2) reasons [1]. The general scheme of BA formation and two alternative PA biosynthesis pathways are shown in Figure 41.2.

41.2 Toxicological Importance

Tyramine is not only quantitatively (see Section 41.3) but also toxicologically (together with histamine) the most important BA in dairy foods. Toxicological importance of tyramine is based on its effect similar to that of the indirect sympathomimetic drugs: vasoconstriction with a possible consequence of hypertension, migraine, brain hemorrhage, and heart failure in more sensitive individuals [2].

Histamine, an endogenous BA, can be released from the stores mainly by a reaction of IgE antibody with an antigen. In sensitive individuals, exogenous histamine in higher concentration

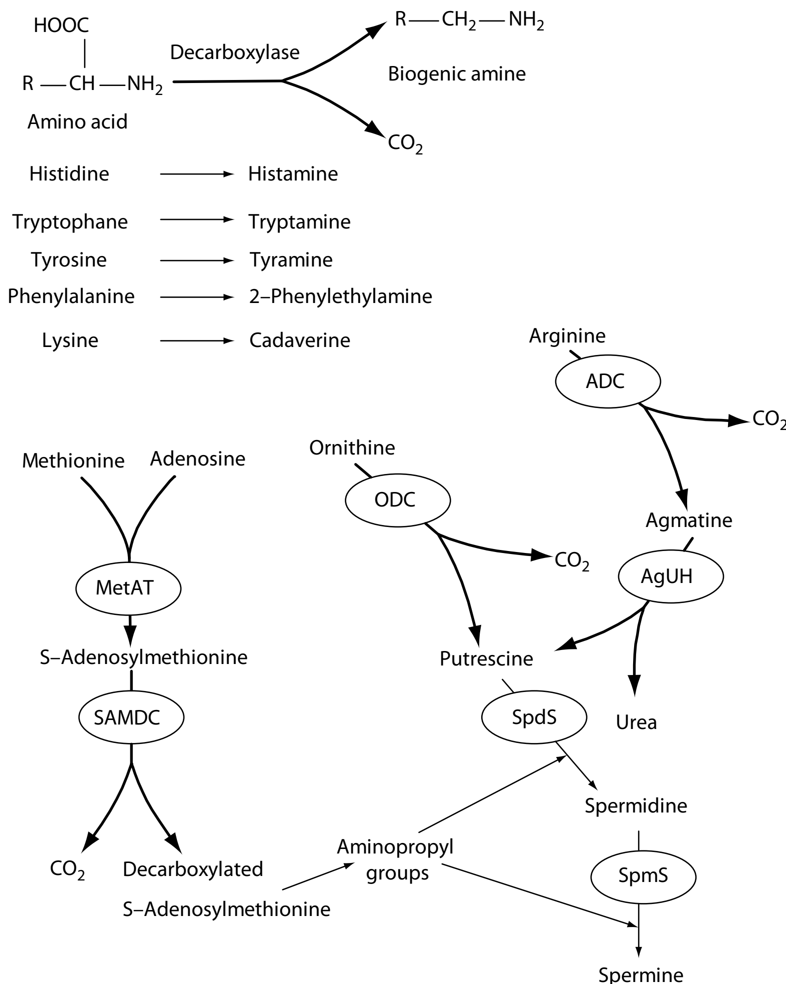

Figure 41.2 General scheme of BA formation and alternative PA (putrescine, spermidine, spermine) biosynthesis pathways; ODC, ornithine decarboxylase; ADC, arginine decarboxylase; AgUH, agmatine ureohydrolase; SpdS, spermidine synthase; SpmS, spermine synthase; MetAT, methionine adenosyltransferase; SAMDC, S-adenosylmethionine decarboxylase. (Adapted from Wolter, F. et al., *J. Nutr.*, 134, 3219, 2004; Komprda, T. et al., *Eur. Food Res. Technol.*, 227, 29, 2008.)

(e.g., from dairy foods) can cause the same clinical symptoms as an early allergic reaction of endogenous histamine: asthma bronchiale, rhinitis, conjunctivitis, urticaria, edema, hypotension, headache, flushing, and abdominal cramps.

Toxicological importance of other BA present in dairy foods is based on their potentiation of the action of tyramine and/or histamine (via monoamine oxidase [MAO] saturation) rather than on their own adverse health effects.

PA that are physiologically required for the cell growth and proliferation are selectively taken up by the tumor cells and can consequently facilitate the growth of a tumor. Moreover,

putrescine stimulates tyrosine kinases and the expression of particular nuclear protooncogens, and thus, is also involved in cancer pathogenesis [3]. PA are also able to form stable carcinogenic *N*-nitroso compounds and enhance the growth of chemically induced aberrant crypt foci in the intestine [4].

Amines are broken down in a mammal by the oxidative deamination catalyzed by MAO, diamine oxidase, or PA oxidase, resulting in the formation of aldehyde, ammonia, and hydrogen peroxide. However, human detoxification mechanisms are insufficient in the case of very high amine intake in the diet or in more sensitive consumers, such as allergic individuals and patients consuming drugs with an action of the MAO inhibitors (some antiparkinsonian drugs and some antidepressants).

Toxicological limits for tyramine or histamine are difficult to establish owing to the great differences between people regarding robustness of the detoxification system and the presence of various amounts of other amines in the food matrix. Nevertheless, the value above 100 mg tyramine and histamine, respectively, per one kg of food are supposed to be deleterious [5].

Concentrations of PA in dairy foods, especially ripening cheeses (see Section 41.3), cannot be properly assessed from the toxicological viewpoint, before further research elucidates both the recommended dietary intake (e.g., wound healing) and the limit which when exceeded would have deleterious effects in cancer patients.

41.3 Expected Amounts of Biogenic Amines and Polyamines in Dairy Foods

Before beginning the analysis, it is useful to have some idea about the possible BA and PA content in dairy foods to be analyzed. According to the literature data, both BA and PA content in milk, curd, whey, yogurt, and unripened cheese is usually lower than 1 mg kg^{-1} [6,7]. Therefore, the only dairy food important from the viewpoint of toxicologically relevant BA and PA content is ripening cheese. The recently published data regarding BA and PA content in various types of cheeses are presented in Tables 41.1 and 41.2, respectively.

41.4 Current Analytical Methods for the Determination of Toxicologically Important Amines in Dairy Foods

41.4.1 Supplementary Analysis: Screening for Amine Producers

When analyzing BA and PA, it should be taken into account that these substances are formed in the dairy foods mainly by the following groups of bacteria possessing genes for particular decarboxylating enzymes: nonstarter lactic acid bacteria, other adventitious bacteria, and even starter bacteria [8,9]. Therefore, an integral part of BA/PA determination is often a microbiological analysis also [7,10,11].

A possible protocol for determining the amine-producing bacterial strains is as follows (the ability to decarboxylate amino acids is not genus- or species-specific, but it is a property of only a small number of strains within a given species [9,12]): cultivate the bacterial groups or genera with a presumed potential to decarboxylate amino acids in dairy foods (*Enterobacteriaceae*, lactic acid bacteria, genus *Enterococcus*); screen an aliquot of colonies in an appropriate decarboxylating medium (e.g., according to [13]); in the positive samples (change of color based on transition from

Table 41.1 Content of Quantitatively Important Biogenic Amines in Different Types of Cheeses

	Biogenic Amine (mg kg^{-1})					
Cheese (Time of Ripening; Weeks)	*Histamine*	*Tryptamine*	*Tyramine*	*Phenylethyl-amine*	*Cadaverine*	*Reference*
Swiss-type (24)	750–1290	—	64–910	—	—	[17]
Processed	n.d.	n.d.	4–160	8–400	12–120	[50]
Processed	1	1	1–29	1–2	1	[51]
Spanish retail cheeses (unripened)	n.d.	n.d.	<1	n.d.	<1	[19]
Spanish retail cheeses (ripened)	2–164	n.d.–45	n.d.–242	n.d.–29	4–215	[19]
Goat cheese (13)	10	22	68	37	4	[7]
Raw-milk goat cheese (13)	43–83	12	325–428	27–92	196–314	[52]
Semisoft cheese; pasteurized milk (20)	n.d.–124	—	39–770	5–179	6–109	[11]
Semisoft cheese; raw milk (20)	226–573	—	400–1478	29–223	280–2369	[11]
Semihard Italian; unpasteurized milk (21)	117–378	2–5	128–394	8–20	5–30	[53]
Greek ovine/goat brined Feta cheese; thermized milk (17)	85	6	246	5	83	[54]
Portuguese Azeitao cheese; raw ovine milk	644–682	—	358–445	—	161–231	[25]
Gouda (12)	178–418	—	337–776	—	—	[27]
Spanish traditional cheeses	n.d.–928	—	10–1807	—	—	[8]
Portuguese traditional; raw ovine milk (9)	16	56	176	9	207	[55]
Dutch-type hard; pasteurized milk (22)	2–17	1–2	3–310	1–54	1–2	[18]
Blue-vein; pasteurized milk (7)	n.d.–90	n.d.–6	10–185	n.d.	3–491	[56]

n.d., not detected.

Table 41.2 Content of Polyamines in Different Types of Cheeses

Cheese (Time of Ripening; Weeks)	*Polyamine (mg kg⁻¹)*			*Reference*
	Putrescine	*Spermidine*	*Spermine*	
Swiss-type (24)	17–360	—	—	[17]
Processed	4–60	8–100	n.d.	[50]
Processed	1–2	1	<1	[51]
Cheddar (young)	10–20	77–104	23–37	[1]
Cheddar (ripened)	650	190	22–38	[1]
Spanish retail cheeses (unripened)	n.d.–1	<1	<1	[19]
Spanish retail cheeses (ripened)	n.d.–612	n.d.–43	n.d.–19	[19]
Goat cheese (13)	34	1	5	[7]
Raw-milk goat cheese (13)	86–175	—	—	[52]
Semisoft cheese; pasteurized milk (20)	1–13	—	—	[11]
Semisoft cheese; raw milk (20)	76–308	—	—	[11]
Semihard Italian cheese; (unpasteurized milk) (21)	129–1105	—	—	[53]
Greek ovine/goat brined cheese; thermized milk (17)	193	—	—	[54]
Portuguese Azeitao cheese; raw ovine milk	110–137	—	17–49	[25]
Gouda (12)	4–42	—	—	[27]
Portuguese traditional; raw ovine milk (9)	218	—	—	[55]
Dutch-type hard; pasteurized milk (22)	6–61	<1	<1	[18]
Dutch-type semihard; pasteurized milk (25)	1–132	n.d.–4	2–9	[57]
Blue-vein; pasteurized milk (7)	n.d.–117	n.d.–29	n.d.–12	[56]

n.d., not detected.

an acidic [amino acid] to a basic [amine] form), confirm the presence of BA/PA in a medium by a chemical analysis (see Section 41.4.2); isolate the bacterial DNA from the positive colonies (procedure see [14]); and multiply a putative gene sequence for a given amino acid decarboxylase using PCR and a pair of corresponding primers [15].

41.4.2 Overall Scheme of Biogenic Amines and Polyamines Analysis

First, when analyzing the amines content in dairy foods, it is necessary to consider the number of samples for a proper statistical evaluation. It should be taken into account that in dairy food technology, the batch, e.g., the cheese vat, and not each single block of cheese obtained from it, is usually considered as the experimental unit [16].

It should also be noted that BA and PA can be distributed unevenly in dairy foods. This is evident especially in ripening cheeses, where conspicuous differences in amines concentration are observed between the core and the edge part [6,17,18]. When the goal is to determine the average amines content, the aliquot parts from several layers of the cheese should be taken and properly homogenized; on the other hand, sometimes, the outer and inner part of the cheese are analyzed separately.

BA/PA determination is analytically demanding owing to the high requirements for sensitivity and precision. In addition, the effect of the matrix is also very important. Dairy foods, especially cheeses, have a complex matrix because of their high proportion of fat and protein, which hinders the extraction of amines [19]. Therefore, the choice of an appropriate analytical method should consider precision and recovery data. The data for evaluation of precision (analytical repeatability) are obtained by measuring the BA/PA content in the same sample by a given method several times; precision is usually expressed as a relative standard deviation (RSD, in %), which numerically corresponds to the coefficient of variation. Recovery data are obtained by spiking the dairy food sample with mixed BA/PA standard at one or two concentration levels. The comparison of precision and recovery values of various analytical protocols differing in the method of extraction, derivatization, and separation of amines in different types of cheeses is presented in Table 41.3.

The general scheme of BA and PA determination is as follows: amines extraction (CCl_3COOH; $HClO_4$; HCl) → derivatization (dansyl chloride; *o*-phthaldialdehyde) → separation (chromatographic; electrophoretic) → detection (UV; MS).

The consecutive steps of amine analysis are described in more detail in the following text.

41.4.3 Amines Extraction from the Matrix of Dairy Foods, Including Cleaning Procedures

Prior to the extraction, the exact amount of internal standard (1,7-diaminoheptane) is added to the sample to improve the reproducibility of amines determination. During the extraction, the samples are disintegrated and the amines are dissolved in the extraction solvent. Ultra-Turrax homogenizers are typically used for sample disintegration.

Apart from amines, the dairy food matrix includes other molecules containing amino groups, such as free amino acids or proteins (including enzymes) that can react with a derivatization agent with a consequence of nonquantitative derivatization of amines (derivatization see Section 41.4). Therefore, one of the main concerns of the cleaning procedure is the removal of amino acids to assure excess of derivatization agent (most of which reacts with the amino acids) to reach the quantitative derivatization of BA and PA. Amino acids can be removed prior or after the derivatization step. A frequently used method is the extraction of amine derivatives into diethylether [20].

Moreover, high content of free amino acids (especially in ripening cheeses) can cause problems in chromatographic determination owing to the elution of these amino acids in the first minutes of analysis, which can interfere with early eluted BA.

Table 41.3 Precision and Recoveries of Various Methods Used for the Determination of Biogenic Amines/Polyamines in Cheeses

	Precision (Analytical Repeatability; RSD, %)[a] [Reference]						*Recovery (%) [Reference]*				
Amine	*[28][b]*	*[29][c]*	*[19][d]*	*[18][e]*	*[44][f]*	*[20][g]*	*[28][b,h]*	*[29][c]*	*[19][d]*	*[18][e,i]*	*[20][g,j]*
Histamine	1.3	6.1	1.1	5.8	9.4	2.7	100	67	97	94	78
Tryptamine	13.9	—	2.0	1.2	5.5	5.5	64	62	92	95	72
Tyramine	2.3	3.4	1.8	5.0	4.5	3.3	72	59	93	60	84
Phenylethylamine	2.2	—	1.0	6.8	—	4.5	72	79	91	90	58
Agmatine	12.1	—	1.2	—	4.7	—	57	—	96	—	—
Putrescine	1.6	6.1	1.3	3.0	6.6	2.0	70	2	97	65	98
Cadaverine	1.5	4.8	1.0	2.9	2.1	4.3	54	61	98	75	95
Spermidine	1.9	7.8	2.1	1.8	3.4	2.4	84	49	97	63	79
Spermine	2.4	12.5	1.6	5.4	9.0	21.0	63	44	97	67	62

[a] Relative standard deviation (numerically corresponds to the coefficient of variation).
[b] Parmesan cheese; HPLC determination after HCl extraction; based on three determinations of the same sample.
[c] Grana cheese; HPLC determination after HCl extraction; based on three determinations of the same sample.
[d] Unripened cheese, retail sample; extraction by perchloric acid, determination by ion-pair HPLC; based on eight determinations of the same sample.
[e] Dutch-type hard cheese; five HCl extracts of the cheese sample with low amines content, HPLC determination.
[f] Dessert Romadour-type cheese; trichloroacetic acid extraction followed by ion-exchange chromatography determination; based on five determinations of the same sample.
[g] Parmigiano Reggiano cheese; 0.1 M HCl extraction, HPLC determination; six repetitions.
[h] Parmesan cheese spiked with 10 mg of amines per 100 g of sample during extraction and purification.
[i] Dutch-type cheese spiked at the concentration level of 2 mg kg–1; measured five times.
[j] Parmigiano Reggiano cheese samples spiked with 1 mL of a standard amines solution (concentration of each amine, 1 mg mL–1); derivatization procedure with 20 mg mL–1 of DCl.

On the other hand, when the content of free amino acids in the extract is low, it is possible to use the derivatized extract directly for the analytical determination of amines, without any other cleaning procedure, thus, avoiding the losses of amines in additional cleaning steps.

Another quantitatively important part of the dairy food matrix, which can interfere with the amines determination in dairy foods, is fat. The lipid fractions are removed from the extracts using low temperature in freezer or in a cooled (usually at 4°C) centrifuge [21–23].

The procedures for amines extraction and extract deproteinization prior to derivatization mainly use acids or organic solvents. Hydrochloric acid (0.1 M) or perchloric acid (0.2 and 0.6 M, respectively) is used most often for amines extraction from dairy products.

Typically, 25 or 50 mL of 0.1 M hydrochloric acid per 5.0–10.0 g of the sample is used for the extraction of amines from dairy foods. Extraction is commonly performed in a homogenizer, with an option of 1–2 repetitions [18], or using an ultrasonic bath [24].

Alternatively, the solution of 0.2 M perchloric acid can be used for amines extraction from dairy foods [25]. Lanciotti et al. [26] extracted amines from ovine and bovine Italian cheeses using 15 mL of 0.2 M $HClO_4$/4 g of cheese. The extraction was performed in an Ultra-Turrax macerator at medium speed. The homogenate was then centrifuged at 10 000 rpm for 10 min at 4°C, the supernatant was removed, and the extraction of the solid part was repeated. The combined extracts were adjusted to 50 mL with 0.2 M perchloric acid. Leuschner et al. [27] used 0.6 M perchloric acid for amines extraction from cheeses in a ratio of 40 mL/10 g of cheese.

Liquid–liquid extraction (LLE). For LLE, the raw extract should be alkalized to transform BA/PA into neutral form in which they are extractable to nonpolar organic phase, such as butanol, butanol/chloroform mixture, or diethylether [28]. Despite the different dissociation constants of particular amines and therefore the different optimum pH for extraction of each of them, it is necessary to maintain and strictly control one fixed pH value during extraction to reach reproducible recovery for all amines [29].

Solid-phase extraction (SPE). Typically, the C18 sorbents are used for the extract purification [21,22]. It is necessary to adjust the pH of a solution to alkaline range (e.g., to 11.0 with NH_4OH) to reach the neutral form of amines and to improve the amine retention. Subsequently, the acidified solution (e.g., pH 3.0 using formic acid) is applied for ionization of amines and their quantitative elution from the SPE column.

Calbiani et al. [30] introduced the method of matrix solid-phase dispersion (MSPD) for the determination of BA. MSPD is a relatively new extraction technique suitable for solid samples, which combines homogenization, analyte extraction, and purification in one step. An aliquot of the sample is placed into a mortar and pretreated C18 or CN-silica sorbent is added and blended. The homogenized sample is introduced into a cartridge, and MSPD column is eluted using formic-acid aqueous solution/methanol.

The recovery of amines extraction can be significantly enhanced by using surfactants [31].

41.4.4 Derivatization

Chromatographic methods with UV/Vis or fluorometric detection are generally used (apart from electrophoretic methods) for the determination of amines (see Sections 41.5 and 41.6). The absence of chromophore or fluorophore group in BA/PA molecules (except tyramine and 2-phenylethylamine) has led to the need for the insertion of this group into the amine molecule using a derivatization step.

The applicable derivatization agents are as follows: dansyl chloride (DCl); dabsyl chloride; *o*-phthaldialdehyde (OPA); derivatives of fluorescein (dichlorotriazinylamino-fluorescein); benzoyl chloride; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; 9-fluorenylmethyl-chloroformate (9-fluorenylmethyloxycarbonyl chloride, FMOC); phenylisothiocyanate [21]; 4-fluor-7-nitro-2,1,3-benzoxadiazole [32]; and 3-(2-furoyl)quinoline-2-carboxaldehyde [33]. Other derivatization agents used specifically in capillary electrophoresis are listed in the review by Oguri [34].

The difference in the use of the two most frequently applied derivatization agents, DCl and OPA, is as follows: dansyl derivatives allow rapid separation in combination with the UV detection (at 254 nm), while OPA derivatives require longer separation time and use fluorometric detection.

1-Dimethylaminonaphtalene-5-sulfonyl chloride (DCl) is the most common derivatization agent for amines determination in dairy food samples, especially cheeses [18,26]. It is the most widely used reagent for precolumn derivatization of amines prior to HPLC separation. However, some limitations in the use of DCl are its light sensitivity and limited stability.

The derivatization step is usually performed using DCl dissolved in acetone under slightly alkaline conditions ($NaHCO_3$ solution) and at a higher temperature (typically 40°C–60°C) for 20–60 min. Lower temperature (20°C) and shorter derivatization time (20 min) leads to poor reproducibility and derivatization recovery.

Dansyl derivatives are detected in the UV region of the spectra (at 254 nm).

The main limitation of OPA application is the fact that the derivatization is insufficient in the case of amines with secondary amino groups (spermidine, spermine). On the other hand, OPA was successfully applied as a derivatization agent for the determination of histamine, tyramine, tryptamine, putrescine, 2-phenylethylamine, and cadaverine in Dutch-type hard cheese [18]. The online derivatization with OPA can also be performed during capillary electrophoretic determination of amines [35].

The OPA derivatives of BA are usually quantified using a fluorometric detector. The excitation and emission wavelength for the detection of amine derivatives is 330 and 440 nm, respectively.

9-Fluorenylmethyloxycarbonyl chloride is a derivatization agent for both amino acids and PA with secondary amino groups, spermine, and spermidine. The derivatives are stable at room temperature and in acidic conditions. The major advantage is a possibility to detect very low concentrations of the analytes. The derivatization is performed in sodium hydrogen carbonate/sodium carbonate buffer (pH = 10.2) at 40°C for 10 min. The reaction is completed by the addition of concentrated hydrochloric acid, and the derivatives can be stored at 4°C until its use in the analysis. Fluorescence detection with excitation and emission wavelength 262 and 615 nm, respectively, is applied. The method has been used for the determination of putrescine, cadaverine, spermine, spermidine, and other amines in biological fluids [36], but can also be applied for dairy foods.

Dabsyl chloride (4-dimethylaminoazobenzene-4′-sulfonyl chloride) was used by Bockhardt et al. [37] as a derivatization agent in connection with reversed-phase HPLC determination of BA in cheeses. Dabsyl chloride reagent (12.4 mmol L^{-1}) was prepared by dissolving 40 mg of dabsyl chloride in 10 mL of acetone. An automated precolumn device was used for the derivatization performed at 70°C for 15 min.

When the determination of both amino acids and amines with primary and secondary amino groups is required, the two-step derivatization can be performed using a combination of OPA, ethanethiol, and 9-fluorenylmethyloxycarbonyl chloride. The primary amino groups are derivatized by OPA in the first step; while the derivatization of secondary amino groups by FMOC is followed in the second step. Ornithine, lysine, putrescine, cadaverine, 1,7-diaminoheptane, spermidine, and spermine were determined by this approach by Körös et al. [38].

For the sake of completeness, one of the less frequently applied derivatization agents, 4-chloro-7-nitrobenzofurazan, was used for the determination of tyramine in cheese [39]. The derivatized product was detected at 458 nm using an UV detector.

41.4.5 Separation

Önal provided a review of the current methods for BA/PA determination in various foods, including dairy foods [40]. The analytical methods can be divided into screening, semiquantitative, and quantitative methods. Thin-layer chromatography is often used for screening or semiquantitative determination (densitometric quantification) of amines. Furthermore, chromatographic (gas chromatography [GC]; liquid chromatography equipped with various types of detectors, including mass spectrometric (MS) detection; ion-exchange chromatography) and electromigration (capillary electrophoresis, capillary electrochromatography, micellar electrokinetic chromatography with optical or contactless conductivity detectors) methods have been applied for more precise determination of BA/PA in dairy foods.

Thin-layer chromatography is a simple and fast separation method used for amines determination and semiquantification. The quantification is performed by densitometers. Owing to the absence of a chromophore in most amine molecules, it is necessary to use a sorbent with UV/Vis absorption properties or to derivatize the analyte [41].

GC is rarely used for the analysis of complex BA/PA mixtures. The volatility of amines is relatively low (GC is a separation method suitable for the determination of volatile analytes), and their direct determination by GC is complicated owing to the poor peak shapes (inherent tailing). To increase volatility, amines should be derivatized, e.g., with heptafluorobutyric anhydride [42].

Liquid chromatography of nonderivatized, precolumn, or postcolumn derivatized amines is the most frequently used separation method for amines determination in all types of dairy food samples. Precolumn derivatization directly influences the separation properties of the amines, while postcolumn derivatization allows only an increase in the sensitivity of the detection.

Reversed-phase HPLC is the most frequently applied method for the determination of derivatized BA. Both DCl [18,26] and OPA derivatives are less polar compounds than the original amines, and therefore, they can be easily separated using columns with nonpolar (reversed) stationary phase.

HPLC procedures were recently used for amines determination in cheeses (stationary phase, elution, mobile phase, flow rate [FR] in mL min^{-1}): Phenomenex Luna RP-18, isocratic elution, methanol/H_2O 70/30, FR 1 [39]; Luna C18, gradient elution, 0.1% TFA/methanol, FR 0.2 [30]; reversed-phase Kromasil KR 100-5 C18, acetonitrile/H_2O, FR 0.8 [20]; Zorbax Eclipse XDB C18, gradient elution, H_2O/acetonitrile, FR 0.8 [18].

Micellar liquid chromatography (MIC), an alternative to a conventional HPLC, uses a mobile phase with surface-active compounds. The application of micellar environment reduces the requirements for sample preparation. Moreover, it improves the chromatographic parameters, such as peak resolution and sensitivity of UV detection in determination of aromatic BA, owing to enhancement of benzene-ring absorption.

However, the problematical part of this method is the impossibility to use a MS detector owing to the very low ion production in ion source in the presence of surfactants, and consequently, low sensitivity of mass detection. MIC with electrochemical detection at 0.8 V with mobile phase of 0.15 M sodium dodecyl sulfate and 5% 1-propanol at pH 3 was used for the determination of tryptamine and tyramine in wine samples, but it can also be successfully applied for the determination of these BA in other food matrices, including dairy products [43].

Owing to the polar properties of BA, hydrophilic interaction liquid chromatography (HILIC) can be successfully used for their determination. The chromatographic column contains a polar stationary phase, which can interact with polar compounds, including BA that are protonized under acidic conditions. Using this approach, Gianotti et al. [22] determined cadaverine, histamine, spermidine, spermine, tryptamine, tyramine, and putrescine in Castemagno cheese.

The separation principle of ion chromatography (IC) is based on cation (or anion) exchange on the stationary phase. Because BA are present as cations in acidic conditions, the derivatization step can be omitted. The disadvantage of IC is strong retention of amines on a column owing to the strong hydrophobic interactions, with a consequence of long retention times and poor peak shapes. Traditionally used conductometric detection is not suitable, owing to the high content of acid in a mobile phase; and hence, amperometric detection should be preferred for amines determination.

Standara et al. [44] separated histamine, tyramine, putrescine, cadaverine, tryptamine, agmatine, spermidine, and spermine (after extraction from cheeses by trichloroacetic acid) using the ion-exchanger column and automatic amino acid analyzer.

Weak acidic ion exchangers have recently been developed, which minimize the hydrophobic interaction between amines and stationary phase, and mobile phase with better compatibility with conductometric detection can be used [45].

In contrast to the chromatographic methods, where the flow of the mobile phase is generated by a pump, the driving force in electrophoretic methods is electroosmosis. The wall of silica capillary contains dissociable siloxane groups that charge the wall negatively. The negative charge is compensated by ions from the solution, and when the electric potential is applied, the liquid moves as bulk to the oppositely charged electrode. The value and polarity of capillary surface charge can be modified by surface-active compounds, such as tensides.

Sun et al. [46] used capillary electrophoresis with pulsed amperometric detection for the determination of BA in milk. Furthermore, capillary zone electrophoresis, in combination with conductometric detection, was applied by Kvasnička et al. [24].

Oguri et al. [47] modified the above-mentioned method, capillary electrochromatography, as follows: they first formed a frit inside a capillary and then filled the rest of this capillary with the C18 stationary phase. This arrangement allowed separation of histamine, serotonine, tyramine, putrescine, and cadaverine as OPA derivatives in 23 min. In another modification, micellar electrokinetic chromatography, applied by Křížek and Pelikánová [48], an uncoated fused-silica capillary column was used for the separation of putrescine, cadaverine, spermidine, spermine, tryptamine, histamine, and tyramine as *N*-substituted benzamides.

41.4.6 Detection

The most frequently used detection method for BA/PA is a measurement of absorbance in the UV range. The majority of amines do not contain chromophores allowing the detection by absorption in the UV/Vis area. Therefore, derivatization is necessary for introducing a chromophore into the amine molecule (for the list of applicable derivatization agents, see Section 41.4). Similarly, fluorometric detection is more selective and sensitive than the UV/Vis one.

As far as determination of BA/PA in dairy foods (cheeses) is concerned, the following combinations of derivatization/UV detection wavelength were recently applied: 4-chloro-7-nitrobenzofuran/458 nm [39]; DCl/254 nm [18,20].

Currently MS is being widely applied in chromatography of BA/PA. Amines can be detected as positively charged ions, either as derivatives (precolumn derivatization with DCl) or without previous derivatization. However, a disadvantage of the MS detection is the high cost of instrumentation.

Gosetti et al. [21] used electrospray ionization-tandem mass spectrometry (ESI-MS/MS) for the determination of amines in the three typical semihard Italian cheeses. Amines were detected in a positive-ion mode (ESI^+) in the following parent → secondary ion mass/charge (*m/z*) ratios: cadaverine 103 → 86; histamine 112 → 95, 112 → 68; spermidine 146 → 129, 146 → 112; spermine 203 → 129, 203 → 112, 203 → 84; tryptamine 161 → 144, 161 → 117; and tyramine 138 → 121, 138 → 93. Similarly, Calbiani et al. [30] performed selected reaction-monitoring analyses of histamine, tyramine, and 2-phenylethylamine (extracted from cheese using the MSPD method, see Section 41.3) as follows: *m/z* 112 → 68 and 112 → 95, *m/z* 138 → 103 and 138 → 121, and *m/z* 122 → 77 and 122 → 130, respectively.

Finally, a possibility of detection of total BA content using an amperometric biosensor with commercial diamino oxidase (from porcine kidney) can be mentioned [49].

Limit of detection for individual BA and PA in a standard solution is usually in the range from 0.2 to 0.45 $\mu g\ L^{-1}$ (ion-pair chromatography using reversed-phase column with postcolumn OPA

derivatization and fluorometric detection [27]) to 5.1–35.0 μg L^{-1} (HPLC followed by tandem MS detection [21]). The detection limits for amines extracted from cheeses vary between 0.05 and 0.25 mg kg^{-1} (MSPD followed by LC–electrospray MS/MS [30]) and 0.7–1.5 mg kg^{-1} [27].

41.4.7 Example of a Protocol

Sample. One of the objectives of the analysis was to evaluate the amine distribution within the Dutch-type hard or semihard cheese produced from pasteurized milk [12,18].

Laboratory equipment and apparatuses:

1. Liquid chromatograph HP 1100 (Agilent Technologies, Wilmington, DE) consisting of a quaternary pump (G1311A), vacuum degasser (G1322A), automatic sampler (G1313A), and a photometric UV/Vis detector with the variable wavelength (G1314A).
2. Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, particle size of 5 μm; Agilent Technologies, Wilmington, DE).
3. Guard column Meta Guard ODS-2 (30 mm × 4.6 mm, particle size of 5 μm; MetaChem Technologies, Torrance, CA).
4. Disintegrator Heidolph Diax 900 (Heidolph Instruments, Germany).
5. Centrifuge Hettich Universal 32R (Hettich, Germany).
6. Minishaker MS2 IKA (IKA Werke GmbH, Staufen, Germany).
7. Thermostat EVATERM for 25 vials, volume of 4 mL (Labicom, Olomouc, Czech Republic).
8. Compressed nitrogen container.
9. Nylon membrane filter, 13 mm, 0.45 μm (Chromatography Research Supplies, Addison, TX).

Reagents

1. Internal standard: dissolve 100 mg of 1,7-diaminoheptane (Sigma-Aldrich, Prague, Czech Republic) in 100 mL of deionized water (concentration 1 mg mL^{-1}).
2. Amines standard stock solution: prepare the solution of the mixed standard of all BA and PA to be analyzed: dissolve 100 mg of each amine (use respective hydrochlorides as standards, Sigma-Aldrich, Prague, Czech Republic) in 100 mL of deionized water (concentration of each amine standard, 1 mg mL^{-1}).
3. Mixed working standard solution: mix 0.5 mL of amines standard stock solution with 0.5 mL of internal standard solution, and adjust the volume exactly to 50 mL; the final concentration of BA is 10 μg mL^{-1}.
4. Extraction agent: 0.1 M HCl; dissolve 3.5 mL of 35% HCl in 1 L of deionized water.
5. Derivatization agent: dissolve 5 mg of DCl (5-dimethylaminonaphthalene-1-sulfonyl chloride) in 1 mL of 2-propanone (Sigma-Aldrich, St. Louis, MO).
6. Na_2CO_3 (saturated solution; pH adjusted to 11.2).
7. 10 mM ammonia solution: dissolve 1.48 mL of 26% ammonia solution in 1 L of deionized water.
8. Diethylether (p.a., Sigma-Aldrich, Prague, Czech Republic).
9. Acetonitrile (ACN; HPLC gradient grade, Sigma-Aldrich, Prague, Czech Republic).

Sample preparation. Cross-cut each block of the cheese in the middle, and divide it into two parts: edge part, 3 cm thick, and the rest is the core part. Analyze each part separately.

Extraction. Grate or cut the cheese sample into small pieces. Weigh the sample (10 g), place it in the 85 mL plastic centrifuge tube, add 20 mL of 0.1 M HCl and 0.5 mL of an internal standard solution, and extract the sample for 2 min using a disintegrator. Centrifuge the suspension at 755 × *g* for 10 min at 4°C to separate the solid part and fats. Filter the supernatant through the paper filter (Filtrak, No. 390) and extract the solid residue for the second time following the same procedure as mentioned earlier, for the quantitative extraction of BA. Make the combined extracts up to 50 mL with deionized water and filter the extracts through a disposable nylon membrane filter.

Derivatization. Mix 1 mL of an extract (or standard) with 0.5 mL of saturated Na_2CO_3 (pH adjusted to 11.2) in 4 mL amber vial for 1 min, add 1 mL of the derivatizing agent, and shake for 1 min in a shaker. Put the vials in a thermostat. Let the derivatization proceed for 1 h in dark at 40°C; shake the vial content repeatedly after 15 min. Let the mixture stay for 15 min after the derivatization reaction is completed, and subsequently, add 250 μL of 10 mM ammonia solution to remove the excess of unreacted DCl, shake for 1 min and wait for 30 min. Extract the amine derivatives by diethylether (3 × 1 mL). Evaporate the organic phase to dryness under nitrogen, and dissolve the solid residue in 0.5 mL of acetonitrile. Filter the solution through the 0.45 μm nylon membrane filter, and inject the aliquot onto the chromatographic column.

Separation. Carry out the separation of BA and PA by gradient elution with H_2O/ACN (time, 0–23 min: H_2O, 35%–0%; ACN, 65%–100%) on the Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, particle size of 5 μm) with the guard column Meta Guard ODS-2 (30 mm × 4.6 mm, particle size of 5 μm) at the FR 0.8 mL min^{-1}.

Identify the separated amines using a photometric UV/Vis detector at 254 nm by comparing the retention times of the particular BA/PA standards (an example of a chromatogram of the BA/PA standard and of amines separated from the typical Dutch-type semihard cheese after HCl extraction is presented in Figure 41.3). Express BA/PA concentrations after DCl derivatization in mg kg^{-1} of the original (fresh) cheese.

Data evaluation. Correct the concentration of BA in the sample (c_x; in mg kg^{-1}) according to the concentration of internal standard, based on the equation:

$$c_x = c_{IS} \times A_x / A_{IS} \times RF_x,$$

where

c_{IS} is the concentration of an internal standard (in mg kg^{-1})
A_{IS} is the peak area of the internal standard (in area units)
A_x is the peak area of the BA/PA (in area units)

RF_x is a response factor of the amine:

$$RF_x = c_{xr} / c_{ISr} \times A_{ISr} / A_{xr},$$

where

c_{xr} is concentration of BA in the reference sample (mg kg^{-1})
c_{ISr} is the concentration of an internal standard added to the reference sample (mg kg^{-1})
A_{ISr} is the peak area of the internal standard in the reference sample (in area units)
A_{xr} is the peak area of the BA in the reference sample (in area units)

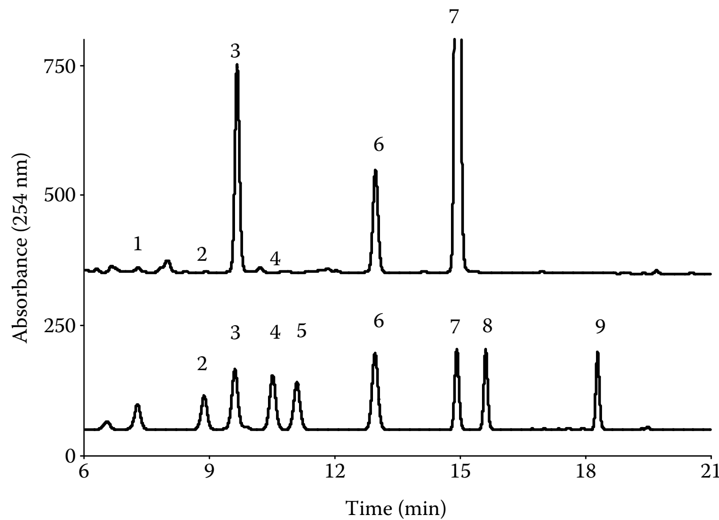

Figure 41.3 Separation of BA and PA by gradient elution with H_2O/acetonitrile on the Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, particle size 5 μm) at the FR 0.8 mL min^{-1} using photometric UV/Vis detector at 254 nm; bottom: standard mixture, top: HCl extract of the typical Dutch-type semihard cheese; 1, tryptamine; 2, 2-phenylethylamine; 3, putrescine; 4, cadaverine; 5, histamine; 6, internal standard (1,7-diaminoheptane); 7, tyramine; 8, spermidine; 9, spermine (for a better comparison, both the dependences are put in a single time-absorbance plot; therefore, the absolute values on the absorbance axis do not correspond, and only the values relative to the respective baseline are relevant).

Evaluate the repeatability of the analytical process (expressed as a RSD), by injecting a mixture of the BA/PA standards after derivatization for 10 times and injecting five extracts of the selected cheese sample with a low BA/PA content after derivatization, respectively.

Evaluate the recoveries by measuring a cheese sample five times with added mixture of BA/PA standards with the concentration level of 2 mg kg^{-1}. Calculate the recovery (R) as

$$\%\ R = [(\mathrm{CF} - \mathrm{CU})/\mathrm{CA}] \times 100,$$

where

CU is the concentration in the original sample

CA is the concentration of the added analyte

CF is the concentration in the spiked sample (all concentrations in mg kg^{-1})

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Publikace II

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ORIGINAL PAPER

Some factors influencing biogenic amines and polyamines content in Dutch-type semi-hard cheese

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Abstract Biogenic amines (BA; histamine, tyramine, tryptamine, phenylethylamine, cadaverine) and polyamines (PA; putrescine, spermidine, spermine) content was determined in Dutch-type semi-hard cheese (ripening 176 days) using an experimental design two producers × two starter cultures × two fat contents. Content of quantitatively most important BA (tyramine and histamine) and PA (putrescine) in the cheese samples at the end of ripening varied in the range 5–392 and 22–59, and 1–132 mg kg^{-1}, respectively. Time of ripening accounted for ($P < 0.01$) 67, 67 and 76% of the explained variability of histamine, tyramine, and putrescine content, respectively. Histamine, tyramine, and putrescine content in cheese (Y, mg kg^{-1}) increased ($P < 0.01$) linearly with increasing time of ripening (X, days): $Y = 0.24X–1.3$ ($R^2 = 0.28$), $Y = 0.69X–18.9$ ($R^2 = 0.24$) and $Y = 0.16X–1.3$ ($R^2 = 0.04$), respectively. Biogenic amines (sum of BA) were produced more than three times more rapidly with increasing time of ripening in the edge part of the cheese than in the core part. Both histamine and tyramine content in cheese was negatively correlated ($P < 0.01$) with counts of lactic acid bacteria (LAB) and counts of total anaerobes, respectively. Accordingly, only 3% of both LAB and enterococci (established tyramine producers) isolates were positive on tyrosine decarboxylase gene sequences using the PCR method.

Keywords Tyramine · Putrescine · Polyamines · Enterococci · Ripening cheese

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Introduction

Biogenic amines (BA; histamine, tyramine, tryptamine, phenylethylamine, cadaverine, putrescine) are the low-molecular organic bases formed mainly by decarboxylation of amino acids [1]. Ripening and/or long-term stored hard and semi-hard cheese, where the degradation of proteins during ripening leads to the accumulation of free amino acids, which can be converted into amines due to the activity of bacterial decarboxylases [2], is the next (after fish) most commonly implicated food item associated with BA poisoning [3].

Polyamines (putrescine, spermidine, and spermine, traditionally constituents of the BA family) are currently classified as a distinct group because they can be formed also by an alternative metabolic pathway ("deureation" instead of direct decarboxylation, with consequent incorporation of the aminopropyl groups originating from methionine; Fig. 1) and due to their specific physiological (and pathophysiological) importance (putrescine belongs to both biogenic amines and polyamines due to its dual roles) [4].

Quantitatively and toxicologically most important BA in ripening cheeses are tyramine and histamine. Tyramine is a potent vasoconstrictor; its higher levels in an organism can lead to hypertension and migraine and can induce brain haemorrhage and heart failure [5]. Histamine (also vasoactive substance) can cause urticaria, hypotension, headache, flushing, and abdominal cramps [3]. Tyramine and histamine are broken down in the mammalian organism by the oxidative deamination catalyzed by monoamine oxidase (MAO; [6]). However, human detoxification mechanisms are insufficient in the following cases: too high intake in a diet; in the allergic individuals; in patients consuming drugs with an action of the MAO inhibitors (antiparkinsonian drugs and antidepressants).

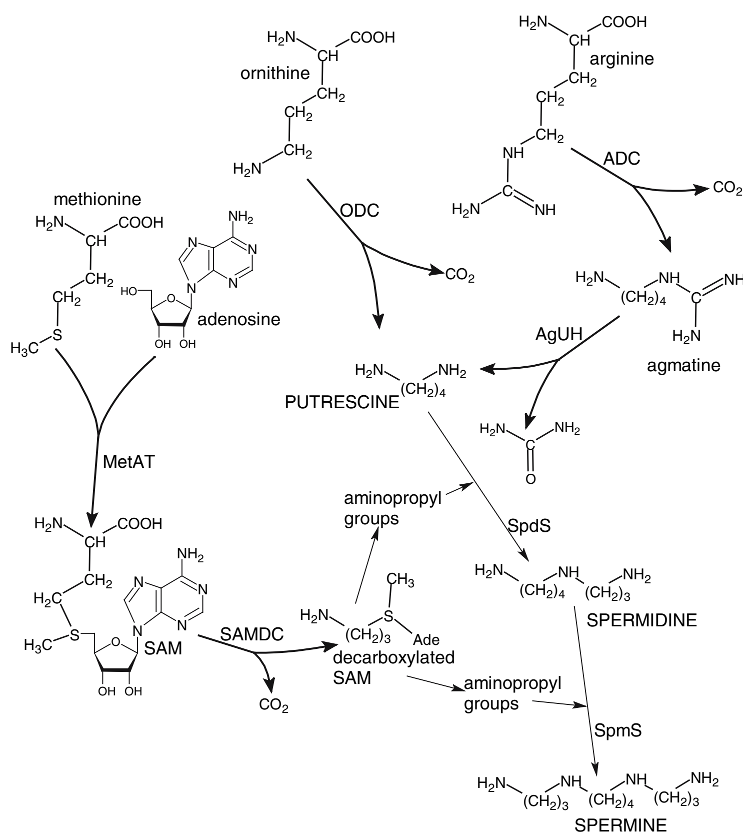

Fig. 1 Reported polyamine biosynthetic pathway in bacteria. *SAM* S-adenosylmethionine; *ODC* ornithine decarboxylase; *ADC* arginine decarboxylase; *AgUH* agmatine ureohydrolase (agmatinase); *SpdS* spermidine synthase; *SpmS* spermine synthase; *MetAT* methionine adenosyltransferase; *SAMDC* S-adenosylmethionine decarboxylase (adapted from Wolter et al. [8], and Ohnuma et al. [19])

Toxicological importance of polyamines is based on their ability to form stable carcinogenic N-nitroso compounds and to enhance the growth of chemically induced aberrant crypt foci in the intestine [7]. Polyamines are required for cell growth and proliferation, and are taken up by tumor cells; strict control of polyamine content in the diet of the cancer patients is therefore a matter of the utmost importance [4]. Putrescine stimulates tyrosine kinases and the expression of particular nuclear protooncogenes and is in this sense involved in cancer pathogenesis [8].

Following factors can influence biogenic amine content in ripening cheese: milk pasteurization (or lack of it [9]), general hygienic conditions of the cheese production [10], starter culture [11], time of ripening [10], and part of the cheese [9, 10].

The main factor affecting biogenic amine content in a cheese is presence of microorganisms with the decarboxylase activity; it can be non-starter lactic acid bacteria and other spontaneous microflora [11], and starter microorganisms [12]. However, it is difficult to find a straight correlation between microorganisms counts and BA content in cheese, because amine-producing abilities of different strains of various bacteria differ widely [2, 13]. Methods of molecular biology (PCR; [14, 15]) are currently most suitable for early detection of particular BA and polyamine producers in cheese.

The main objective of the present experiment was to evaluate the following factors presumably influencing biogenic amine and polyamine content in the ripening semi-hard Dutch-type cheese: producer (general hygienic conditions of the cheese production), fat content in the cheese, starter culture, part of the cheese (core, edge) and time of ripening.

Materials and methods

Material

Ripening Dutch-type semi-hard cheeses produced from pasteurized milk were used in the experiment. An experimental design 2 (two producers, designated R and H) × 2 (two starter cultures) × 2 (cheeses with different fat content, 30 and 45%, respectively) was used. Both dairies have been producing in the highest-ranking hygienic conditions. Each dairy produced four different batches of the cheese: two fat contents × two starter cultures. The composition of the starter cultures was as follows: a mixture of *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*; designated as “*Y*”); a

mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*; designated as "*L*". Starter cultures were dosed in an amount ensuring pH 5.2 in an instant when the cheese production was finished.

Within each batch, 21 blocks of cheese weighing approximately 13 kg were produced in each dairy; each block was vacuum-packed in a polyethylene casing and let to ripen in the ripening chamber at 10°C. Three randomly chosen blocks within each producer–starter–fat content set were taken at the production day (day 0) and consequently after 26, 54, 88, 119, 146 and 176 days of ripening.

After receiving at the laboratory (delivery at 4 °C), each block was cross-cut (in aseptic conditions) in the middle and divided into two parts: 3 cm edge part (designated as "E") and the rest core part ("C"). Biogenic amine and polyamine content and microbiological traits were measured in each part of the cheese.

Biogenic amine and polyamine determination

The sample (10 g) was weighed into the 85 mL test tube, 0.5 mL of an internal standard (1,7-diaminoheptane; concentration 1 mg mL^{-1}) was added and the sample was extracted for 2 min with 20 mL of 0.1 M hydrochloric acid (HCl) using a disintegrator Heidolph Diax 900 (Heidolph Instruments, Germany). Suspension was centrifuged at 755*g* 10 min at 4 °C (Hettich Universal 32R; Hettich, Germany). The supernatant was filtered through paper filter and the solid residue was extracted for the second time as above. The combined extracts were made up to 50 mL with deionized water and filtered through a disposable nylon membrane filter (13 mm, 0.45 µm; Chromatography Research Supplies, Addison, USA).

An extract was derivatized by dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride, DCl). The derivatizing agent was prepared by dissolving of 5 mg of dansyl chloride in 1 mL of acetone (Sigma-Aldrich, St. Louis, USA). The derivatization proceeded as follows: 1 mL of an extract (or standard) was mixed with 0.5 mL of saturated Na_2CO_3 (pH adjusted to 11.2), 1 mL of the derivatizing agent was added and the mixture was shaken for 1 min (MS2 Minishaker IKA; IKA Werke GmbH, Staufen, Germany). Derivatization proceeded for 1 h in dark at 40 °C, and the amine derivatives were extracted by diethylether (3 × 1 mL). The organic phase was evaporated to dryness under the nitrogen and the solid residue was dissolved in 0.5 mL of acetonitrile (ACN). The solution was filtered through the nylon membrane filter 0.45 µm and injected onto the chromatographic column.

Biogenic amines and polyamines were separated using a liquid chromatograph HP 1100 (Agilent Technologies, Wilmington, USA) consisting of a quarternary pump (G1311A), vacuum degasser (G1322A), automatic sampler (G1313A), and UV/VIS detector with the variable wavelength (G1314A). Separation after DCl derivatization was carried out by gradient elution with H_2O/ACN (time 0–23 min: H_2O 35–0%, ACN 65–100 %) on the Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, particle size 5 µm) with the guard column Meta Guard ODS-2 (30 mm × 4.6 mm, particle size 5 µm) at the flow rate 0.8 mL min^{-1} using photometric UV/VIS detector at 254 nm.

Concentration of biogenic amines and polyamines in the sample was corrected based on the method of internal standard according to Komprda [10].

The separated biogenic amines (histamine, tyramine, tryptamine, and cadaverine) and polyamines (putrescine, spermidine, and spermine) were identified by comparison of the retention times of the particular amine standards (all the amine standards were used as the respective hydrochlorides; supplied by Sigma-Aldrich), and their concentrations after DCl derivatization were expressed in mg kg^{-1} of original (fresh) cheese.

Microbiological analyses

Immediately after receiving at the laboratory 10 g of the cheese were taken aseptically from the slice, homogenized in the stomacher with 90 mL of Ringer solution (Noack, Austria) preheated to 45 °C, and serial decimal dilutions were prepared. The following groups of microorganisms were determined: total anaerobic counts (TAN) on Plate Count Agar (PCA; Biokar Diagnostics, France) after 72 h at 30 °C (samples were incubated in an anaerostat using generator and indicator of anaerobic conditions); lactic acid bacteria (LAB) on De Man-Rogosa-Sharpe medium (MRS; Biokar; 1 mL of an inoculum was poured over by the MRS medium, agar was let to fix and the second layer of MRS was applied in order to obtain microaerophilic conditions) after 72 h at 30 °C; enterococci on Slanetz-Bartley medium enriched with triphenyltetrazolium chloride (TTC; Noack) after 48 h at 37 °C.

Screening of the histamine- and tyramine-forming microorganisms

Bacteria within the above groups were tested using cheese samples from ripening day 26. An aliquot (square root) of the total amount of countable bacterial colonies grown on the dish within each group of bacteria was purified (by the three times repeated consecutive streaking) and inoculated into the liquid decarboxylase screening medium (DCM) according to [16], containing tyrosine (di-sodium salt) and histidine, respectively, in an amount of 1%. All isolates were inoculated in duplicate in DCM with and without (negative control) particular amino acid and incubated

4 days at 37 °C. Samples after incubation were centrifuged (at 750*g* 10 min at 4 °C; Hettich Universal 32R; Hettich, Germany), 1 mL of supernatant was mixed with 1 mL of 0.1 M HCl and 20 μL of internal standard 1,7-diaminoheptane (Sigma-Aldrich), the solution was vortexed on mini-shaker (MS2 Minishaker, IKA Werke, Germany) and centrifuged again. The supernatant was filtered through nylon membrane filter (13 mm, 0.45 μm; Chromatography Research Supplies, Addison, USA) and tyramine and histamine, respectively (if formed) was determined as previously mentioned.

Samples of original purified colonies that were tested positive in DCM and in which particular biogenic amine was consequently confirmed by HPLC, were used for DNA isolation and subsequent PCR analysis. DNA was isolated (including all standard DNA manipulations) according to [17]. PCR was carried out in the final volume of 25 μL containing approximately 10 ng of genomic DNA, 1U *Taq* DNA polymerase, an appropriate amount of Qiagen HotStar Master Mix (Qiagen, Hilden, Germany) and 10 pmol of either primers TD5/TD2 (for tyrosine decarboxylase gene, *tyrdc*) or primers HDC3/HDC4 (for histamine decarboxylase gene, *hdc*; [14]). DNA was completely denaturated by incubation at 95 °C for 15 min and consequently amplified by 30 cycles of denaturation at 95 °C for 45 s, primer annealing at 52 °C for 45 s, and elongation at 72 °C for 75 s using thermal cycler PTC-150 HB (MJ Research, Waltham, USA). In the last amplification cycle, the samples were incubated at 72 °C for 10 min for complete elongation of the final PCR products. After PCR, amplified DNA fragments were separated by the agarose gel electrophoresis (1% agarose; 5 V cm^{-1}; 60 min) in 1 × TBE buffer and visualized by ethidium bromide staining.

Statistical evaluation

All chemical and microbiological traits were measured in duplicate in each part (core, edge) of each block within each batch of the cheese. Means of these two measurements, representing a particular part of the block, were used in statistical evaluation.

The Unistat package, version 4.53 (Unistat Ltd., London, England) was used for calculation of the basic statistical characteristics, regressions (including significance testing of the linear and quadratic terms, respectively), differences between the sets of cheeses in biogenic amine and polyamine content and microbial counts, respectively (one-way classification of the variance-ratio test, including Duncan's multiple range test), and for calculation of the correlations between particular traits. Percentage of total variability in biogenic amines content, polyamines content and microbial counts, explained by the producer, starter culture, fat content, part of the cheese and time of ripening, respectively, was calculated using multiple-way classification of the variance-ratio test.

The linear regressions equality test [18] was used for the comparison of the particular dependences on the time of ripening. The independent variable (*X*, time of ripening) and the number of measurements being the same for both compared regressions, the decisive criterion (*F*-value) was calculated according to the simplified formula:

$$F = \frac{n(y_1^2 + y_2^2) - \frac{(y_1+y_2)^2}{2} + \frac{s_x(b_1^2+b_2^2-2b_1b_2)}{2}}{s_{y1}^2 + s_{y2}^2}$$

where y_1 and y_2 are the means of compared dependent variables, s^2_{y1} and s^2_{y2} their variances, b_1 and b_2 corresponding coefficients of the linear term, and s_x is a standard deviation of the mean of the independent variable (time of ripening).

Results and discussion

Quantitatively most important biogenic amines and polyamines were tyramine (5–392 mg kg^{-1}) and histamine (22–59 mg kg^{-1}), and putrescine (1–132 mg kg^{-1}), respectively. Effect of the tested variability factors (producer, starter culture, fat content in the cheese, part of the cheese, and time of ripening) on the content of these amines (and counts of the particular groups of microorganisms) is shown in Table 1. Time of ripening was the most important factor, which accounted for most of the explained variability of all traits ($P < 0.01$), except counts of enterococci. Different distribution of tyramine (and consequently of the sum of BA), and also of the counts of total anaerobes and enterococci, between the core and edge part of the cheese is also apparent from Table 1.

Starter culture and especially fat content had only limited effect on the production of biogenic amines and growth of microorganisms. Important effect of the producer was found in the case of histamine and putrescine content (but not in the case of tyramine and sum of BA content), and counts of all tested microorganisms.

No correlation ($P > 0.05$) between putrescine content and any of the microbial traits was established in the present experiment. On the other hand, we found highly significant negative relationships (calculated for the case when all samples irrespective of producer, fat content, starter culture, part of the cheese, and ripening time were taken as one set) between histamine content and LAB and TAN (r = –0.42 and –0.36, respectively; $P < 0.001$), and between tyramine content and LAB and TAN (r = –0.18 and –0.18, respectively; $P < 0.01$). No correlation between any biogenic amine or polyamine content on the one hand and enterococci counts on the other hand was found. In the

Table 1 Comparison of factors affecting content of quantitatively most important biogenic amines and polyamines and counts of microorganisms in Dutch-type semi-hard cheese (multiple-way analysis of the variance ratio test)

Trait	Variability factor (% of explained variability, significance[a])					Explained variability in % of total variability
	Producer	Fat content	Starter culture	Part of the cheese	Time of ripening	
Histamine	21.4 ***	1.0 NS	6.0 **	4.7 NS	66.6 ***	48.7
Tyramine	0.5 NS	0.5 NS	4.0 **	28.0 ***	67.0 ***	40.4
Σ of BA[b]	0.0 NS	0.4 NS	3.7 **	22.8 ***	73.0 ***	45.3
Putrescine	18.5 *	1.7 NS	3.6 NS	0.0 NS	76.1 **	9.2
Σ of PA[c]	11.1 NS	9.3 NS	3.0 NS	3.2 NS	73.5 **	7.3
TAN[d]	10.7 ***	3.8 **	1.9 *	27.2 ***	56.4 ***	39.4
LAB[e]	7.1 ***	2.1 *	2.5 *	9.1***	79.3 ***	44.6
Enterococci	45.5 ***	4.1 NS	10.1 **	29.2 ***	11.1 NS	17.8

[a] NS not significant, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

[b] Sum of biogenic amines (histamine + tyramine + tryptamine + cadaverine)

[c] Sum of polyamines (putrescine + spermidine + spermine)

[d] Total anaerobic counts

[e] Lactic acid bacteria

case of polyamines, the lack of relationship can partially be explained by the fact that spermidine and spermine, which are not very probably formed by the microbial action in cheese but pass to the product from milk [4, 9], constituted in average 50% of polyamines.

The reason for an often-missing straight correlation between biogenic amines content and counts of associated microorganisms in particular foods [2] is the fact that biogenic amine decarboxylase activity is not a function of bacterial genera or species, but a function of particular strains within a given species.

Roig-Sagués et al. [11] identified the main tyramine-forming bacteria as enterococci and some LAB. In the present experiment, 17% of the LAB colonies tested (square root of all countable colonies), that is 123 isolates, were positive in the tyrosine-decarboxylase medium. However, tyramine formation was confirmed by HPLC only in 15 LAB isolates; this figure corresponded with 15 *tyrdc*-positive isolates using PCR, which was in average 3% of total LAB colonies. Following figures were obtained regarding enterococci: 48 tyrosine-DCM+ isolates (7% of total tested colonies), and 20 *tyrdc*+ isolates (3% of total tested colonies).

As far as histamine-forming bacteria are concerned, we failed both to find positive reaction in DCM and confirm histamine production in this medium by HPLC and detect corresponding *hdc*+ isolates in any sample in the present experiment.

We did not tried to test the cheese samples for the presence of bacterial putrescine producers based on the particular gene sequences using the PCR method; as follows from Fig. 1, finding of not only ornithine decarboxylase gene sequences [15], but also of gene sequences for arginine decarboxylase and agmatine ureohydrolase [19] would probably be necessary for a reliable screening. According to Ohnuma et al. [19], apart from production of putrescine by decarboxylation of ornithine (which proceeds in animals and bacteria), alternative pathway of putrescine biosynthesis (decarboxylation of arginine followed by hydrolysis of agmatine, which is usual in plants) can take place also in bacteria.

As follows from Table 1, contents of all quantitatively (and toxicologically) important amines, and counts of microorganisms (except of enterococci) changed significantly ($P < 0.01$) with the time of ripening. The generalized dependences (all samples irrespective of producer, fat content, starter culture, and part of the cheese were taken as a one set; $n = 336$) of tyramine, histamine, and putrescine content, respectively, on the time of ripening are presented in Fig. 2. Despite the fact that the difference between producers regarding dependences of tyramine content (Y, mg kg^{-1}) on the time of ripening (X, days) was not established (due to the high inner variability) using the linear regressions equality test ($F = 0.4$, $P > 0.05$), coefficient of the linear term was in the case of the producer R by 60% higher ($Y = 0.88X–31.4$, $R^2 = 0.30$, $P < 0.001$) in comparison with the producer H ($Y = 0.50X–6.3$, $R^2 = 0.18$, $P < 0.001$). Komprda et al. [10] found even sixfold difference in the value of the coefficient of the linear term when comparing Dutch-type cheeses from two producers (different from those evaluated in the present experiment) regarding dependence of tyramine content on the time of ripening.

Despite the fact, that a producer accounted for more than 20% of histamine content variability in the present exper-

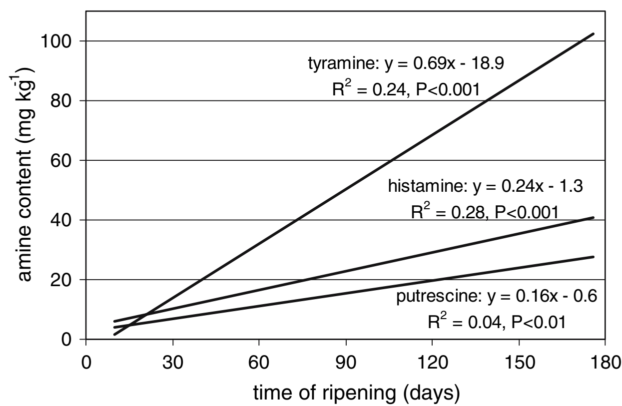

Fig. 2 Dependence of tyramine, histamine and putrescine content, respectively, in cheese on the time of ripening. All samples irrespective of producer, fat content, starter culture, and part of the cheese were taken as one set (*n* = 336)

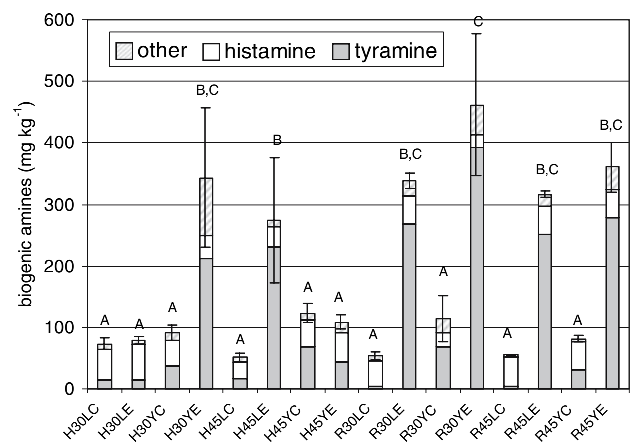

Fig. 3 Biogenic amine content at the end of ripening (176th day) in the core (C) and edge (E) part of cheeses from two dairies (R, H), produced with two different fat contents (30 and 45%) using two starter cultures (*Y*: a mixture of *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*; *L*: a mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, respectively). *A*, *B*, *C* means (*n* = 3) with different superscript differ significantly ($P < 0.01$)

iment (Table 1), the coefficient of the linear term was the same for each producer as far as comparison of the dependence of histamine content on the time of ripening is concerned. The results regarding the dependence of putrescine content on time of ripening were similar. The sum of all biogenic amines also only tended ($P > 0.05$) to increase more rapidly in the R-cheeses ($Y = 1.53X–30.9$, $R^2 = 0.33$, $P < 0.001$) than in H-cheeses ($Y = 0.95X + 7.7$, $R^2 = 0.25$, $P < 0.001$) in the present experiment (inclusion of the quadratic term was not significant, $P > 0.05$).

Comparison of all particular cheese samples (two producers × two starter cultures × two fat contents × two parts of the cheese) regarding content of the sum of all biogenic amines at the end of ripening (176 days) is shown in Fig. 3. Histamine was the most abundant biogenic amine in some cheeses (both H30L samples, and core samples of R30L, L45L, R45Y cheese; range 22–59 mg kg^{-1}). The upper limit is substantially higher (3.6 times) in comparison with our previous experiment with ripening Dutch-type cheese (two producers different from those in the present experiment [10]), but still much lower than in Swiss-type cheese stored 5 months at 5 °C in an experiment of Petridis and Steinhart (250 mg kg^{-1}; [20]).

However, tyramine was dominant BA in most cheese samples in the present experiment (5–392 mg kg^{-1}). In this case, the upper limit is comparable both with the data of Petridis and Steinhart ([20]; 320 mg kg^{-1}) and the results of our previous experiment ([10]; 3–310 mg kg^{-1}). Similarly to the results of the present experiment reported Roig-Sagués et al. [11] significantly higher amount of tyramine (mean value 10–1085 mg kg^{-1}) than histamine (0–494 mg kg^{-1}) in Spanish traditional cheeses from retail markets.

The toxicological limits for histamine and tyramine are difficult to establish due to the great differences between people regarding robustness of the detoxification system and possible synergistic effects of other biogenic amines. Therefore very different values have been published; 100 mg kg^{-1} of food for both histamine and tyramine [1] is one of more commonly suggested upper limits. From this viewpoint, nearly 40% of the cheese samples at the end of ripening in the present experiment should be approached with caution regarding tyramine content, but all samples were safe as far as histamine content is concerned (Fig. 3). In average represented tyramine and histamine 49 and 40%, respectively, of the sum of all biogenic amines in the present experiment.

The most conspicuous tendency apparent from Fig. 3 regards higher BA content in the edge samples in comparison with the core ones. This is in accordance with the data of Table 1 (a column regarding part of the cheese) and it is also illustrated by the particular dependences of the sum of BA (*Y*; mg kg^{-1}) on the time of ripening (*X*; days). The coefficient of the linear term of the regression depicting the edge part of the cheese ($Y = 1.65X–31.7$; $R^2 = 0.48$, $P < 0.001$) was more than three times higher in comparison with the core part ($Y = 0.48X–1.4$; $R^2 = 0.31$, $P < 0.001$). The dependences of tyramine content in the edge and core samples (*Y*, mg kg^{-1}) on the time of ripening (*X*, days; $Y = 1.19X–34.3$, $R^2 = 0.43$, $P < 0.001$ and $Y = 0.19X–3.5$, $R^2 = 0.17$, $P < 0.001$, respectively), were even statistically different (*F*-value = 21.6, $P < 0.05$). These results confirmed the previous findings regarding Dutch- [10] and Swiss-type [21] ripening cheese, that the

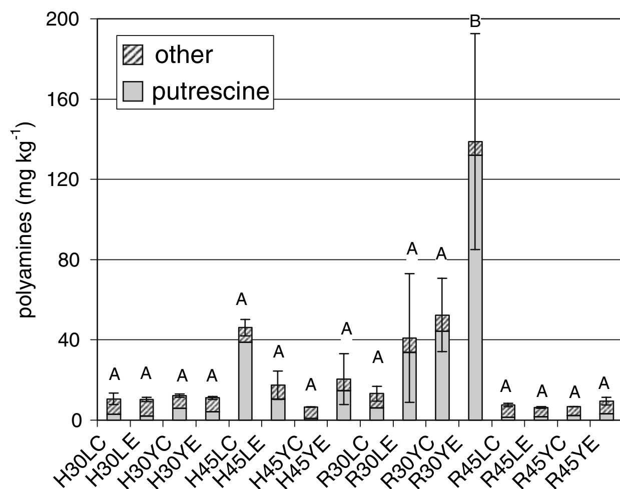

Fig. 4 Polyamines content at the end of ripening (176th day) in the core (C) and edge (E) part of cheeses from two dairies (R, H), produced with two different fat contents (30 and 45%) using two starter cultures (*Y*: a mixture of *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*; L: a mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, respectively). *A*, *B* means ($n = 3$) with different superscript differ significantly ($P < 0.01$)

bulk of tyramine content can be expected in the outer parts of the cheese. Still, tyramine distribution can be different in other types of cheese [9].

The higher ($P < 0.05$) rate of tyramine formation in the edge parts of cheese found in the present experiment can be related to a presumable contamination of cheeses with enterococci during production. Higher ($P < 0.05$) enterococci counts in the edge parts of the cheese (total mean of all E-samples 3.14 log cfu g^{-1}) in comparison with the core parts (2.36 log cfu g^{-1}) suggest contamination of cheeses from the environment of the production plant. Moreover, more massive enterococci ($P < 0.01$) contamination of cheeses from the producer R as compared to the producer H (total means of all R- and H-samples 3.18 and 1.95 log cfu g^{-1}, respectively) was found in the present experiment.

Polyamines content in cheeses at the end of ripening is presented in Fig. 4. The highest polyamine ($P < 0.01$) content was found in the R30YE sample (the mean 139 mg kg^{-1}), which also tended to have the highest biogenic amine content (461 mg kg^{-1}; Fig. 3). Putrescine constituted in average 50% of all polyamines. The mean (median; minimum–maximum) value for putrescine, spermidine and spermine was 19 (3; 1–132), 2 (2; 0–4), and 5 (5; 2–9) mg kg^{-1}, respectively, in the cheese samples after 176 days of ripening in the present experiment. For comparison, Novella-Rodrígues et al. [9] reported the corresponding median (minimum–maximum) values in hard-ripened Spanish cheeses from pasteurized milk to be 5.0 (0–612), 5.7 (0–43) and 1.6 (0–19) mg kg^{-1}, respectively. The mean of putrescine, spermidine, and spermine content in matured Norvegia cheese was 7, 0 (not detected) and 3 mg kg^{-1} in an experiment of Eliassen et al. [22].

The above values are difficult to assess from the viewpoint of food safety. According to Smith et al [23], toxicity of polyamines increases with molecular weight and charge. Similarly, spermine was the most toxic polyamine based on the subacute toxicity assay in rats according to Til et al. [5]. These authors found no-observed-adverse-effect level for putrescine, spermidine, and spermine to be 180, 83, and 19 mg kg^{-1} body weight day^{-1}, respectively. However, these values cannot be probably reached even in ripened cheeses, which ranks among the foods with the highest content of polyamines, mainly putrescine, according to the review by Kalač and Krausová [4]. Based on the various literature data estimated Eliassen et al. [22] daily polyamine intake to be 100–500 μmol (recalculated to the weight units, these figures correspond to 9–44 mg of putrescine). However, a derivation of dietary polyamine levels pertinent to their beneficial effects regarding growth and development of the digestive system and wound healing after surgery or injury on the one hand, and their harmful effects in cancer patients (where polyamine intake should be restricted) on the other hand, is still missing. In conclusion, an amount of polyamines found in the cheese samples in the present experiment cannot be properly assessed before further research elucidates both recommended dietary intake of polyamines (growth and development of the digestive system, wound healing) and the limit whose exceeding would have deleterious effects in cancer patients.

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Publikace III

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Tyramine production in Dutch-type semi-hard cheese from two different producers

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Abstract

Tyramine content and counts of lactic acid bacteria (LAB) and enterococci were measured (including tyrosine-decarboxylase activity testing, and testing of the presence of the tyrosine-decarboxylase gene sequence, *tyrdc*, by PCR) during ripening (0, 26, 54, 88, 119, 146, and 176 days) in the core (C)- and edge (E)-samples of Dutch-type semi-hard cheese produced from pasteurized milk by two dairies (R, H) with two levels of fat content (30 and 45%) using two different starter cultures (Y, L), respectively. Tyramine content (y, mg kg^{-1}) increased ($P<0.001$) with increasing time of ripening (x, days) in the cheeses of both producers (R: $y=0.88x-31.4$, $R^2=0.30$; H: $y=0.50x-6.3$, $R^2=0.18$), and its content was higher ($P<0.01$) in E-samples in comparison with C-samples. Time of ripening, part of the cheese and starter culture accounted for 67%, 28%, and 4% of explained variability of tyramine content in the cheese, respectively. After 26 days of ripening, using decarboxylase screening medium (DCM), tyrosine-decarboxylase positive LAB isolates constituted 7–27% and 6–32% of the square root of total countable colonies of LAB isolates of the producer R and H, respectively; tyrosine-decarboxylase positive enterococci were present only in R-cheeses (4–26% of the square root of total countable colonies). *Tyrdc* was confirmed only in 13% and 42% of the tyrosine-decarboxylase positive LAB and enterococci isolates, respectively (presumably due to the tendency of DCM to give false-positive results). *Lactobacillus curvatus* subsp. *curvatus* and *Enterococcus durans*, *Enterococcus faecalis*, and *Enterococcus casseliflavus* were identified as *tyrdc*-positive LAB and enterococci in the cheeses, respectively.
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Keywords: Tyramine; Tyrosine-decarboxylase; Tyrosine-decarboxylase gene; Lactic acid bacteria; Enterococci; PCR; Ripening cheese

1. Introduction

Tyramine, one of the toxicologically important biogenic amines (BA), is formed in foods by the action of tyrosine-decarboxylase produced by bacteria associated with the foods (Silla-Santos, 1996). Tyramine is broken down in the mammalian organism (to corresponding ketone, NH_3, and H_2O_2) in mitochondrion of neurons, hepatocytes and enterocytes (and other tissues) by the oxidative deamination catalyzed by monoamine oxidase (MAO; Morgan, 1998). However, the detoxification mechanisms in man are insufficient in the following cases: too high intake in a diet; in the allergic individuals; in patients consuming drugs with an action of the MAO inhibitors (anti-Parkinsonian drugs and anti-depressants inhibiting MAO-B and MAO-A, respectively). As a potent vasoconstrictor, tyramine can induce hypertension, migraine, brain haemorrhage and heart failure when present in higher concentration in an organism (Til et al., 1997). Toxicological limits are difficult to establish due to the great differences between people regarding robustness of the detoxification system. However, concentrations above 100 mg kg^{-1} of food are supposed to be deleterious (Silla-Santos, 1996), especially in the above-mentioned risky groups of consumers.

To the fermented foods, in which tyramine can be present in toxicologically relevant levels, belong fermented vegetables (sauerkraut; Špička et al., 2002), alcoholic beverages (Kalač et al., 2002), fermented dry sausages (Komprda et al., 2004), and especially ripened cheeses (after scombroid fish the second most important food-borne source of biogenic amines; Stratton et al., 1991).

Apart from the basic prerequisites for tyramine production (availability of free tyrosine, presence of microorganisms

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with active tyrosine-decarboxylases and conditions enabling growth of these microorganisms; Silla-Santos, 1996), the following factors can influence tyramine content in ripening cheese: milk pasteurization (or lack of it; Novella-Rodrígues et al., 2003), general hygienic conditions of the cheese production (Komprda et al., 2007), starter culture (Fernández-García et al., 2000), time of ripening (Komprda et al., 2007), and possibly fat content (Komprda et al., 2007). Contrary results were recently presented regarding tyramine distribution within the cheese: higher (Novella-Rodrígues et al., 2003) or lower (Komprda et al., 2007) tyramine concentration in the core parts of the cheese in comparison with the edge parts.

From the viewpoint of the food safety, it is important that only a fraction of strains within a given microbial genus or species has ability to decarboxylate tyrosine. That is the reason for an often missing straight correlation between tyramine content and counts of associated microorganisms in particular foods (Innocente and D'Agostin, 2002). Therefore, despite the known fact that tyramine-producing microorganisms belong predominantly to the starter lactic acid bacteria (LAB) (Fernández-García et al., 2000), non-starter LAB, and other spontaneous microflora, especially enterococci (Roig-Sagués et al., 2002), routine (i.e., only genus- or species-aimed) microbiological analysis is not suitable for early detection of particular tyramine-producers in cheese. Methods of molecular biology (PCR) are currently available to solve the task (Fernández et al., 2006; Coton et al., 2004; Coton and Coton, 2005).

There were two main objectives of the present experiment. First, to evaluate the following factors presumably influencing tyramine content in the ripening semi-hard Dutch-type cheese: producer (general hygienic conditions of the cheese production), fat content in the cheese (in our previous experiment, Komprda et al., 2007, we were not able to separate an effect of the fat content from that of a producer), starter culture, part of the cheese (core, edge) and time of ripening, and second, to verify the possibility to detect tyramine-producing bacteria in Dutch-type cheeses by molecular methods (PCR).

2. Materials and methods

2.1. Cheese-making

Dutch-type semi-hard cheeses produced from pasteurized milk were made for the experiment. An experimental design 2 (two producers, designated R and H) × 2 (two starter cultures) × 2 (cheeses with different fat content, 30% and 45%, respectively) was used. We have included fat content in the design because in our previous experiment (Komprda et al., 2007), the differences in tyramine content were found between the cheese with 30% fat produced by one dairy and the cheese with 45% fat produced by a second dairy, but an effect of fat content and producer, respectively, could not be distinguished. Both dairies participating in the present experiment met the highest hygienic standards. Each dairy produced four different batches of the cheese: two fat contents × two starter cultures. The composition of the starter cultures was as follows: *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*, and *Lactobacillus helveticus* designated as "Y"); *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* designated as "L". Y and L starters were chosen because they have been routinely in use in the plant H and R, respectively. Neither producer of a particular starter communicated exact proportion of each species in the culture. Counts of starter microorganisms in the cheese milk were approximately 8.8 and 8.2 log cfu mL^{-1} in the case of the producer H and R, respectively (a dosage differed between producers, but was roughly the same regarding both cultures used by a particular producer). However, the above figures are not exact target values in the cheese milk, because the decisive criterion was reaching pH-value 5.2 in an instant when the cheese production was finished, and more or less culture was added into the vat accordingly.

Within each batch, 21 blocks of cheese weighting approximately 13 kg were produced in each dairy; each block was vacuum-packed in a polyethylene casing and let to ripen in the ripening chamber at 10 °C. Three randomly chosen blocks within each producer–starter–fat content set were taken at the production day (day 0), and consequently after 26, 54, 88, 119, 146, and 176 days of ripening.

After being received at the laboratory (delivery at 4 °C), each block was cross-cut (in aseptic conditions) in the middle and divided into two parts: 3 cm edge part (designated as "E") and the rest core part ("C"). Tyramine content, microbiological counts and water activity were measured in each part of the cheese.

Water activity was measured using AW Sprint TH-500 apparatus (Novasina, Switzerland) at 24.5 °C, proximal composition and pH of the cheeses were not recorded.

2.2. Tyramine determination

The sample (10 g) was weighted into a 85 mL test tube, 0.5 mL of an internal standard (1,7-diaminoheptane; concentration 1 mg mL^{-1}) were added and the sample was extracted for 2 min with 20 mL of 0.1 M hydrochloric acid (HCl) using a disintegrator Heidolph Diax 900 (Heidolph Instruments, Germany). Suspension was centrifuged at 755 × *g* for 10 min at 4 °C (Hettich Universal 32R; Hettich, Germany). The supernatant was filtered through paper filter and the solid residue was extracted for the second time as above. The combined extracts were made up to 50 mL with deionized water and filtered through a disposable nylon membrane filter (13 mm, 0.45 μm; Chromatography Research Supplies, Addison, USA). The aliquot of an extract was precolumn derivatized (in the liquid chromatograph autosampler) by *o*-phthaldialdehyde (OPA) in borate

buffer (pH 9.5; Sigma-Aldrich) in the presence of 2-sulfonylethan-1-ol (Merck, Germany); the same procedure was applied on tyramine standard (tyramine hydrochloride; Sigma-Aldrich).

Tyramine was separated using a liquid chromatograph HP 1100 (Agilent Technologies, USA) consisting of a quarternary pump (G1311A), vacuum degasser (G1322A), automatic sampler (G1313A), and UV–vis detector with the variable wavelength (G1314A). Separation after OPA derivatization was carried out by gradient elution with H_2O/ACN (time, 0–23 min; H_2O, 35–0%; ACN, 65–100%) on the Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, particle size 5 μm) with the guard column Meta Guard ODS-2 (30 mm × 4.6 mm, particle size 5 μm) at the flow rate 0.8 mL min^{-1} using photometric UV–vis detector at 254 nm. Because of the several steps of the sample preparation, tyramine concentration in the sample was corrected based on the method of internal standard (Komprda et al., 2007).

The separated tyramine was identified by comparison of the retention time with that of the standard (tyramine hydrochloride, Sigma-Aldrich), and its concentration after OPA derivatization was expressed in mg kg^{-1} of cheese.

2.3. Microbiological counts

Immediately after being received at the laboratory (Department of Food Technology, Mendel University Brno), 10 g of the cheese were taken aseptically, homogenized in a stomacher with 90 mL of Ringer solution (Noack, Austria) preheated to 45 °C, and serial decimal dilutions were prepared. The following groups of microorganisms were determined: total anaerobic counts (TAN) on Plate Count Agar (PCA; Biokar Diagnostics, France) after 72 h at 30 °C (samples were incubated in an anaerostat using generator and indicator of anaerobic conditions); LAB on De Man–Rogosa–Sharpe medium (MRS; Biokar; 1 mL of an inoculum was poured over by the MRS medium, agar was let to fix and the second layer of MRS was applied in order to obtain microaerophilic conditions) after 72 h at 30 °C; *Enterobacteriaceae* on Endo agar (Noack) after 24 h at 37 °C. Apart from LAB determination as a whole group, enterococci (which belong also to LAB) were enumerated separately on Slanetz–Bartley medium enriched with triphenyltetrazolium chloride (TTC; Noack) after 48 h at 37 °C.

2.4. Screening of microorganisms with tyrosine-decarboxylase activity and confirmation of tyramine formation

Presence in cheeses of microorganisms with presumptive tyrosine-decarboxylase activity was tested within the groups of LAB, enterococci and *Enterobacteriaceae*. Cheese samples after 26 days of ripening were used. A number of colonies equal to the square root of the total number recorded in countable Petri dishes of each group of bacteria assayed was purified (i.e., colonies were three times repeatedly streaked) and inoculated into the liquid decarboxylase screening medium (DCM) according to Bover-Cid and Holzapfel (1999), containing tyrosine disodium salt (1%). All isolates were inoculated in duplicate in DCM with and without (negative control) tyrosine and incubated 4 days at 37 °C. DCM positive samples (color transition to violet) after incubation were centrifuged (at 755 × *g* 10 min at 4 °C; Hettich Universal 32R; Hettich, Germany), 1 mL of supernatant was mixed with 1 mL of 0.1 M HCl and 20 μL of internal standard 1,7-diaminoheptane (Sigma-Aldrich), the solution was vortexed on mini-shaker (MS2 Minishaker, IKA Werke, Germany) and centrifuged again. The supernatant was filtered through nylon membrane filter (13 mm, 0.45 μm; Chromatography Research Supplies, Addison, USA) and tyramine (if formed) was determined as previously mentioned.

2.5. Isolation of DNA and PCR screening of the tyrosine-decarboxylase (tyrdc) gene fragment

Samples of all original purified colonies within the groups of LAB and enterococci that were tested positive in DCM and in which tyramine was consequently confirmed by HPLC were used for DNA isolation and subsequent PCR analysis. DNA was isolated (including all standard DNA manipulations) according to Sambrook and Russel (2001). PCR was carried out in the final volume of 25 μL containing approximately 10 ng of genomic DNA, 10 pmol of primers TD5 (5′-CAAATGGAAGAAGAAGTAGG-3′)/TD2 (5′-ACATAGTCAACCATRTTGAA-3′; Coton et al., 2004), 1U *Taq* DNA polymerase and an appropriate amount of Qiagen HotStar Master Mix (Qiagen, Hilden, Germany). DNA was completely denatured by incubation at 94 °C for 15 min and subsequently amplified by 30 cycles of denaturation at 95 °C for 45 s, primer annealing at 52 °C for 45 s, and elongation at 72 °C for 75 s using thermal cycler PTC-150HB (MJ Research, Waltham, USA). In the last amplification cycle, the samples were incubated at 72 °C for 10 min for complete elongation of final PCR products. After PCR, amplified DNA fragments were separated by agarose gel electrophoresis (1% agarose; 5 V cm^{-1}; 60 min) in 1 × TBE buffer and visualized by ethidium bromide staining.

2.6. Specification of tyrdc-positive isolates

The original colonies, in which the sequence for tyrosine-decarboxylase gene was identified by PCR, were further specified using morphological traits and biochemical assays. The isolates within the genus *Enterococcus* were identified using Gram staining, optical microscopy, growth on Columbia blood agar (Merck, Germany), catalase production test, methyl-α-D-glukopyranosid utilization test (MGP), pyruvate kinase production test (PYU) and ENcoccus test (Pliva-Lachema, Brno, Czech Republic).

Gram staining, optical microscopy, morphology on the M17 medium (Biokar, France) and MRS medium, and

API-test (BioMerieux, France) were applied within the group of LAB.

2.7. Statistical evaluation

All microbiological and chemical traits were measured in duplicate in each part (core, edge) of each block within each batch of the cheese. However, because the batch (the cheese vat), not each single block of cheese obtained from it, is usually considered the experimental unit in the cheese technology (Hunter et al., 1997), means of the six measurements representing a particular batch were used in statistical evaluation in the present experiment. On the other hand, if more cheeses are produced from a given experimental unit, the cheeses can be looked upon as subunits (Hunter et al., 1997). This possibility was employed when cheeses from only one sampling interval were compared in the present experiment.

The Unistat package, version 4.53 (Unistat Ltd., London, UK) was used for calculation of the basic statistical characteristics, regressions (including significance testing of the linear and quadratic terms, respectively), differences between the sets of cheeses in tyramine content and microbial counts, respectively (one-way classification of the variance-ratio test, including Duncan's multiple range test), and for calculation of the correlations between particular traits. Percentage of total variability in tyramine content explained by the producer, starter culture, fat content, part of the cheese, and time of ripening, respectively, was calculated using multiple-way classification of the variance-ratio test.

The linear regressions equality test (Rod and Vondráček, 1973) was used for the comparison of producers and parts of the cheese, respectively, regarding the dependence of tyramine content on the time of ripening. The independent variable (X, time of ripening) and the number of measurements ($n = 56$) being the same for both producers (parts of the cheese), the decisive criterion (F-value) was calculated according to the simplified formula:

$$F = \frac{n(y_1^2 + y_2^2) - ((y_1 + y_2)^2/2) + s_x(b_1^2 + b_2^2 - 2b_1b_2)/2}{s_{y1}^2 + s_{y2}^2},$$

where y_1 and y_2 are the means of compared tyramine contents, s_{y1}^2 and s_{y2}^2 their variances, b_1 and b_2 corresponding coefficients of the linear term, and s_x is a standard deviation of the mean of the independent variable (time of ripening).

3. Results and discussion

3.1. Tyramine content in cheeses

The factors with potential effect on tyramine content, which were tested in the present experiment, are compared in Table 1. Quantitatively most important factors were time of ripening and part of the cheese, which accounted for 67% and 28% of explained variability, respectively. Effect of the starter culture was still significant ($P<0.01$), but this factor accounted for only 4% of explained variability. However, as it is also apparent from Table 1, all factors tested in the present experiment explained only 41% of total variability in tyramine content.

Table 1
Comparison of factors effecting tyramine content in Dutch-type semi-hard cheese (multiple-way analysis of the variance ratio test)

Variability factor	% of explained variability	% of total variability	P-value
Producer	<1	<1	0.250
Fat content	<1	<1	0.317
Starter culture	4	2	0.004
Part of the cheese	28	12	0.000
Time of ripening	67	27	0.000
Explained	100	41	

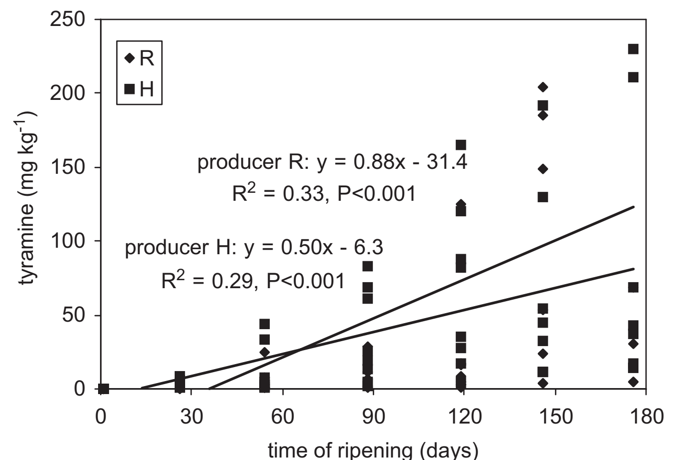

Fig. 1. Dependence of tyramine content in cheeses on the time of ripening. All samples from the particular producer (R, H) irrespective of fat content, starter culture and part of the cheese were taken as a one set ($n = 56$).

Tyramine content in cheese increased significantly with increasing time of ripening in the present experiment (Fig. 1). This finding is in full agreement with the literature data (Petridis and Steinhart, 1996b; Komprda et al., 2007). The coefficient of the linear term was nearly 80% higher in the case of the producer R in comparison with the producer H. Particular relationships shown in Fig. 1 were generalized for each producer: all samples of a given producer irrespective of fat content, starter culture and part of the cheese were taken as a one set.

Regarding estimated toxicological limit 100 mg kg^{-1} (Silla-Santos, 1996), the cheese of the producer R (but not the producer H) can be considered unsafe when ripened more than 150 days.

From comparison of tyramine content in particular cheeses at the end of ripening (Fig. 2), only one clear

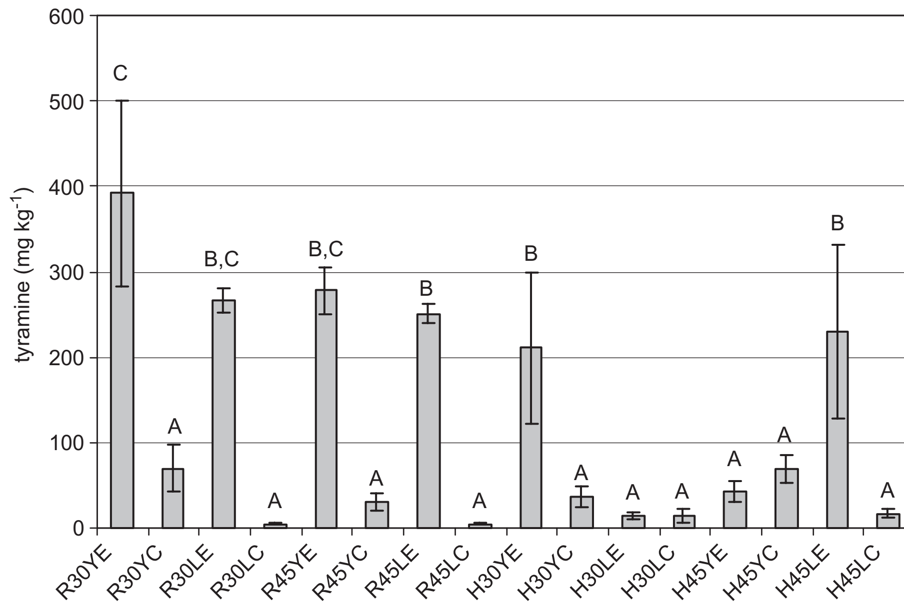

Fig. 2. Tyramine content at the end of ripening (176th day) in the core (C) and edge (E) part of cheeses from two dairies (R, H), produced with two different fat contents (30% and 45%) using two starter cultures (Y: *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*; L: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, respectively). Means ($n = 3$) with different superscripts (A, B, C) differ significantly ($P<0.01$).

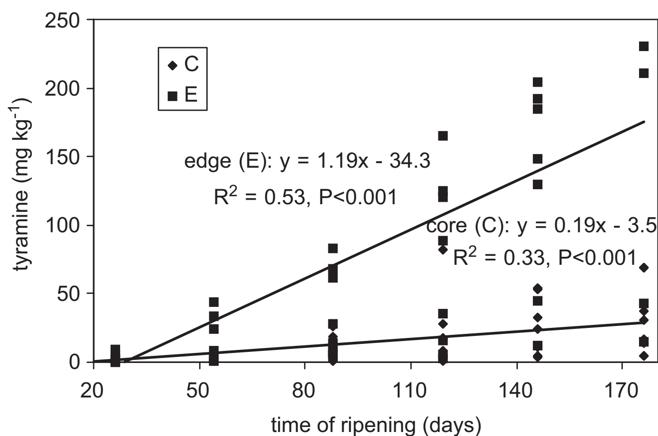

Fig. 3. Dependence of tyramine content in core (C) and edge (E) parts of the cheese on time of ripening. All core and edge samples, respectively, irrespective of producer, fat content and starter culture, were taken as a one set ($n = 56$).

tendency is apparent at first sight: there was a significantly higher ($P<0.01$) tyramine content in the edge part of the cheese in comparison with the core part in most samples. The range of the values presented in Fig. 2 (5–392 mg kg^{-1}, R30LC–R30YE, respectively) is similar to the results of our previous experiment with another two producers of Dutch-type ripening cheese (tyramine content 16–300 mg kg^{-1} at 22 weeks of ripening; Komprda et al., 2007). Petridis and Steinhart (1996b) reported tyramine content in Swiss-type cheese stored 5 months 320 mg kg^{-1}.

The uneven tyramine distribution in cheese (core × edge polarization) in the present experiment is confirmed by the data presented in Fig. 3; to generalize an effect of the part of the cheese on tyramine content during the ripening, all core and edge samples, respectively, regardless of producer, fat content and starter culture, were taken as two sets. The dependences were statistically different based on the linear regression equality test ($F = 21.6$, $P<0.05$). This finding confirms the data both of Petridis and Steinhart (1996a) in a Swiss-type cheese and of our previous experiment in a Dutch-type cheese (Komprda et al., 2007), contrary to the results of Novella-Rodrígues et al. (2003), who found higher tyramine content inside the hard-ripened raw-milk goat cheese as compared to the edge. All the three mentioned research groups admitted they had not been able to present satisfactory explanations for their respective differences in tyramine distribution within the cheese, mentioning unspecified different external and internal microenvironmental conditions, possible different O_2 (Novella-Rodrígues et al., 2003) or water activity (a_w; Komprda et al., 2007) requirements of tyramine producers. We have measured a_w in the present experiment, but with inconclusive results: a_w in the edge part (0.955) was significantly ($P<0.01$) lower (therefore, this part of the cheese was less suitable for the growth of microorganisms from this viewpoint) in comparison with the core part (0.961). However, the absolute differences in the a_w values were small; the more probable explanation of higher tyramine concentration in the E-parts of the cheese was higher counts of contaminant enterococci, some of which possessed tyrosine-decarboxylase activity.

Table 2
Counts of total anaerobes, lactic acid bacteria, enterococci, and *Enterobacteriaceae*, respectively, in sets of cheeses at the end of ripening (176th day); $n = 3$

Set	Counts of microorganisms (log cfu g^{-1}; mean ± S.E.M.)			
	Total anaerobes	Lactic acid bacteria	Enterococci	*Enterobacteriaceae*
R30YE	$7.85^{a,b,c,d} \pm 0.34$	$8.13^{b,c} \pm 0.24$	$3.51^{d} \pm 0.23$	$2.16^{b,c} \pm 0.15$
R30YC	$8.34^{d,e} \pm 0.19$	$8.47^{c} \pm 0.10$	$0.02^{a} \pm 0.01$	$1.53^{a,b,c} \pm 0.77$
R30LE	$8.17^{b,c,d,e} \pm 0.14$	$7.91^{a,b,c} \pm 0.06$	$3.18^{c,d} \pm 0.08$	$2.26^{b,c} \pm 0.26$
R30LC	$8.29^{d,e} \pm 0.01$	$8.04^{b,c} \pm 0.08$	$0.90^{a,b} \pm 0.89$	$0.69^{a,b} \pm 0.66$
R45YE	$7.73^{a,b,c,d} \pm 0.14$	$7.34^{a} \pm 0.30$	$3.03^{c,d} \pm 0.17$	$1.65^{a,b,c} \pm 0.87$
R45YC	$7.57^{a,b} \pm 0.08$	$7.73^{a,b} \pm 0.10$	$1.49^{a,b,c,d} \pm 0.76$	$1.83^{b,c} \pm 0.93$
R45LE	$8.21^{d,e} \pm 0.12$	$7.81^{a,b} \pm 0.16$	$1.53^{a,b,c,d} \pm 0.75$	$1.53^{a,b,c} \pm 0.76$
R45LC	$8.08^{b,c,d} \pm 0.06$	$7.98^{a,b,c} \pm 0.06$	$0.67^{a,b} \pm 0.65$	$2.85^{c} \pm 0.43$
H30YE	$8.20^{c,d,e} \pm 0.05$	$7.85^{a,b,c} \pm 0.13$	$1.57^{a,b,c,d} \pm 0.78$	$0.01^{a} \pm 0.00$
H30YC	$8.63^{d,e} \pm 0.09$	$7.93^{a,b,c} \pm 0.11$	$2.37^{b,c,d} \pm 0.11$	$0.57^{a,b} \pm 0.55$
H30LE	$7.45^{a} \pm 0.23$	$7.53^{a,b} \pm 0.18$	$1.47^{a,b,c,d} \pm 0.78$	$2.11^{b,c} \pm 0.05$
H30LC	$8.01^{a,b,c,d} \pm 0.08$	$7.52^{a,b} \pm 0.16$	$1.13^{a,b,c} \pm 0.56$	$0.01^{a} \pm 0.00$
H45YE	$8.07^{b,c,d,e} \pm 0.08$	$7.77^{a,b} \pm 0.04$	$1.79^{a,b,c,d} \pm 0.89$	$1.98^{b,c} \pm 0.10$
H45YC	$7.92^{a,b,c,d} \pm 0.36$	$7.61^{a,b} \pm 0.33$	$2.35^{b,c,d} \pm 0.09$	$2.28^{b,c} \pm 0.31$
H45LE	$7.72^{a,b,c,d} \pm 0.30$	$7.69^{a,b} \pm 0.43$	$1.71^{a,b,c,d} \pm 0.88$	$2.37^{b,c} \pm 0.37$
H45LC	$7.59^{a,b,c} \pm 0.03$	$7.37^{a} \pm 0.07$	$1.13^{a,b,c} \pm 0.56$	$1.49^{a,b,c} \pm 0.74$

R, H—producers; 30, 45—fat content 30% and 45%, respectively; Y—starter culture: *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*; L—starter culture: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*; E—edge, C—core.
Means with different superscripts (^{a,b,c,d,e}) differ at $P<0.05$.

3.2. Microorganisms associated with the cheeses

Tyramine-producing bacterial strains were expected to be present especially within the groups of LAB (lactobacilli), *Enterobacteriaceae* and enterococci, as indicated in the paper of Martuscelli et al. (2005). Tyramine-forming isolates from Spanish traditional cheeses in the experiment of Roig-Sagués et al. (2002) belonged mostly to the groups of enterococci and LAB. On the other hand, Schneller et al. (1997) reported only enterococci and *Enterobacteriaceae* as tyramine producers, lactobacilli did not seem important from this viewpoint in semi-soft ripening cheeses. The above-quoted authors (and similarly the present experiment) make a distinction between LAB as a group and enterococci, which are otherwise an integral part of this group. The reason is that genus *Enterococcus* is the most controversial part of LAB: on one hand, it plays an important role in the cheese ripening (proteolysis, lipolysis, citrate breakdown), produces bacteriocins and is used as probiotics; on the other hand, it has been associated with a number of human infections (Foulquié Moreno et al., 2006) and it is an established tyramine producer in cheese (Roig-Sagués et al., 2002).

Regarding the present experiment, counts of LAB, total anaerobes (TAN), *Enterobacteriaceae* and enterococci in 16 tested sets of cheeses at the end of ripening (176th day) are presented in Table 2. Dependences of LAB counts on the time of ripening are shown in Fig. 4. The regressions were calculated for the sets of cheeses irrespective of the fat content (similarly to tyramine content, Table 1, an effect of fat percentage was negligible also in this case). When calculated as total means for either producer (the whole ripening interval taken as a one set), LAB counts in the H-cheeses were higher ($P<0.01$) than in the R-samples (8.06 log cfu g^{-1} vs. 7.84 log cfu g^{-1}; however, despite the significant difference, the values are very close).

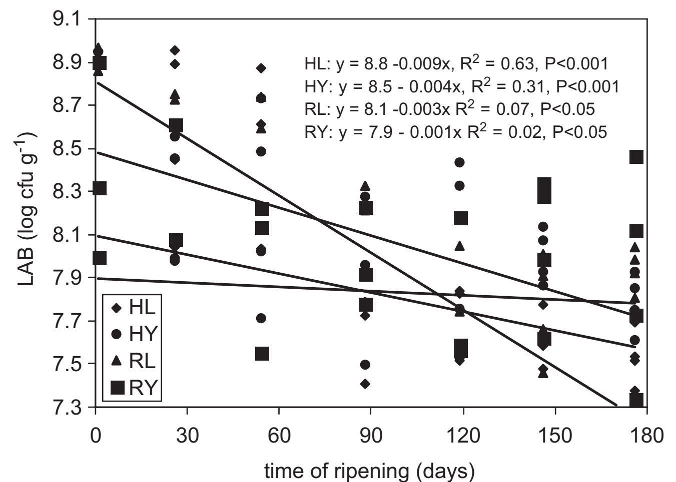

Fig. 4. Counts of lactic acid bacteria (LAB) in the course of ripening in cheeses produced by two different dairies (R, H) using different starter cultures (Y: *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*; L: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*); all samples within particular producer–starter combination (irrespective of fat content and part of the cheese) were taken as a one set ($n = 14$).

The dependences of enterococci counts in cheeses on the time of ripening (Fig. 5) were calculated using the sets irrespective not only of fat content, but also of starter

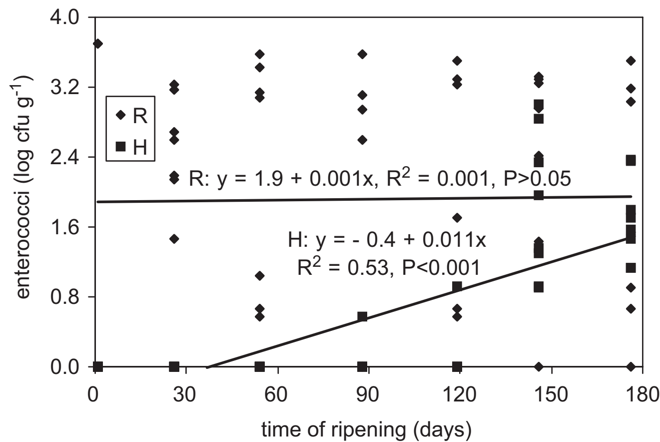

Fig. 5. Counts of enterococci in the course of ripening in cheeses from two producers (R, H). All samples from the particular producer (R, H) irrespective of fat content, starter culture and part of the cheese were taken as a one set ($n = 56$).

culture (enterococci were not present in any starter culture). More massive initial enterococci contamination of the R-cheeses, presumably from an ambient environment of the dairy R, follows from Fig. 5. On the other hand, enterococci counts in the R-cheeses did not change during ripening, contrary to the significant ($P<0.001$) increase in the H-cheeses.

However, enterococci counts at the end of ripening in the most contaminated sample (R30YE, 3.51 log cfu g^{-1}; Table 2) were still substantially lower in comparison with the data of Schneller et al. (1997) regarding semi-soft ripening cheese: 6.93 log cfu g^{-1}.

We were unable to investigate in more detail the source of the enterococci contamination. However, in fact milk used in the present experiment was pasteurized and the enterococci presumably originating in cows' gastrointestinal tract and present in raw milk (generally assumed fact; Rea et al., 2004) would have been destroyed, which corresponds with the findings of Gelsomino et al. (2002) that bovine faeces was not the source of the enterococci, despite the fact, that corresponding strains were found in milk, cheese, human faeces and milking equipment; the quoted authors were unable to find the ultimate source of enterococci. The same conclusion can be drawn from the data of Schneller et al. (1997), who reported similar enterococci counts after 2 months of ripening in semi-soft cheese produced from either raw or pasteurized milk. The enterococci distribution within the cheese in the present experiment (total means of the respective core and edge sample sets within the whole experiment 0.78 log cfu g^{-1} vs. 1.66 log cfu g^{-1}, $P<0.01$) is indicative of the secondary contamination during cheese production, but it may be also a consequence of different environmental conditions in the cheese.

Correlation coefficients of the relationships between tyramine content and TAN, LAB, *Enterobacteriaceae* and enterococci counts in the cheeses were low and in some cases even significantly negative in the present experiment: −0.18 ($P<0.001$), −0.18 ($P<0.001$), 0.06 ($P>0.05$), and 0.09 ($P>0.05$), respectively. This is in agreement with the data of Fernández-García et al. (2000) that the population of tyrosine-decarboxylase positive bacteria represented less than 1% of the total bacterial population. Similarly, Schneller et al. (1997) reported that semi-soft cheeses with comparable microbial profiles differed enormously in their tyramine concentrations during ripening.

3.3. Screening of tyrosine-decarboxylase activity and tyrosine-decarboxylase gene

Due to the capacity of the laboratory, only LAB and enterococci were tested in DCM and on the presence of the *tyrdc* gene sequences. Number of LAB and enterococci isolates from cheeses after 26 days of ripening, positive in DCM, is shown in Table 3. Despite the fact, that LAB were predominant in the core part of the cheeses, no similar tendency is apparent from Table 3. On the other hand, 69 Y- and only 43 L-isolates of LAB were DCM-positive, which could correspond with positivity of the starter culture Y (see below). However, from the sum of 112 DCM-positive LAB isolates, tyramine was confirmed only in 15 samples (Table 3) using the HPLC method. Roig-Sagués et al. (2002) found very similar proportion between number of all LAB isolates and tyramine-positive samples in Spanish traditional cheeses.

As far as enterococci are concerned in the present experiment, the data in Table 3 confirm dominant presence of this group of bacteria in the edge part of the cheeses. Presence of decarboxylase-positive enterococci strains in the environment of dairy R, and consequent contamination of the R-cheeses during production presumably follows from the data of Table 3. However, based on the results of the present experiment, it is difficult to unequivocally prove if the above-mentioned enterococci distribution was a consequence of the environmental conditions prevailing in the given part of the cheese or an external contamination once the cheese had been molded, or during molding/pressing/brining stages.

The proportion between the DCM-positive enterococci isolates (together 48) and number of samples in which tyramine was confirmed by HPLC (20; Table 3) was higher in comparison with the LAB isolates, similarly to the results of Roig-Sagués et al. (2002).

Isolates of LAB and enterococci presented in Table 3 were concurrently tested on the presence of tyrosine-decarboxylase gene sequences (by the PCR method using TD5/TD2 primers; Coton et al., 2004). The 1100 bp fragment characteristic for the presence of the *tyrdc* gene was found in all LAB and enterococci isolates (15 and 20, respectively), in which tyramine was confirmed by HPLC after the DCM-testing (Table 3). Superiority of the PCR method over the classical microbiological methods (DCM) follows from the results of the present experiment: high percentage of the false-positive results using DCM. On the

Table 3
Total number and percentage of isolates of lactic acid bacteria and enterococci in cheeses taken after 26 days of ripening, tested positive in decarboxylase screening medium (DCM), positive for tyramine presence in medium by the HPLC method, and positive for *tyrdc* gene sequence by the PCR method, respectively

Set of cheeses	Lactic acid bacteria						Enterococci					
	Positive in DCM[a]		Tyramine confirmed by HPLC		*tyrdc*-positive[b]		Positive in DCM[a]		Tyramine confirmed by HPLC		*tyrdc*-positive[b]	
	n[c]	%[d]	*n*[c]	%[d]	*n*[c]	%[d]	*n*[c]	%[d]	*n*[c]	%[d]	*n*[c]	%[d]
R30YE	10	27	5	14	5	14	12	25	4	8	4	8
R30YC	5	13	3	8	3	8	0	–	0	–	0	–
R30LE	5	9	0	–	0	–	12	24	6	12	6	12
R30LC	5	8	0	–	0	–	1	2	0	–	0	–
R45YE	9	24	0	–	0	–	12	26	3	7	3	7
R45YC	8	22	3	9	3	9	3	6	0	–	0	–
R45LE	8	18	0	–	0	–	6	13	5	11	5	11
R45LC	4	7	0	–	0	–	2	4	2	4	2	4
H30YE	7	19	0	–	0	–	0	–	0	–	0	–
H30YC	9	27	0	–	0	–	0	–	0	–	0	–
H30LE	4	6	0	–	0	–	0	–	0	–	0	–
H30LC	6	13	0	–	0	–	0	–	0	–	0	–
H45YE	12	32	4	11	4	11	0	–	0	–	0	–
H45YC	9	24	0	–	0	–	0	–	0	–	0	–
H45LE	6	9	0	–	0	–	0	–	0	–	0	–
H45LC	5	8	0	–	0	–	0	–	0	–	0	–

Total numbers and percentages refer to an aliquot (square root) of total countable colonies within each group of bacteria. R, H—producers; 30, 45—fat content 30% and 45%, respectively; Y—starter culture: *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*; L—starter culture: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*; E—edge, C—core.

[a]Decarboxylase screening medium (Bover-Cid and Holzapfel, 1999).

[b]Tyrosine-decarboxylase gene sequence (based on the primers TD5/TD2; Coton et al., 2004).

[c]Total number of positive isolates.

[d]Percentage of positive isolates from an aliquot of total countable colonies.

other hand, tyramine was not confirmed by HPLC in any tested DCM-negative sample (however, because only small number of DCM-negative samples was tentatively tested by HPLC, the possibility that DCM also gives false negatives cannot be excluded based on the results of the present experiment). It is also worth mentioning in this context the results of Rea et al. (2004), who found no relationship between tyramine production in a tyrosine containing broth and tyramine production in the cheese.

Based on the evaluation of the morphological and biochemical markers, *tyrdc*-positive enterococci belonged to the species *Enterococcus durans*, *Enterococcus faecalis*, and *Enterococcus casseliflavus*. In an experiment of Rea et al. (2004), strains of *E. durans* and *E. faecalis*, but not *E. casseliflavus* produced tyramine in Cheddar cheese. *E. casseliflavus*, *E. durans*, *E. faecalis* and *Enterococcus faecium* isolates represented 4%, 8%, 53%, and 12% of all tyramine-positive isolates from traditional Spanish cheeses, respectively, as reported by Roig-Sagués et al. (2002).

Tyrdc-positive LAB were all lactobacilli in the present experiment; from the fifteen isolates, ten were classified as *Lactobacillus curvatus* subsp. *curvatus* (five isolates from R30YE cheese, two isolates from R45YC, three isolates from H45YE), one was classified only as *Lactobacillus* spp. (R30YC). Remaining four lactobacilli isolates were morphologically and biochemically *L. curvatus* subsp. *curvatus*, but repetitive sequence-based PCR using (GTG) 5 primer (carried out in the laboratory of the Czech Collection of Microorganisms, Masaryk University Brno, Czech Republic) did not confirm this finding unequivocally.

In addition to the cheese samples, also starter cultures were tested on tyrosine-decarboxylase activity and a presence of *tyrdc* gene. Results regarding the culture L were negative, but the culture Y was positive in DCM. After separation of single components of the culture, particular strain of *L. helveticus* was found to possess tyrosine-decarboxylase activity. This strain was consequently confirmed as *tyrdc*-positive using the PCR method.

4. Conclusions

It follows from the results of the present experiment that tyramine content in Dutch-type semi-hard cheese produced from pasteurized milk in best hygienic conditions can nevertheless reach toxicologically relevant levels after 5 months of ripening. Tyramine-producing bacterial strain was found in one of the starter cultures used; the producers of the starter cultures should test their products from this viewpoint to decrease the risk of negative health effects on susceptible consumers.

Methods of molecular biology (PCR) are faster and more reliable in comparison with the methods of classical

microbiology (testing tyrosine-decarboxylase activity) as far as the detection of tyramine-producing microorganisms in cheese is concerned. On the other hand, an often-recommended early detection of *tyrdc*-positive strains by PCR may be questionable from the following reasons: secondary contamination of the cheese with adventitious bacteria, especially enterococci, during production seems unavoidable even in dairies applying the best hygienic principles; the expression of *tyrdc* cannot be evaluated by PCR; even if the gene is expressed, the enzymatic activity may be influenced by the environmental conditions prevailing in the cheese.

At any rate, consumers from risky groups (patients consuming drugs with an effect of MAO-inhibitors) should avoid consumption of Dutch-type cheese ripening more than 5 months and are recommended to remove 3 cm outer part of the cheese (which contains higher tyramine concentration) before consumption.

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Publikace IV

Contents of Some Biologically Active Amines in a Czech Blue-vein Cheese

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Abstract

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Biogenic amines (histamine, tyramine, tryptamine, phenylethylamine, cadaverine) including biologically active polyamines (putrescine, spermidine and spermine) were determined by HPLC method after 7, 21, 35, and 49 days of ripening in the core (C) and edge (E) samples of a blue-veined cheese, popular in the Czech Republic under the trade mark Niva, produced in the three consecutive months (October, November, December) from pasteurised cow milk using *Penicillium roqueforti* spores; two vats were produced in each month. The cheese vat, including the production period, accounted for ($P < 0.05$) one third and two thirds of the explained variability of the sum of biogenic amines and the sum of polyamines, respectively. The ripening time was significant ($P < 0.05$) from this viewpoint only in the case of the sum of biogenic amines (nearly half of the explained variability). Putrescine and spermidine contents in cheese did not change ($P > 0.05$), spermine content even decreased ($P < 0.05$) with increasing time of ripening. Tyramine content (Y, mg/kg) in the core samples increased linearly with increasing time of ripening (X, days), $Y = 6.3 + 11.69X$, $R^2 = 0.26$, $P < 0.001$, contrary to the edge part where tyramine content did not change ($P > 0.05$). At the end of ripening (49 days), tyramine was quantitatively the most abundant amine (the mean and median 380 mg/kg and 289 mg/kg, respectively), its content in different cheeses (vats) varying from 10 mg/kg to 875 mg/kg. Cadaverine concentration varied between 3 mg/kg and 491 mg/kg (the mean 114, median 56 mg/kg). The levels of other biogenic amines and polyamines (with the exception of putrescine in the edge part of one of the December vats: 117 mg/kg) were very low even at the end of ripening. Tyramine contents at the end of ripening in the core-samples were higher ($P < 0.01$) in comparison with those in the edge-samples, contrary to histamine, cadaverine, putrescine, and spermine contents.

Keywords: tyramine; cadaverine; polyamines; blue-vein cheese; *Penicillium roqueforti*

Niva is a typical Czech variant of the internal-mould cheeses, produced under the use of specific strains of the *Penicillium roqueforti*. As a high-protein-containing ripening foodstuff, it belongs to the products where the degradation of proteins during ripening leads to the accumulation of free amino acids, which can be converted (due to the activity of bacterial decarboxylases) into biogenic amines (Innocente & D'Agostin 2002). Ripening cheeses are the next (after fish) most commonly implicated food item associated with biogenic amine poisoning (Stratton *et al*. 1991).

Quantitatively and toxicologically the most important biogenic amines (histamine, tyramine, tryptamine, phenylethylamine, cadaverine) in ripening cheeses are tyramine and histamine. Tyramine is a potent vasoconstrictor; its higher levels in an organism can lead to hypertension

and migraine and can induce brain haemorrhage and heart failure (Til *et al.* 1997). Histamine (also vasoactive substance) can cause urticaria, hypotension, headache, flushing, and abdominal cramps (Stratton *et al.* 1991). Tyramine and histamine are broken down in the mammalian organism by oxidative deamination catalysed by monoamine oxidase (MAO). However, human detoxification mechanisms are insufficient in the following cases: too high intake in a diet; in the allergic individuals; in patients consuming drugs acting as MAO inhibitors (antiparkinsonian drugs and antidepressants).

Within the group of biogenic amines, some polyamines (putrescine, spermidine and spermine; but not cadaverine, from the chemical viewpoint also a polyamine) are currently classified as a distinct group for the following reasons. Putrescine, and subsequently its higher metabolites spermidine and spermine, are synthesised in a mammalian cell from ornithine and therefore are already present in the raw materials (cheese milk; Coleman *et al.* 2004). Toxicological importance of polyamines is based on their ability to form stable carcinogenic N-nitroso compounds and to enhance the growth of chemically induced aberrant crypt foci in the intestine (Paulsen *et al.* 1997). Polyamines are required for normal cell growth and proliferation, but are readily taken up by tumor cells; a strict control of the polyamine content in the diet of the cancer patients is therefore a matter of the utmost importance (Kalač & Krausová 2005). Putrescine stimulates tyrosine kinases and the expression of particular nuclear protooncogenes and is in this sense involved in cancer pathogenesis (Wolter *et al.* 2004).

Apart from the milk pasteurisation (or the lack of it; Novella-Rodríguez *et al.* 2003), general hygienic conditions of the cheese production (Komprda *et al.* 2007) and starter culture (Roig-Sagués *et al.* 2002), the following factors can influence biogenic amine content in ripening cheese: the time of ripening (Komprda *et al.* 2007) and part of the cheese (Novella-Rodríguez *et al.* 2003; Komprda *et al.* 2007). The data regarding the assessment of these factors in blue-vein cheeses (Novella-Rodríguez *et al.* 2003) are surprisingly scarce. Therefore, the objective of the present study was to evaluate the biogenic amine and polyamine contents in one of the blue-vein cheese varieties from the viewpoint of the distribution within the cheese and of the dependence on the time of ripening.

MATERIALS AND METHODS

Cheese-making. The samples of the blue-veined cheese Niva were prepared in a production plant under the standard technological and hygienic conditions. The basic raw materials for the cheeses production were pasteurised full-fat cow milk, the mold *Penicillium roqueforti*, rennet, butter starter culture (*Lactococcus lactis*, subsp. *lactis*, and *L. lactis*, subsp. *cremoris*), and common salt. Milk was pasteurised at 73°C for 30 seconds. Cheese milk in a vat was cooled to 29°C, and before renneting, $CaCl_2$ (15 g/100 l), starter culture (0.9%), and mould culture (commercial *P. roqueforti* spores) were added (unfortunately, the producer refused to communicate both the particular *P. roqueforti* strain and the amount inoculated per ml of milk). The time of renneting was 60 min, that of curd forming 110 minutes. NaCl was added into the curd in the amount of 8 kg per 1000 l of milk. Loaves weighing 2 kg, with a diameter of 15 cm, height 12 cm, and the final fat content of 52% in dry matter were produced. Cheeses were subsequently soaked in a salt solution (20% NaCl) for 48 h (final NaCl content in the cheese was 4.8%), let to dry, pierced, and transferred to the cheese cellar, where they ripened at the temperature of 8°C.

Altogether six vats of blue-veined cheese were produced, two vats in each of the three different time periods: October (Vats 1 and 2), November (Vats 3 and 4), and December (Vats 5 and 6). Within each vat, 12 loaves were produced. Three randomly chosen loaves were taken from each cheese vat after 7, 21, 35, and 49 days of ripening.

Amines determination. At each sampling, the loaf was cross-cut in the middle, and 100 g of the edge part (3 cm thick outer part of the cheese, designated as "E") and 100 g of the remaining core part (designated as "C") were taken, respectively.

The E and C samples were homogenised separately. 10 g of each sample was weighed into a 85 ml test tube, 0.5 ml of the internal standard (1,7-diaminoheptane; concentration 1 mg/ml) was added and the sample was extracted with 20 ml of 0.1M hydrochloric acid (HCl) for 2 min using a disintegrator Heidolph Diax 900 (Heidolph Instruments, Germany). The suspension was centrifuged at 755 × g at 4°C for 10 min (Hettich Universal 32R; Hettich, Germany). The supernatant was filtered through a paper filter and the solid residue was extracted once again as described above. The combined extracts were made up to 50 ml with

deionised water and filtered through a disposable nylon membrane filter (13 mm, 0.45 µm; Chromatography Research Supplies, Addison, USA).

The extract was derivatised with dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride, DCl). The derivatising agent was prepared by dissolving 5 mg of dansyl chloride in 1 ml of acetone (Sigma-Aldrich, St. Louis, USA). The derivatisation was performed as follows: 1 ml of the extract (or standard) was mixed with 0.5 ml of saturated Na_2CO_3 (pH adjusted to 11.2), 1 ml of the derivatising agent was added and the mixture was shaken for 1 min (MS2 Minishaker IKA; IKA Werke GmbH, Staufen, Germany). The derivatisation proceeded in the dark at 40°C for 1 hour. The amine derivatives were then extracted with diethylether (3 × 1 ml). The organic phase was evaporated to dryness under nitrogen and the solid residue was dissolved in 0.5 ml of acetonitrile (ACN). The solution was filtered through the nylon membrane filter 0.45 µm and a volume of 10 µl was injected onto the chromatographic column.

Dansylated amines were separated using a liquid chromatograph HP 1100 (Agilent Technologies, Wilmington, USA) consisting of a quarternary pump (G1311A), vacuum degasser (G1322A), automatic sampler (G1313A), and UV/VIS detector with variable wave-length (G1314A). The separation after DCl derivatisation was carried out using gradient elution with H_2O/ACN (time 0–23 min: H_2O 35–0%, ACN 65–100%) on the Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, particle size 5 µm) with the guard column Meta Guard ODS-2 (30 mm × 4.6 mm, particle size 5 µm) at the flow rate of 0.8 ml/min. Photometric UV/VIS detector was used at 254 nm. The concentration of amines in the sample was corrected based on the method of internal standard according to Komprda *et al.* (2007).

The separated dansylated amines were identified by comparing their retention times with those of the particular dansylated amine standards (all the amine standards were used as the respective hydrochlorides supplied by Sigma-Aldrich and subsequently derivatised in the same way as the amines proper), and their concentrations after DCl derivatisation were expressed in mg/kg of cheese.

Two groups of amines are distinguished in the following text: "biogenic amines" (histamine, tyramine, tryptamine, and cadaverine,) and "polyamines" (putrescine, spermidine, and spermine). Despite the fact that the latter substances traditionally belong to biogenic amines, they are currently classified as a distinct group based on their biological properties (not their chemical structure); for the same reason, cadaverine (chemically also polyamine) is not included in these biologically active polyamines.

Statistical evaluation. Biogenic amines and polyamines were determined in duplicates in each part (core, edge) of each loaf within each vat of the cheese. As far as the number of replications is concerned, despite the fact that the cheese vat is usually considered an experimental unit in the cheese technology, if more cheeses are produced from a given experimental unit, the loaves can be looked upon as sub-units (Hunter *et al.* 1997). Therefore, the experimental data measured were assessed from four different viewpoints, and, correspondingly, four different sets of data were evaluated based on the following number of measurements: comparison of the vats in a given time of ripening, 3 measurements (n = 3); comparison of all cheeses produced in a given month: 3 loaves × two vats (n = 6); comparison of the parts of cheeses: all core parts and all edge parts, respectively, from all loaves from all vats produced within all three months were taken as two particular sets (n = 18); dependence on the time of ripening: 3 loaves × 6 vats × 4 samplings (n = 72).

The Unistat package, version 4.53 (Unistat Ltd., London, England), was used for the calculation of the basic statistical characteristics, regressions (that is the dependences of particular amine or group of amines contents on the time of ripening), including significance testing of the linear and quadratic terms, respectively, and the differences between the sets of cheeses (vats; loaves) in the amine contents (one-way classification of the variance-ratio test, including Duncan's multiple range test). The percentage of total variability in the amine content explained by particular tested variability factor (vat, part of the loaf, time of ripening) was calculated as the percentage of the sum of squares belonging to the particular factor from the total sum of squares using multiple-way classification of the variance-ratio test.

The P-level was used in the tables, figures, and within the text in the usual meaning of the probability of error that is involved in accepting the observed results as valid. R-square value (R^2) was used (in Figure 1 and within the text) in the sense of the coefficient of determination, the measure of

the variability of the dependent variable around the regression line, that is the percentage of the explained variability from the original variability.

RESULTS AND DISCUSSION

The contents of biogenic amines and polyamines, respectively, were affected differently by the tested variability factors in the present experiment (Table 1). The differences between the vats, including the differences between the production periods (October × November × December), accounted for one third of the explained variability of the sum of biogenic amines, but nearly for two thirds in the case of the sum of polyamines. This finding might seem paradoxical, because the differences in the amine contents between various production periods are supposed to originate from different microbial contamination of the raw milk and/or the environment of the production plant, and biogenic amines are most likely formed by the contaminant bacteria possessing the amino acid-decarboxylase activities (Novella-Rodríguez *et al.* 2003), contrary to polyamines which are mainly synthesised in eukaryotic cells (in dairy cow in this case; Coleman *et al.* 2004).

The difference between the sum of biogenic amines (nearly half of explained variability) and the sum of polyamines (only 4% of explained variability; Table 1) was even greater as far as the time of ripening is concerned. The likely reason is the known fact that the content of biogenic amines increases during cheese ripening (Komprda *et al.* 2007, 2008a), which was confirmed also in the present experiment, see below); the content of polyamines usually changes less conspicuously with increasing time of ripening (Komprda *et al.* 2008a). However, the percentages of the explained variability due to the time of ripening found in blue-vein cheese in the present experiment differ from the corresponding data of our previous experiment with a Dutch-type semi-hard cheese (Komprda *et al.* 2008a), where the time of ripening accounted for 73% of the explained variability both of the sum of biogenic amines and the sum of polyamines.

Moreover, despite the above clear trends concerning the sums of biogenic amines and polyamines, respectively, great differences existed between the individual amines regarding the percentages of the explained variability, and, as also apparent from Table 1, a greater part the of total variability remained unexplained by the factors tested in the present experiment.

As already mentioned above, the difference between biogenic amines and polyamines regarding the changes during ripening can be expected (Komprda *et al.* 2008a). In the present experiment (when the relationships for a given amine using all values irrespective of the vat or production period were calculated), the content of no polyamine, with the only exception of spermine,

Table 1. Effect of particular tested factors on biogenic amines and polyamines contents in blue-vein cheese Niva (general linear model of the multiple-way analysis of the variance ratio test)

Amine	% of explained variability by the tested factors			Residual variability
	vat	part of the cheese	time of ripening	
Tyramine	30	39	31	59
Histamine	14	55	31	84
Tryptamine	54	1	44	91
Cadaverine	36	18	46	85
Phenylethylamine	69	14	17	90
Sum of biogenic amines[1]	36	18	46	73
Putrescine	61	26	12	87
Spermidine	70	0	30	96
Spermine	28	33	39	84
Sum of polyamines[2]	62	34	4	90

[1]tyramine + histamine + tryptamine + cadaverine + phenylethylamine; [2]putrescine + spermidine + spermine

significantly changed during ripening. Spermine content (Y, mg/kg) even decreased ($P < 0.05$) with increasing time of ripening (X, days) according to the equation $Y = 7.7 - 0.11X$ ($R^2 = 0.06$). Even putrescine, polyamine that (apart from the pathway from ornithine functioning in mammalian cells) can be alternatively synthesised from arginine in some bacteria (potentially contaminating cheese milk; Ohnuma *et al.* 2005) did not change during ripening of blue-vein cheese in the present experiment, contrary to the Dutch-type semi-hard cheese used in our previous experiment (Komprda *et al.* 2008a), where putrescine significantly ($P < 0.01$), though slowly, increased with increasing time of ripening.

The content of tryptamine (which belongs to biogenic amines) also tended ($P > 0.05$) to decrease during ripening in the present experiment. On the other hand, the contents of all other biogenic amines tended to increase with increasing time of ripening; significant ($P < 0.01$) relationships are shown in Figure 1.

As far as tyramine is concerned (tyramine was quantitatively the most important biogenic amine in the present experiment, see below), it is interesting that the content of this amine did not change ($P > 0.05$) in the edge part of the cheese (contrary to the core samples, Figure 1) during ripening.

Based on the constant of the linear term (11.7; Figure 1), the tyramine content in the core samples increased seventeen-times more rapidly during ripening in the present experiment than the tyramine content in the Dutch-type semi-hard cheese in our previous experiment (Komprda *et al.* 2008a). It should be underlined that this difference regards only different tyramine distribution between the core and edge parts of the cheese, not the total tyramine content in the compared kinds of cheeses as a whole. The authors of the experiment cited (Komprda *et al.* 2008a) ascribed the substantially higher increase of the tyramine content during ripening in outer parts of the Edam cheese to higher counts of contaminant enterococci in this part of the cheese. Without microbiological analysis, we can only presume higher counts of tyrosine-decarboxylating microorganisms in the inner part as compared to the edge part of the blue-vein cheese in the present experiment.

As far as the possible explanation for the uneven tyramine distribution within the blue-vein cheese in the present experiment is concerned, the conclusions of several other research groups that focused on this question can be mentioned: thus Petridis and Steinhart (1996), Novella-Rodríguez *et al.* (2003), and Komprda *et al.* (2007) admitted in agreement that they had not been able to present

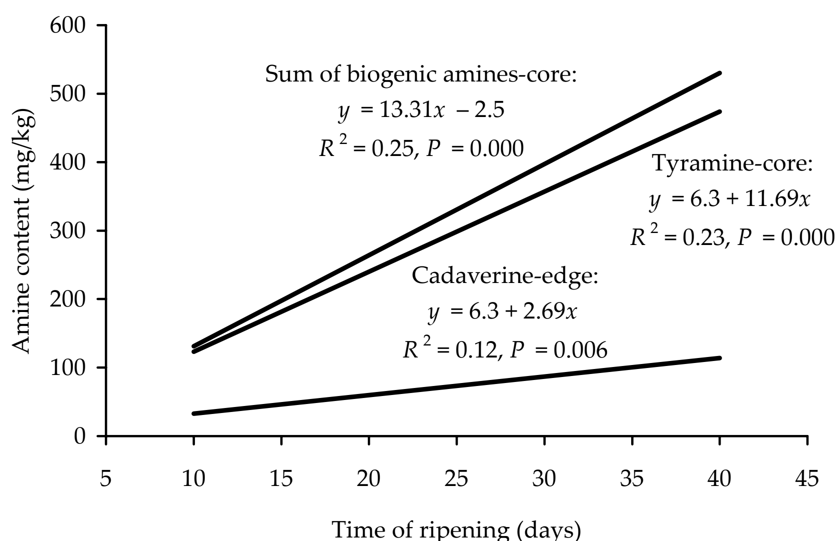

Figure 1. Dependence of biogenic amine content on the time of ripening (only significant relationships, $P < 0.01$, are presented); samples irrespective of vat or production period were taken as one set: $n = 72$ (3 loaves × 6 vats × 4 samplings); R^2 – coefficient of determination (interpretation: e.g. $R^2 = 0.25$ means that the variability of the amine content around the regression line is [1–0.25] times the original variance; in other words 25% of the original variability has been explained, 75% is residual variability)

any satisfactory explanation for their respective differences in tyramine distribution within the cheese, mentioning unspecified different external and internal microenvironmental conditions, possible different access of O_2 or different water activity (a_w). The latter factor (a_w) was pursued in greater detail by Komprda *et al.* (2008b), again with inconclusive results.

Apart from the dependences presented in Figure 1, the histamine content as well as the contents of the sum of all biogenic amines in the edge-samples, respectively, also significantly increased ($P < 0.05$) with increasing time of ripening ($Y = 8.4 + 0.49X$, $R^2 = 0.05$ and $Y = 66.1 + 4.43X$, $R^2 = 0.09$, respectively). The sum of all amines (biogenic amines + polyamines) increased much more rapidly in the core samples ($Y = 15.4 + 13.2X$, $R^2 = 0.25$, $P < 0.001$) in comparison with the edge ones ($Y = 87.3 + 4.48X$, $R^2 = 0.08$, $P < 0.05$).

All the results regarding the amines content in the cheeses are presented in fresh matter. We determined dry matter (DM) content, but only in mixed core/edge samples. Therefore, any differences between the amines contents in the core and edge parts of the cheeses due to the different DM content cannot be inferred from the results of the present experiment. On the other hand, the statements regarding the changes in biogenic amines contents during cheese ripening were not apparently influenced by this fact because the dry matter content did not change ($P > 0.05$) with increasing time of ripening (the mean of DM content of all cheese samples after 7 and 49 days of ripening was 56.8% and 57.4%, respectively).

As follows from Table 1, the factor of the vat affected substantially the contents of both biogenic amines and polyamines in the cheeses, this effect being partly caused by the time period in which the cheese was produced. However, when the three periods were directly compared, only the differences in the content of tyramine (quantitatively the most important biogenic amine) and in the sum of biogenic amines, respectively, between the cheeses produced in October, November, and December were demonstrated ($P < 0.01$), contrary to the sum of polyamines ($P > 0.05$; Figure 2). The variability due to the factors not tested in the present experiment likely outweighed the differences in the polyamine content (residual variability was 90% of total variability in this case, Table 1).

The cheese producer has used mixed milk from different suppliers and therefore it was not possible to evaluate during the experiment one important factor that can affect the amines content in the cheeses, namely the dairy cow's feed mixture composition. The months October to November can be the period of transition from the summer to the winter diet; the high silage content in the dairy cow's diet could introduce higher counts of different lactic acid bacteria into the cheese milk, including enterococci, proved tyramine producers in cheese (Martuscelli *et al.* 2005).

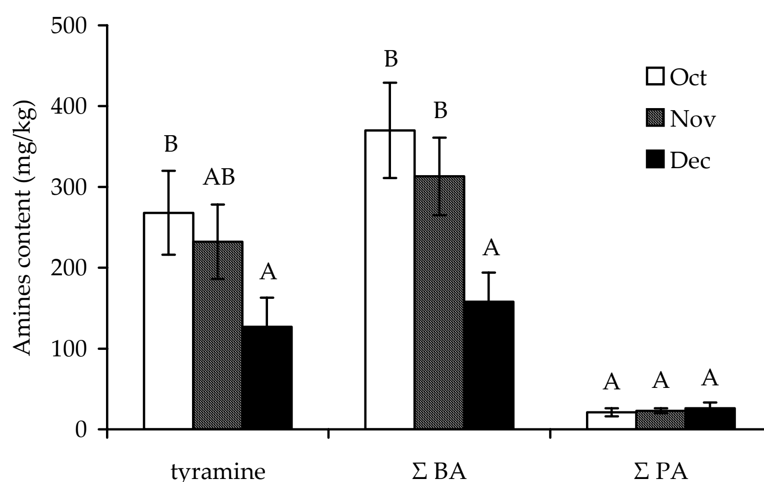

Figure 2. Effect of the production date (cheese produced in October, November and December, respectively) on the content of tyramine, sum of biogenic amines (Σ BA: tyramine + histamine + tryptamine + cadaverine + phenylethylamine) and polyamines (Σ PA: putrescine + spermidine + spermine), respectively; all loaves from both vats produced in a given month were taken as one set, $n = 6$; A, B – means with different superscripts differ at $P < 0.01$

The difference between biogenic amines and polyamines (Figure 2) can be also discussed from the following viewpoint. If it is true that putrescine (an essential precursor of the higher polyamines, spermidine and spermine) can be synthesised by alternative pathways from arginine in some bacteria, it is presumed that the substantial part of putrescine (and subsequently of spermidine and spermine) enters the milk via the mammalian (dairy cow in this case) putrescine synthesis pathway from ornithine (Coleman *et al.* 2004). The possible explanation for the same concentrations of the sum of polyamines in cheeses produced from milk originating in different months (Figure 2) may be the relatively constant rate of polyamine synthesis in a dairy cow. However, this explanation does not correspond with the data of Motyl *et al.* (1995), who found considerable variability in polyamine (spermidine and spermine) content in cow's milk due to the individual dairy cow, phase of lactation, milk yield, and age.

On the other hand, biogenic amine producers are mainly amino acid-decarboxylase positive strains within contaminant bacterial genera and species (Schneller *et al.* 1997; Öner *et al.* 2004). It can be suggested, based on the data of Figure 2, that the extent of secondary contamination by adventitious bacteria of the cheese milk or/and of the produced cheese differed substantially in various months in the present experiment. Bacterial counts were not determined in the present experiment, but based on our previous results (Komprda *et al.* 2008b), the secondary contamination of the pasteurised milk by bacteria with proteolytic and/or decarboxylating activities cannot be excluded regarding some vats produced within the present experiment.

As mentioned above, the main reason for the formation of biogenic amines in cheeses is the presence of microorganisms possessing decarboxylase activity (non-starter lactic acid bacteria, other spontaneous microflora, Roig-Sagués *et al.* 2002; starter microorganisms, Fernández-García *et al.* 2000). However, despite the fact that bacterial producers of biogenic amines in cheeses are well established: lactic acid bacteria (LAB), enterococci (in this regard an important group within LAB) and *Enterobacteriaceae* (Schneller *et al.* 1997; Roig-Sagués *et al.* 2002; Martuscelli *et al.* 2005), we found only either insignificant or even significantly negative correlations between biogenic amine content and the counts of above-mentioned bacteria in our previous experiment (Komprda *et al.* 2008b). In general, it is difficult to find direct correlation between microorganisms counts and biogenic amine content in cheese, because the amine-producing abilities of different strains of various bacteria differ widely (Valsamaki *et al.* 2000; Innocente & D'Agostin 2002). For these reasons, we resigned to the microbiological analysis in the present experiment. Similarly, one of the most distinguished research groups in the field (Novella-Rodríguez *et al.* 2003), reported the distribution of biogenic amines and polyamines

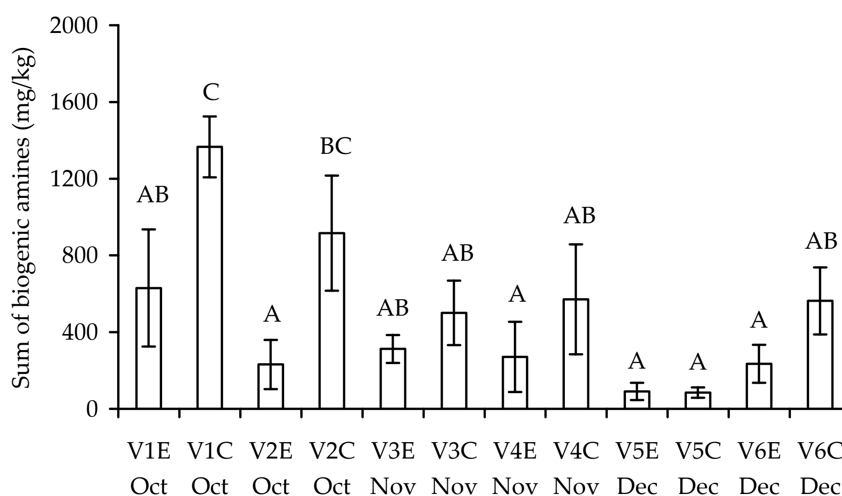

Figure 3. Sum of biogenic amines (tyramine + histamine + tryptamine + cadaverine + phenylethylamine) at the end of ripening (49 days) in the cheeses (vats; V1–V6) produced in October (Oct; V1, V2), November (Nov; V3, V4) and December (Dec; V5, V6); E – edge; C – core; *n* = 3; A, B, C – means with different letters differ at $P < 0.01$; *n* = 3

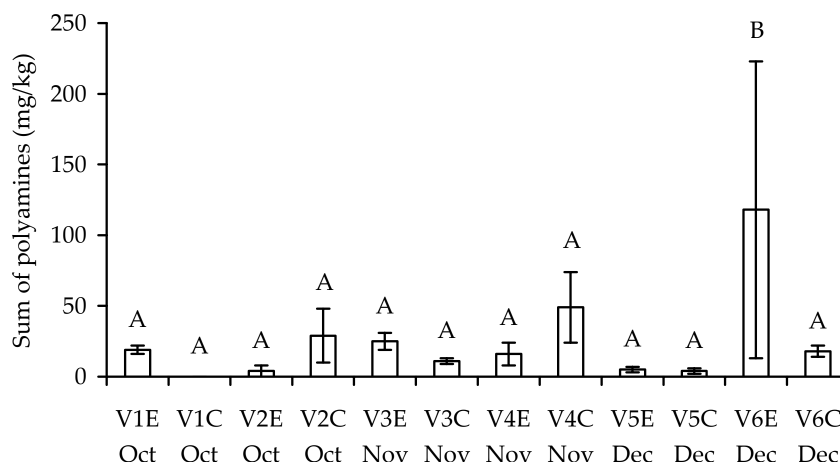

Figure 4. Sum of polyamines (putrescine + spermidine + spermine) at the end of ripening (49 days) in particular cheeses (vats; V1–V6) produced in October (Oct; V1, V2), November (Nov; V3, V4) and December (Dec; V5, V6); E – edge; C – core; $n = 3$; A, B – means with different letters differ at $P < 0.01$; $n = 3$

in various types of cheese without microbiological analysis.

The contents of individual biogenic amines and polyamines in the cheeses at the end of the experiment (after 7 weeks of ripening) are presented in Table 2; the sums of biogenic amines and polyamines at the end of ripening are shown in Figures 3 and 4, respectively.

It is apparent from Table 2 that, firstly, tyramine was quantitatively by far the most abundant amine (the mean and median being 380 mg/kg and 289 mg/kg, respectively) and, secondly, its content in different cheeses (vats) varied widely, from 10 mg/kg, to 875 mg/kg. Novella-Rodríguez *et al.* (2003) found the median of the tyramine content within 20 samples of blue cheeses obtained from Spanish retail stores to be 14 mg/kg with the range from not detected to 1585 mg/kg. Though the upper limits (875 mg/kg and 1585 mg/kg, respectively) are different, both values found in blue-vein cheeses are higher in comparison with the corresponding values in other kinds of ripening cheeses produced from pasteurised milk: Swiss-type (320 mg/kg; Petridis & Steinhart 1996) or Dutch-type (392 mg/kg; Komprda *et al.* 2008a). On the other hand, Roig-Sagués *et al.* (2002) reported maximum value of tyramine within the set of 44 samples of soft, semi-soft and hard traditional Spanish cheeses to be 1807 mg/kg; however, it is not apparent from the paper cited whether or not some cheeses were produced from unpasteurised milk.

Due to the great differences between people regarding the robustness of their detoxification system and possible synergistic effects of other biogenic amines, the toxicological limit for tyramine is difficult to establish. Among very different data having been published on the topic to date, the value of 100 mg/kg of food suggested in the review article of Silla-Santos (1996) is one of the more commonly used upper limits. Nine cheese samples from twelve tested in the present experiment (75%) can be considered unsafe from this viewpoint.

The median value of the histamine content (14 mg/kg; based on the figures in Table 2) in the blue cheese in the present experiment is comparable with the data of Novella-Rodríguez *et al.* (2003) regarding this type of cheese (7 mg/kg). However, the range of histamine values was much broader in the experiment by Novella-Rodríguez *et al.* (2003) (from not detected to 377 mg/kg) than in the present experiment (14–62 mg/kg), likely due to the fact that the cited authors evaluated cheese samples obtained from retail stores, presumably from different producers. In the present experiment, we used samples of blue cheese from only one producer (though the cheeses were produced in three different months). The histamine content exceeded 30 mg/kg in only two cheeses produced in October (Table 2). At any rate, no cheese tested in the present experiment exceeded the suggested (Silla-Santos *et al.* 1996) toxicological limit of 100 mg/kg.

Cadaverine was the second most abundant amine in the present experiment. Its concentration, simi-

Table 2. Biogenic amine and polyamine contents (mg/kg) in blue-vein cheese Niva at the end of ripening (49 days); mean ± standard error of the mean; $n = 3$

Amine	Cheese produced in											
	October				November				December			
	vat 1		vat 2		vat 3		vat 4		vat 5		vat 6	
	edge	core	edge	core	edge	core	edge	core	edge	core	edge	core
Tyr[1]	$411^{ab} \pm 313$	$875^{b} \pm 328$	$166^{ab} \pm 110$	$849^{b} \pm 367$	$66^{a} \pm 14$	$452^{ab} \pm 178$	$153^{ab} \pm 62$	$823^{b} \pm 22$	$10^{a} \pm 2$	$58^{a} \pm 39$	$158^{ab} \pm 65$	$553^{ab} \pm 178$
His[2]	$90^{b} \pm 48$	$0^{a} \pm 0$	$21^{ab} \pm 11$	$59^{ab} \pm 28$	$25^{ab} \pm 5$	$8^{ab} \pm 4$	$19^{ab} \pm 4$	$0^{a} \pm 0$	$14^{ab} \pm 3$	$0^{a} \pm 0$	$13^{ab} \pm 1$	$0^{ab} \pm 0$
Try[3]	$0^{a} \pm 0$	$0^{a} \pm 0$	$1^{a} \pm 1$	$6^{a} \pm 5$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$
Cad[4]	$132^{a} \pm 63$	$491^{a} \pm 285$	$45^{a} \pm 22$	$3^{a} \pm 2$	$222^{a} \pm 60$	$41^{a} \pm 9$	$235^{a} \pm 147$	$35^{a} \pm 5$	$68^{a} \pm 48$	$24^{a} \pm 19$	$66^{a} \pm 36$	$10^{a} \pm 5$
Phe[5]	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$
Put[6]	$9^{a} \pm 4$	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$13^{a} \pm 6$	$0^{a} \pm 0$	$24^{a} \pm 0$	$44^{a} \pm 33$	$3^{a} \pm 2$	$0^{a} \pm 0$	$117^{b} \pm 105$	$12^{a} \pm 1$
Spd[7]	$4^{a} \pm 3$	$0^{a} \pm 0$	$0^{a} \pm 0$	$29^{b} \pm 19$	$0^{a} \pm 0$	$8^{a} \pm 0$	$0^{a} \pm 0$	$18^{ab} \pm 17$	$0^{a} \pm 0$	$4^{a} \pm 2$	$0^{a} \pm 0$	$6^{a} \pm 2$
Spm[8]	$7^{ab} \pm 3$	$0^{a} \pm 0$	$4^{ab} \pm 3$	$0^{a} \pm 0$	$12^{b} \pm 2$	$3^{a} \pm 2$	$0^{a} \pm 0$	$11^{a} \pm 8$	$2^{a} \pm 1$	$0^{a} \pm 0$	$1^{a} \pm 1$	$0^{a} \pm 0$
ΣBA+ ΣPA[9]	$649^{ab} \pm 308$	$1366^{c} \pm 159$	$236^{a} \pm 128$	$946^{bc} \pm 280$	$339^{ab} \pm 79$	$512^{ab} \pm 165$	$287^{ab} \pm 189$	$620^{ab} \pm 310$	$97^{a} \pm 47$	$89^{a} \pm 28$	$355^{ab} \pm 199$	$581^{ab} \pm 174$

[1]tyramine; [2]histamine; [3]tryptamine; [4]cadaverine; [5]phenylethylamine; [6]putrescine; [7]spermidine; [8]spermine; [9]sum of biogenic amines (tyramine + histamine + tryptamine + cadaverine + phenylethylamine) + sum of polyamines (putrescine + spermidine + spermine)

a, b, c – means with different letters differ at $P < 0.01$

larly to that of tyramine, varied widely: between 3 mg/kg and 491 mg/kg (the mean 114, median 56 mg/kg). It follows from the data of Novella-Rodríguez *et al.* (2003) that, despite the relatively low median value 11 mg/kg, cadaverine can reach very high values in blue-vein cheese: more than 2100 mg/kg.

The level of other biogenic amines were very low in the present experiment, even at the end of ripening (Table 2).

The same was true regarding polyamines, with the exception of putrescine in the edge part of the vat 6-cheeses: 117 mg/kg (Table 2). The same putrescine concentration was found already in the vat 6-edge sample during the initial sampling (7th day of ripening). Putrescine (polyamines) can be synthesised both in mammalian tissues (their biosynthesis being regulated mainly by the activities of ornithine decarboxylase and S-adenosylmethionine decarboxylase; Hillary and Pegg 2003) and in microorganisms (alternative pathway involving L-arginine decarboxylase, which was not confirmed in mammals; Coleman *et al.* 2004). Considering the fact that the putrescine content in fresh milk is usually very low, its atypically high level (117 mg/kg) can be indicative of some hygienic error during the milk treatment before renneting.

The median of the putrescine content in the present experiment, 6 mg/kg (the mean being 18 mg/kg), is comparable with the data of (Novella-Rodríguez *et al.* 2003) for Spanish blue cheeses, 18 mg/kg. However, the quoted authors (Novella-Rodríguez *et al.* 2003) reported more than twice as high upper level of putrescine content (257 mg/kg) than was that found in the present experiment (117 mg/kg). The mean values of not only putrescine content, but also of spermidine and spermine contents found in the present experiment resemble the corresponding means measured in two varieties of Norwegian blue cheese, Saga and Normanna, in an experiment by Eliassen *et al.* (2002): 12 and 16, 15 and 24, and 2 and 0.4 mg/kg, respectively. Novella-Rodríguez *et al.* (2003) reported the highest ($P < 0.05$) spermidine content in blue cheese in comparison with all other tested unripened and ripened semi-hard and hard cheeses tested. The authors explained it by the known fact that mold cells can contain higher levels of polyamines. This could be the case of the *Penicillium roqueforti* mold in the experiment cited, but obviously not in the present experiment. The several times higher ($P < 0.01$) polyamines content in the P6-edge sample in comparison with that in the P6-core sample (Figure 4; it seems paradoxical from the above viewpoint, because *Penicillium roqueforti* spores are supposed to be present predominantly inside the cheese) is indicative of some other polyamine producers, possibly contaminant bacteria possessing enzyme equipment for a parallel polyamine biosynthesis pathway from arginine, via agmatine (not measured in the present experiment) as an intermediate (Sekowska *et al.* 1998).

As far as the comparison with other cheese varieties is concerned, it is interesting (regarding the data shown in Table 2) that we found very similar putrescine distribution among 16 different samples of the Dutch-type semi-hard cheese in our previous experiment (Komprda *et al.* 2008a): most samples below 40 mg/kg, and only one (significantly different) value slightly above 120 mg/kg.

Generally speaking, the content of polyamines was very low in most samples in the present experiment and neither harmful nor favourable (wound healing) effects on the consumers can be expected. The no-observed-adverse-effect levels (a measure of the chronic toxicity) for putrescine, spermidine, and spermine were reported to be 180, 83, and 19 mg/kg body weight per day, respectively (Til *et al.* 1997). Similarly, no harmful effect, due to the consumption of the blue cheese evaluated in the present experiment can be expected in cancer patients where the polyamine intake should be restricted (Eliassen *et al.* 2002).

However, polyamines content measured in the cited papers and in the present experiment cannot be properly evaluated before both the recommended dietary intake of polyamines (growth and development of the digestive system, wound healing) and the upper limit regarding cancer patients has been established by further research (Komprda *et al.* 2008a).

The sum of all amines measured (biogenic amines + polyamines) was in the range of 89 mg/kg (core part of one of the December cheeses) to 1366 mg/kg (core sample of one of the October cheeses) in the present experiment. According to the review of Silla-Santos (1996), the amine level of 1000 mg/kg food is considered dangerous for health. However, the toxicological level of amines is difficult to establish because it depends on individual characteristics and the presence of other amines. The synergistic effects are described with histamine and

other amines (Silla-Santos 1996). However, this was not the case in the present experiment, where the above-mentioned upper value (1366 mg/kg) concerned exclusively tyramine and cadaverine (Table 2). In any rate, if not the healthy consumers, then the patients consuming monoamine oxidase inhibitors should avoid the consumption of such a product.

The values of the sum of biogenic amines at the end of ripening (Figure 3) reflect tyramine and cadaverine contents shown in Table 2. Moreover, the trend of higher biogenic amine content in the core samples in comparison with the edge ones is apparent from Figure 3; the differences were significant (P < 0.01) in the cheeses produced in October.

As far as polyamines are concerned, the content of the sum of all polyamines at the end of ripening did not differ ($P > 0.05$) between cheeses, with the exception of the December vat 6-edge samples (Figure 4) that had a higher ($P < 0.01$) sum of polyamines content in comparison with all other cheeses. This was the consequence of the unusually high putrescine content in this cheese (117 mg/kg; Table 2).

The higher biogenic amine contents in the core parts of the cheeses as compared to those in the edge parts (Figure 3) reflect substantially higher tyramine contents in the C-samples and are rather deceptive as far as the comparison of the other individual amines is concerned. As follows from Figure 5, a higher ($P < 0.01$) content in the core-samples was found only in the case of tyramine. The concentrations of histamine, cadaverine, putrescine, and spermine at the end of ripening (after 49 days of ripening), were higher ($P < 0.01$) in the edge part of the cheese in comparison with the core samples, when all cheeses within all vats produced within all three intervals (October + November + December) were taken as one set.

The different distribution of polyamines (putrescine and spermine) within the cheese in the present experiment (Figure 5) disagrees with the findings by Novella-Rodríguez *et al.* (2003), who reported no difference in the polyamine content in relation to the part of the ripened semi-hard and hard cheese produced from goat milk, which the authors viewed as a reinforcement of the non-microbial origin of these substances.

As far as the distribution of tyramine is concerned, the results of the present experiment contributed to the inconsistence of the data reported in the available literature. Novella-Rodríguez *et al.* (2003) found much higher tyramine content inside ripened goat milk hard cheese than in the edge. The authors suggested that tyramine producers likely prefer anaerobic conditions prevailing in the internal parts of cheese. On the other hand, the tyramine content was higher in he outer parts of

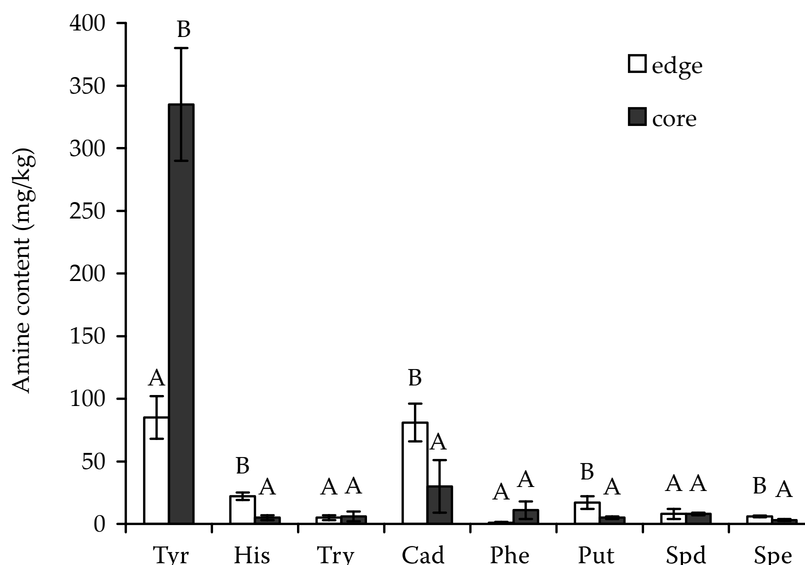

Figure 5. Comparison of the core and edge parts of the cheese at the end of ripening (after 49 days of ripening) regarding the content of particular biogenic amines and polyamines; all loaves from all vats produced within the whole production interval October–December were taken as one set, $n = 18$; A, B – means with different letters differ at $P < 0.01$; Tyr – tyramine, His – histamine, Try – tryptamine, Cad – cadaverine, Phe – phenylethylamine, Put – putrescine, Spd – spermidine, Spe – spermine

both the Swiss-type cheese (Petridis & Steinhart 1996) and the Dutch-type semi-hard cheese in an experiment by Komprda *et al.* (2008a).

The last-mentioned authors attributed this finding to the secondary enterococci contamination during the cheese production and/or to different environmental conditions in the cheese (counts of enterococci, which belong to the confirmed tyramine producers in cheese, were significantly higher in the outer parts of the cheese). To screen various *Penicillium roqueforti* strains from the viewpoint of their putative potential to decarboxylate tyrosine can be viewed as an objective for further research.

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Biogenic amine content in dry fermented sausages as influenced by a producer, spice mix, starter culture, sausage diameter and time of ripening

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ABSTRACT

Sixteen types of dry fermented sausages were commercially produced as combinations of two producers (designated K and R), two starter cultures (*Pediococcus pentosaceus*, C; *Lactobacillus curvatus* + *Staphylococcus carnosus*, F), two spicing mixtures (H; P) and two casing diameters (4.5 cm, T; 7 cm, W), and were sampled at days zero, 14, 28 (end of ripening), 49, 70, 91 and 112 (samples were stored at 15 °C and relative humidity of 70% between days 28 and 112). Tyramine and putrescine content (Y, mg kg^{-1}) increased ($P < 0.01$) with increasing time of ripening/storage (X, days): $Y = 52.0 + 5.19X - 0.0275X^2$ ($R^2 = 0.60$) and $Y = 37.0 + 3.45X - 0.0192X^2$ ($R^2 = 0.23$), respectively. Smaller diameter (T), spice mix containing red pepper (P) and starter culture C decreased ($P < 0.05$) both tyramine and putrescine content in the sausages as compared to the W, H and F counterparts, respectively; content of both amines was lower ($P < 0.05$) in the K-sausages than in the R-sausages. Tyramine content in the sausages at the time interval 28 days of ripening + 21 days of storage was in the range from 170 (KHCU sausage combination) to 382 (RHFS) mg kg^{-1}.

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1. Introduction

Biogenic amines (BA) are the low-molecular organic bases formed mainly by decarboxylation of amino acids (Silla Santos, 1996). Toxicologically most important BAs are tyramine and histamine. Tyramine is a potent vasoconstrictor; its higher levels in an organism can lead to hypertension and migraine and can induce brain haemorrhage and heart failure (Til, Falke, Prinsen, & Willems, 1997). Histamine (also vasoactive substance) can cause urticaria, hypotension, headache, flushing and abdominal cramps (Stratton, Hutkins, & Taylor, 1991). Tyramine and histamine are broken down in the mammalian organism by the oxidative deamination catalyzed by monoamine oxidase (MAO; EC 1.4.3.4.). However, human detoxification mechanisms can be insufficient in the case of too high BA intake in a diet, in the allergic individuals or in patients consuming drugs with an action of the MAO inhibitors (MAOI; some antiparkinsonian drugs and antidepressants).

Polyamines spermidine and spermine, including their precursor putrescine (chemically a diamine), are currently classified as a distinct group within BAs because they can be formed also by an alternative metabolic pathway (deureation instead of direct decarboxylation, with consequent incorporation of the aminopropyl groups by an action of *S*-adenosylmethionine; Wolter, Ulrich, & Stein, 2004) and due to their specific physiological and pathophysiological importance (Kalač & Krausová, 2005). Toxicological importance of polyamines is based on their ability to form stable carcinogenic *N*-nitroso compounds and to enhance the growth of aberrant crypt foci in the intestine (Paulsen, Reistad, Eliassen, Sjaastad, & Alexander, 1997). Polyamines are required for cell growth and proliferation, and are taken up by tumor cells. Putrescine stimulates tyrosine kinases and the expression of particular nuclear protooncogenes and is in this sense involved in cancer pathogenesis (Wolter et al., 2004).

Because dry fermented sausages meet the key prerequisites for BA formation (presence of free amino acids, presence of microorganisms with decarboxylase activities and conditions favourable for the growth of microorganisms; Silla Santos, 1996), they belong to the foods that can arouse safety concerns, especially regarding the above-mentioned risky groups of consumers. Consequently, several factors influencing BA content in dry fermented sausages have been considered, most of them summarized in the reviews of Suzzi and Gardini (2003) and Ruiz-Capillas and Jiménez-Colmenero (2004). Apart from quality of the raw materials (Eerola, Roig-Sagués, & Hirvi, 1998), including thawing time of raw materials (Maijala, Eerola, Lievonen, Hill, & Hirvi, 1995), the most important factor influencing BA content in fermented products is a starter culture (Gencellep, Kaban, & Kaya, 2007; González-Fernández, Santos, Jaime, & Rovira, 2003; Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués, Mariné-Font, & Vidal-Carou, 1997; Komprda, Neznalová, Standara, & Bover-Cid, 2001). As far as physicochemical parameters are concerned, following factors

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were evaluated: pH, a_W (Lorenzo, Martinez, Franco, & Carballo, 2008), high-pressure (Ruiz-Capillas, Jiménez Colmenero, Carrascosa, & Muñoz, 2007), ripening temperature (Maijala et al., 1995), storage temperature (Komprda et al., 2001), sausage diameter (Miguélez-Arrizado, Bover-Cid, Latorre-Moratalla, & Vidal-Carou, 2006). Moreover, several additives were tested from this viewpoint: sugar (Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 2001), NaCl (Lorenzo et al., 2008), sodium sulphite (Bover-Cid, Miguélez-Arrizado, & Vidal-Carou, 2001), nitrite (Gencellep et al., 2007).

In our previous experiment (Komprda et al. (2004)), we have suggested an influence of a spice mixture on BA production in dry fermented sausages, however without being able to distinguish between this effect and influence of a sausage diameter. Therefore the objective of the present study was to evaluate more properly an effect of a spice mixture and a sausage diameter, considering also an influence of different producers and starter cultures on biogenic amine content in commercially produced dry fermented sausages.

2. Materials and methods

2.1. Material

Two commercial dry sausage producers (designated "K" and "R") participated in the experiment. Each of them produced four 50 kg batches using in each case the same basic raw materials: lean beef meat, lean pork and pork fat in equal parts, nitrite salt mixture (2.5%) and dextrose (1%). The best hygienic conditions, including the storage of all raw materials at-5 °C for 48 h before beginning of the production were applied in all cases. Regarding a given producer, each of the four batches contained a unique combination of one spice mixture and one starter culture. The two starter cultures consisted of *Pediococcus pentosaceus* (Christian Hansen, Denmark; designated as "C") and *Lactobacillus curvatus* + *Staphylococcus carnosus* (Alfred Willich GmbH, Germany; starter "F"), respectively. Given starter was admixed to a particular batch in an amount of 25 g/50 kg of the product (both starter culture producers declared total counts of microorganisms to be 4.0×10^9 cfu g^{-1} of the starter; bacteria counts of 2×10^6 cfu g^{-1} of raw materials could therefore be expected). The two spice mixtures were as follows: spice mix used usually in a production of the typical Czech dry sausage "herkules" (mixture "H") and mixture used usually in a production of another Czech dry sausage "paprikáš" (designated in the experiment as "P"). The spice mixtures and starter cultures were applied according to the producers' instructions.

A half (25 kg) of chopped and blended ingredients of each batch was stuffed into the cutisin casing with external diameter 4.5 cm (thin, "T" sausages) and 7 cm (wide, "W" sausages), respectively (cutisin is a DEVRO group, Czech Republic trade mark for edible collagen casing). Weight of both types of sausages was approximately the same, about 1 kg (the T-sausages were somewhat longer than the W-ones). Regarding sausages of both producers, altogether 16 combinations of producer/spice/starter/diameter (KPFT – RHCW) were produced.

All products ripened in the same temperature/humidity conditions (including smoking by the cold smoke): 27 °C/92% 1st day, 20 °C/85% between days 2 and 12, 12 °C/70% until the 28th day (end of the ripening) and subsequently were stored at 15 °C/70% until the expiration of the declared date of the safe use, 112 days. Two 1 kg pieces of the each sausage variant (KPFT – RHCW) were sampled on days 0, 14, 28, 49, 70, 91 and 112. Microbiological analysis and determination of water activity (a_W) and pH was performed immediately after refrigerated transportation from the producer to the laboratory; 150 g of the sausage was homogenised in a Moulinex blender (Moulinex, France) and stored in the dark-glass powder bottles at −18 °C until the successive biogenic amines analyses. Material from the same part of the sausage was taken in each case regarding the particular type of analysis (biogenic amine, microbial, pH, dry matter, a_W). Each type of analysis (chemical, microbiological, physical) was carried out in duplicate for each sausage variant.

2.2. Biogenic amine determination

The sample was thawed overnight (at the temperature of + 5 °C), 10 g (±1 mg) was weighed into the 85 ml test tube, 0.5 ml of an internal standard (1,7-diaminoheptane; concentration 1 mg mL^{-1}) was added and the sample was extracted for two minutes with 15 ml of 5% trichloroacetic acid (TCA) using disintegrator Heidolph Diax 900 (Heidolph Instruments, Schwabach, Germany). Suspension was centrifuged at 755 g for 10 min at 4 °C (Hettich Universal 32R; Hettich, Germany). The supernatant was filtered through paper filter and the solid residue was repeatedly extracted as above. The combined extracts were made up to 50 ml with deionized water and filtered through a disposable nylon membrane filter (13 mm, 0.45 μm, Chromatography Research Supplies, Addison, USA). It follows from our previous paper (Smělá, Pechová, Komprda, Klejdus, & Kubáň, 2004) that extraction repeated three times is more efficient than the twice-repeated one, but the latter procedure is sufficient regarding recovery rates for single biogenic amines (78% for tryptamine to 117% for histamine) as found in other of our previous experiments (Komprda et al., 2004).

An extract was derivatized by dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride, DCl). The derivatizing agent was prepared by dissolving of 5 mg of dansyl chloride in 1 mL of acetone (Sigma–Aldrich, St. Louis, USA). The derivatization proceeded as follows: 1 mL of an extract (or standard) was mixed with 0.5 mL of saturated Na_2CO_3 (pH adjusted to 11.2), 1 mL of the derivatizing agent was added and the mixture was shaken for 1 min (MS2 Minishaker IKA; IKA Werke GmbH, Staufen, Germany). Derivatization proceeded for 1 h in dark at 40 °C, and the amine derivatives were extracted by diethylether (3×1 mL). The organic phase was evaporated to dryness under the nitrogen and the solid residue was dissolved in 0.5 mL of acetonitrile (ACN). The solution was filtered through the nylon membrane filter 0.45 μm (13 mm, 0.45 μm; Chromatography Research Supplies, Addison, USA) and injected onto the chromatographic column.

Biogenic amines were separated using liquid chromatograph HP 1100 (Agilent Technologies, Wilmington, USA) consisting of a quarternary pump (G1311A), vacuum degasser (G1322A), automatic sampler (G1313A) and UV/VIS detector with the variable wavelength (G1314A). Separation after DCl derivatization was carried out by gradient elution with H_2O/acetonitrile (time 0–23 min: H_2O 35–0%, acetonitrile 65–100%) on the Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, particle size 5 μm) with the guard column Meta Guard ODS-2 (30 mm × 4.6 mm, particle size 5 μm) at the flow rate 0.8 mL min^{-1} using photometric UV/Vis detector at 254 nm. The following BAs were determined: tyramine, histamine, phenylethylamine, cadaverine, putrescine, spermidine and spermine. The separated biogenic amines were identified by comparison of the retention times of the particular amines and amine standards after DCl derivatization (all the amine standards were used as the respective hydrochlorides supplied by Sigma–Aldrich), and their concentrations after DCl derivatization were expressed in mg kg^{-1} of original (fresh) sample (i.e. not on dry matter basis in order to better reflect the conditions of consumption; Latorre-Moratalla et al., 2008). Concentration of biogenic amines in the sample was corrected based on the method of internal standard according to Komprda et al. (2004). Limits of detection (LOD) and repeatability for individual amines were determined in our previous experiment (Smělá et al., 2004) using standard amine solutions in the

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range of 1.4 to 509.1 ng in the injection volume of 10 μg (as far as LOD is concerned), and as a relative standard deviation of ten times repeated measurement of the same sample (repeatability). LOD and repeatability was in the range of 0.0009 ng (putrescine) to 0.0015 ng (tyramine), and 2.30% (histamine) to 6.69% (putrescine), respectively.

2.3. Microbiological analysis

Immediately after being received at the laboratory, 10 g of the sausage were taken aseptically, homogenised in a stomacher with 90 mL of Ringer solution (Noack, Austria) preheated to 45 °C, and serial decimal dilutions were prepared. The following groups of microorganisms were determined: total aerobic and facultatively anaerobic counts (TAC) on Plate Count Agar (PCA; Biokar Diagnostics, France) after 72 h at 30 °C; lactic acid bacteria (LAB) on De Man–Rogosa–Sharpe medium (MRS; Biokar; 1 mL of an inoculum was poured over by the MRS medium, agar was let to fix and the second layer of MRS was applied in order to obtain microaerophilic conditions) after 72 h at 30 °C. Apart from LAB determination as a whole group, enterococci (which belong also to LAB) were enumerated separately on Slanetz–Bartley medium enriched with triphenyltetrazolium chloride (TTC; Noack, Czech Republic) after 48 h at 37 °C.

2.4. Testing the decarboxylase activities of the starter cultures

Each starter culture was inoculated into the liquid decarboxylase screening medium (DCM) according to Bover-Cid and Holzapfel (1999), containing tyrosine, histidine and ornithine disodium salts (1%). All cultures were inoculated in duplicate in DCM with and without (negative control) particular amino acids and incubated for 4 days at 37 °C. DCM positive samples (color transition to violet) after incubation were centrifuged (at 755*g* for 10 min at 4 °C; Hettich Universal 32R; Hettich, Germany), 1 ml of supernatant was mixed with 1 mL of 0.1 M HCl and 20 μL of 0.01 mg mL^{-1} internal standard solution 1,7-diaminoheptane (Sigma–Aldrich), the solution was vortexed using a mini-shaker (MS2 Minishaker, IKA Werke, Germany) and centrifuged again. The supernatant was filtered through nylon membrane filter (13 mm, 0.45 μm; Chromatography Research Supplies, Addison, USA) and tyramine, histamine and putrescine, respectively (if formed) was determined as previously mentioned.

2.5. Auxiliary analyses

Water activity was determined by AW Sprint TH-500 apparatus (Novasina, Switzerland) at 24.5 °C. Concentration of H^+ ions was measured in the filtrate of the 60 min water extract of 10 g of homogenised sample using WTW pH 95 apparatus (Weilheim, Germany). An ability of the components of the spice mixture P to inhibit the growth of LAB and enterococci, respectively, was tested using the agar diffusion test according to López-Malo Vigil, Palou, Parish, and Davidson (2005).

2.6. Statistical evaluation

All chemical and microbiological traits were measured in duplicate in each sausage. Means of these two measurements were used in statistical evaluation.

The Statistica 8 package (StatSoft Inc., Tulsa, OK, USA) was used for calculation of the basic statistical characteristics, regressions (including significance testing of the linear and quadratic terms, respectively), differences between the sets of sausages in biogenic amine content and microbial counts, respectively (one-way classification of the variance-ratio test, including *post hoc* Duncan's test), and for calculation of the correlations between particular traits. Percentage of variability in a content of quantitatively most important biogenic amines explained by the producer, starter culture, spice mix, diameter of the sausage and time of ripening/storage, respectively, was estimated (based on mean squares) by means of the multiple-way classification of the variance-ratio test (variance components analysis using general linear model with the fixed effects of independent variables).

3. Results and discussion

3.1. Changes of biogenic amines content and microbial counts during ripening/storage

The particular dependences were calculated based on the set of all sausages irrespective of producer, spice mix, starter culture and sausage diameter, respectively. Content of tyramine and putrescine significantly increased ($P < 0.01$) with increasing time of ripening/storage (Fig. 1). An inclusion of the quadratic term was significant ($P < 0.05$) in both cases. Because these two amines were quantitatively most important in the present experiment, content of the sum of all determined biogenic amines increased ($P < 0.001$) accordingly (Fig. 1). Also phenylethylamine content (Y, mg kg^{-1}) increased during ripening/storage (X, days; $Y = 3.9 + 0.32X - 0.0021X^2$, $R^2 = 0.15$, $P < 0.01$), but histamine content only tended ($P > 0.05$) to increase. Moreover, histamine content was very low in most samples (<5 mg kg^{-1}). However, there was important exception regarding histamine: its content increased from < 4 mg kg^{-1} to 63 mg kg^{-1} in the sample RHFS between days 28 (end of ripening) and 49. Despite the fact that TAC decreased in this sausage within this time interval (7.9–7.2 log cfu g^{-1}), increase (not significant, $P > 0.05$) of both LAB and enterococci counts (6.1–6.8 and 2.7–2.9 log cfu g^{-1}, respectively) was recorded. There were no manipulation with the sausages after ripening has been finished; all samples remained in a drying chamber until the end of the experiment (112th day). However, contamination of the particular product by adventitious LAB apparently cannot be excluded: Maijala and Eerola (1993) identified lactobacilli as histamine producers in dry sausages.

The dependences of counts of the tested microorganisms on the time of ripening/storage are presented in Fig. 2. LAB increased until the end of ripening and then decreased steadily until the end of the experiment ($P < 0.0001$). Interestingly, as follows from comparison of Figs. 3 and 4, which depicts the extreme variants (from the viewpoint of LAB and pH course) of the particular sausages (KPCW and RHFT), higher LAB counts (Fig. 3) paradoxically corresponded with higher pH values (Fig. 4). No relationship ($r = -0.08$, $P > 0.05$)

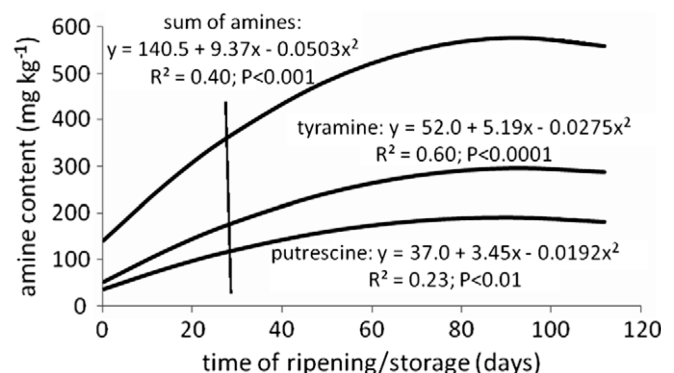

Fig. 1. Dependence of the content of quantitatively most important biogenic amines, including the sum of all determined amines, on the time of ripening/storage ($n = 224$); a vertical line indicates the end of ripening.

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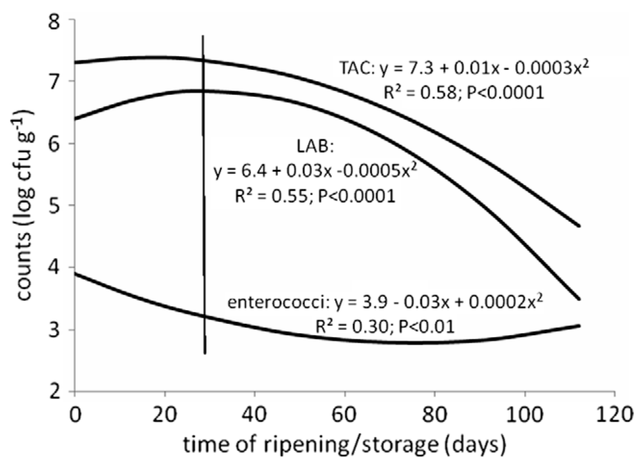

Fig. 2. Dependence of the counts of total aerobes + facultative anaerobes (TAC), lactic acid bacteria (LAB) and enterococci, respectively on the time of ripening/storage ($n = 224$); a vertical line indicates the end of ripening.

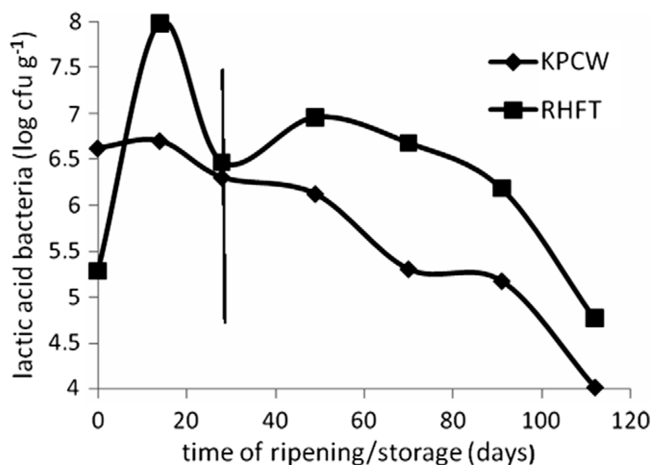

Fig. 3. Dependence of lactic acid bacteria (LAB) counts in the extreme (from the viewpoint of LAB course) sausage variants on the time of ripening/storage (KPCW – sausages of the producer K with spice mix used in the typical Czech dry sausage "paprikáš" and starter culture containing *Pediococcus pentosaceus*, diameter 4.5 cm; RHFT – sausages of the producer R with spice mix used in the typical Czech dry sausage "herkules" and starter culture containing *Lactobacillus curvatus* + *Staphylococcus carnosus*, diameter 7 cm); a vertical line indicates the end of ripening.

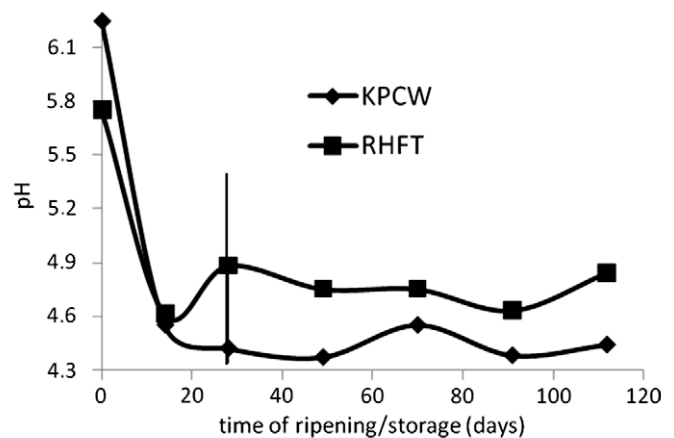

Fig. 4. pH course during ripening/storage; extreme (from the viewpoint of the pH course) sausage variants: KPCW – sausages of the producer K with spice mix used in the typical Czech dry sausage "paprikáš" and starter culture containing *Pediococcus pentosaceus*, diameter 4.5 cm; RHFT – sausages of the producer R with spice mix used in the typical Czech dry sausage "herkules" and starter culture containing *Lactobacillus curvatus* + *Staphylococcus carnosus*, diameter 7 cm; a vertical line indicates the end of ripening.

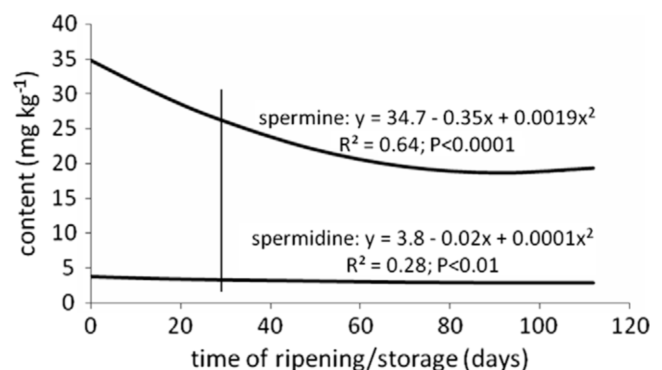

Fig. 5. Dependence of polyamines content on the time of ripening/storage ($n = 224$); a vertical line indicates the end of ripening.

between LAB counts and pH was found in the set of all sausages irrespective of a producer, spice mix, starter culture and sausage diameter. The same was true in the case of TAC. However, relatively strong positive correlation was found between pH and counts of enterococci ($r = 0.41$, $P < 0.01$). Despite nonexistent or positive relationships between counts of microorganisms and pH, significant negative correlation between pH and both tyramine and putrescine content was found ($r = -0.56$ and $r = -0.27$, respectively, $P < 0.01$). These data do not correspond with the results of Lorenzo et al. (2008), who, as far as pH is concerned, reported only positive correlations, namely with histamine and spermidine. On the other hand, according to Miguélez-Arrizado et al. (2006), who (similarly to the present experiment) found higher biogenic amine content in more acid than in low-acid sausages, acid sausages are *a priori* more intensively fermented and therefore higher BA levels could be expected. As far as water activity is concerned, results of Lorenzo et al. (2008) and the present experiment agree in significant negative correlation between a_W and tyramine content ($r = -0.32$, $P < 0.05$ in the present experiment).

Contamination of the raw materials by enterococci at the outset in the present experiment is apparent from Fig. 2. Significant differences ($P < 0.05$) between enterococci counts in particular sausage samples at the day zero were found (the range from 3.41 [RHCS sample] to 4.64 [RHFS] log cfu g^{-1}). Enterococci counts decreased until the day 70 and then slightly increased again.

Polyamines spermidine and spermine are not products of amino acid decarboxylation by microorganisms (in sausages), but are formed in a mammalian cell from putrescine (Wolter et al., 2004). Therefore they are already present in the raw materials and their content does not increase during ripening/storage, which was demonstrated also in the present experiment (Fig. 5).

Despite the fact that LAB and especially enterococci are proved biogenic amine producers in sausages (Ruiz-Capillas & Jiménez-Colmenero, 2004), poor correlation between content of quantitatively important biogenic amines on the one hand and TAC, LAB or enterococci counts on the other hand is apparent from comparison of Figs. 1 and 2. Correlation coefficient between tyramine content and TAC, LAB and enterococci counts was −0.19 ($P < 0.05$), −0.10 ($P > 0.05$) and −0.33 ($P < 0.05$), respectively. Regarding other biogenic amines, similar relationships (insignificant or even negative correlations) were demonstrated (data not shown). Latorre-Moratalla et al. (2008) were similarly not able to find a relationship between technological flora and the increase in BA content, and

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demonstrated that changes in BA content and spoilage microorganisms were in opposite direction using the principal component analysis. The above results are a confirmation of a known fact that ability to decarboxylate amino acids is not a faculty of groups or genera or even species of microorganisms, but only faculty of a small number of strains within a given species (Bover-Cid, Hugas, Izquierdo-Pulido, & Vidal-Carou, 2001a).

On the other hand, correlation coefficients between content of polyamines spermidine and spermine, and microbial counts were in the range + 0.25 to + 0.48 ($P < 0.05$) in the present experiment. However, these correlations are most probably coincidences (compare changes of spermine content during ripening/storage, Fig. 5 with corresponding changes of enterococci counts, Fig. 2), not causal connections.

3.2. Effect of producer, spice mix, starter culture and sausage diameter on biogenic amine content and microbial counts

To evaluate generalized effects of particular traits on biogenic amine content, the two sets of particular sausages within a given factor irrespective of all other parameters were compared in each case (e.g. all sausages of producer K were compared with all sausages of producer R irrespective of spice mix, starter culture, sausage diameter or time of ripening). Despite the fact that the averaging of the data over all time intervals is rather problematic, because biogenic amines content changes during ripening/storage (see Fig. 1), the comparison of the total means nevertheless provided interesting results (Fig. 6). It is apparent from Fig. 6, that each of the tested factors influenced content of qualitatively most important biogenic amines, tyramine and putrescine (with an exception of a producer in the case of putrescine, where only a tendency was found), respectively.

First of all, content of tyramine and putrescine was higher ($P < 0.05$) and tended to be higher ($P > 0.05$), respectively, in sausages of producer R in comparison with producer K. Miguélez-Arrizado et al. (2006) similarly reported that the processing plant (including technological factors and its characteristic in-house microbiota) had a significant effect on biogenic amine composition of Spanish fermented sausages.

Secondly, spice mix P decreased ($P < 0.05$) both tyramine and putrescine content in sausages as compared to H-spice mixture in the present experiment, which is a confirmation of our previous results (Komprda et al., 2004). Following components of the P-mixture can be considered to cause the effect (only components present in the sausages in an amount $\geqslant$ 50 mg kg^{-1} are listed): sweet pepper, sweet pepper oleoresine, chilli pepper, black pepper, garlic granules, caraway essential oil, coriander oleoresine, coriander oil, sodium isoascorbate (antioxidant). However, no substance tested in relevant concentrations was positive (formed no observable inhibition zone) on isolated LAB or enterococci colonies using an agar diffusion technique according to López-Malo Vigil et al. (2005). The same was true regarding each of the complete spice mixtures: no synergistic effect was observed.

As far as a starter culture is concerned, the culture with *P. pentosaceus* used in the present experiment decreased tyramine and putrescine content, respectively ($P < 0.05$) in comparison with starter containing *L. curvatus* + *S. carnosus*. However, despite declaration of the producers that their respective cultures are amino acid-decarboxylase negative, both starter cultures used in the present experiment were tyrosine-decarboxylase positive in decarboxylase screening medium; production of tyramine from tyrosine was confirmed by HPLC in both cases. On the other hand, neither starter formed histamine or putrescine in DCM. We have similarly found a very different ability of purportedly amino acid-decarboxylase negative starter cultures to decrease BA levels in dry sausages also in our previous experiment (Komprda et al., 2004). However, in a usual experimental design, either a truly amino acid-decarboxylase negative starter culture is compared with demonstrably positive culture, or BA formation in sausages with negative culture and without any starter culture is tested (Ayhan, Kolsarici, & Özkan, 1999; Bover-Cid, Hugas, Izquierdo-Pulido, & Vidal-Carou, 2000; Roig-Sagués & Eerola, 1997). Interestingly, Bover-Cid et al. (2000) reported that even positive tyramine producer (*L. curvatus* CTC 371) attenuated tyramine accumulation compared with the control without starter culture. On the other hand, according to Hernández-Jover et al. (1997), starter culture decreased BA production in fermented sausages, bud did not prevent it due to the naturally present background flora that still had a strong influence on BA formation. Nevertheless, Bover-Cid et al. (2000) underlined a

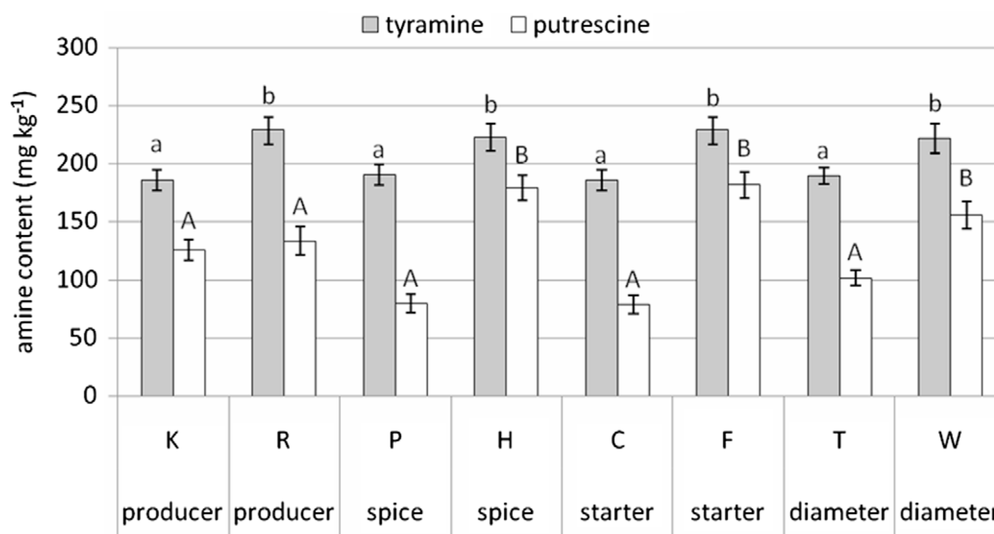

Fig. 6. Effect of the tested parameters (producer, spice mix, starter culture, sausage diameter) on the content of quantitatively most important biogenic amines; P – spice mix used in the typical Czech dry sausage "paprikáš"; H – spice mix used in the typical Czech dry sausage "herkules"; C – *Pediococcus pentosaceus*; F – *Lactobacillus curvatus* + *Staphylococcus carnosus*; T – 4.5 cm ("thin"); W – 7 cm ("wide"); a, b (tyramine) and A, B (putrescine), respectively – means with different letters within a given trait differ at $P < 0.05$; the particular means referring to a given parameter regard sets of all sausages within a given parameter irrespective of all other parameters, $n = 16$.

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necessity of an addition of a proper selected starter culture to produce safer sausages with low BA content; the proper strains from the group of LAB with a simultaneous rapid pH decrease are advisable (González-Fernández et al., 2003).

Finally, both tyramine and putrescine content in thinner sausages was lower ($P < 0.05$) than in the wider ones in the present experiment. In agreement with these results, Miguélez-Arrizado et al. (2006) reported that the diameter of the sausages correlated significantly with content of both tyramine and putrescine, the most abundant BAs in the quoted experiment. Both the results of Miguélez-Arrizado et al. (2006) and the present experiment are a confirmation of the hypothesis that the diameter is a factor that may affect the formation of BAs during sausage fermentation (Bover-Cid, Schoppen, Izquierdo-Pulido, & Vidal-Carou, 1999). The last quoted authors found that amine levels in the biggest diameter sausages were higher than in the thinnest ones, which is in accordance with our results (Fig. 6). A less distinctive preservative effect (i.e. decrease in water activity and increase in NaCl concentration) in the larger-diameter sausages and consequently lower reduction of the microbial growth has been suggested as a plausible explanation; therefore amine production by the present microorganisms could be more intense in comparison with thinner sausages (Bover-Cid et al., 1999). We did not measure salt concentration in the present experiment, but water activity was significantly ($P < 0.05$) higher in the W-sausages (0.863) than in the thinner ones (0.785), when all W- and T-samples, respectively, were considered as the whole sets irrespective of all other tested factors, including ripening/storage interval.

Based on the results of an estimate of the variance components using general linear model with the fixed effects of independent variables, producer, spice mixture, starter culture, sausage diameter and time of ripening/storage accounted for 17, 10, 17, 8 and 48% of explained variability of tyramine content in the sausages in the present experiment. In the case of putrescine, stronger effect of spice mixture (38% of explained variability) and starter culture (42%) was found at the expense of the time factor (8%); sausage diameter and producer accounted for 11 and 1% of explained variability of putrescine content, respectively.

As far as the sum of BAs is concerned, the similar conclusions as in the case of tyramine and putrescine are also valid in the present experiment: BAs content (averaged over the whole time interval, days zero to 112) tended to be lower ($P > 0.05$) in sausages of producer K (387 vs. 432 mg kg^{-1}), and was lower ($P < 0.05$) in P-sausages (333 vs. 486 mg kg^{-1}), in C-samples (323 vs. 496 mg kg^{-1}) and in the thinner sausages (356 vs. 457 mg kg^{-1}), respectively, as compared to the corresponding counterparts. Conclusions from the above results did not changed when spermidine and spermine were excluded from the calculation, because these polyamines originating from raw materials constituted in average only 7% of the sum of BAs when particular whole sets of samples irrespective of the ripening/storage period were considered. On the other hand, at the day zero (immediately after stuffing), spermidine + spermine represented 67% and 96% of all BAs in the sausages of the producer K and R, respectively; in this case, spermidine + spermine content in the samples of the producer R was higher ($P < 0.05$) in comparison with the producer K (45 vs. 37 mg kg^{-1}).

On the other hand, LAB counts were not influenced by any of the tested factors in the present experiment ($P > 0.05$; Fig. 7). However, enterococci counts were lower in sausages of the producer K ($P < 0.05$) in comparison with producer R, and in sausages with the C-starter as compared to the F-culture (Fig. 7).

3.3. Biogenic amine content and counts of microorganisms in particular sausages (producer/spice mix/starter culture/sausage diameter combinations)

Tyramine content at the end of ripening (28th day) was in the range from 147 (KHCU sausage) to 285 (RHFS sample) mg kg^{-1} (the differences were significant, $P < 0.05$). The mean was 197 mg kg^{-1}, which constituted 48% of all BAs determined. On the other hand, histamine content did not change at all at this time interval ($P > 0.05$) and was lower than 5 mg kg^{-1} in nearly all samples with only one exception (RHFS, 13 mg kg^{-1}). Phenylethylamine and cadaverine content changed ($P < 0.05$) from 3 and 1 to 34 and 68 mg kg^{-1}, respectively. After tyramine, quantitatively most important amine was putrescine, whose content differed ($P < 0.05$) widely at the end of ripening: 13 (RPCU)–320 (RHFS) mg kg^{-1} (the mean 144 mg kg^{-1}, 35% of the sum of BAs). Tyramine and putrescine content was therefore decisive for the sum of all amines, whose content was in the range from 240 (KPCS) to 711 (RHFS) mg kg^{-1} (the mean value 412 mg kg^{-1}). According to Latorre-Moratalla et al. (2008), who classified traditional

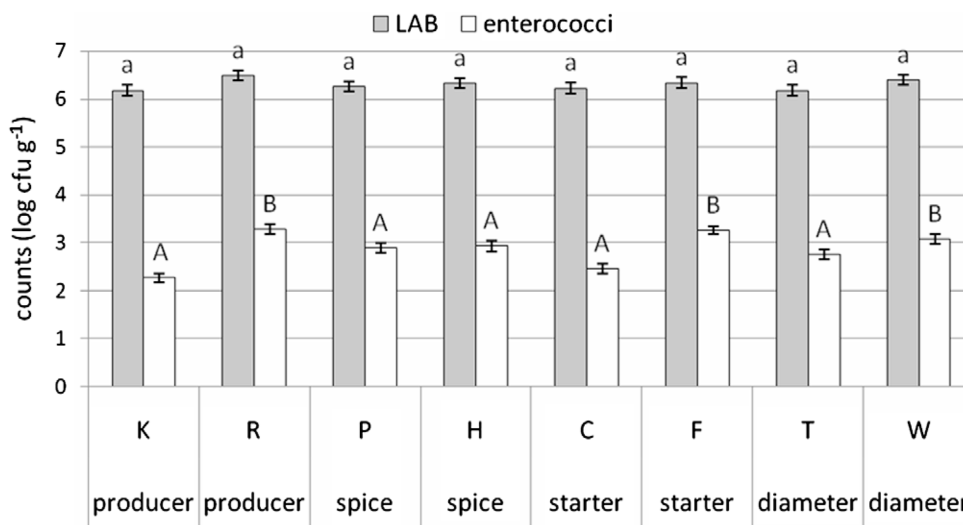

Fig. 7. Effect of the tested parameters (producer, spice mix, starter culture, sausage diameter) on the counts of lactic acid bacteria (LAB) and enterococci, respectively; P – spice mix used in the typical Czech dry sausage "paprikáš"; H – spice mix used in the typical Czech dry sausage "herkules"; C – *Pediococcus pentosaceus*; F – *Lactobacillus curvatus* + *Staphylococcus carnosus*; T – 4.5 cm; W – 7 cm; a (LAB) and A, B (enterococci), respectively – means with the same letters within a given trait do not differ ($P > 0.05$); the particular means referring to a given parameter regard sets of all sausages within a given parameter irrespective of all other parameters, $n = 16$.

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fermented sausages produced in selected European countries in five groups based on total biogenic amines content, the sausages of both the producer K and the producer R in the present experiment (the mean of the sum of BAs 396 and 437 mg kg^{-1}, respectively) would belong to group D characterized by high total BA content at the end of ripening (350–550 mg kg^{-1}). However, in the experiment of Latorre-Moratalla et al. (2008), the most abundant BA in this group was cadaverine, suggesting low hygienic standards in treating the raw materials and sausage manufacture. This clearly was not the case in the present experiment, where cadaverine constituted only 6% of total BAs. Relative representation of individual amines in the sum of BAs in the present experiment exactly corresponded to the group B in the experiment of Latorre-Moratalla et al. (2008), with tyramine as a major amine, putrescine the second, followed by cadaverine, histamine being practically absent.

The time interval 49 days (28 days of ripening + 21 days of the usual expiration date for dry fermented sausages) can be interesting from the consumer's viewpoint. Content of the two quantitatively most important biogenic amines, tyramine and putrescine after 49 days of ripening/storage is presented in Figs. 8 and 9, respectively. As it is apparent from comparison of Figs. 8 and 9, putrescine content varied more widely (13 [RPCU]–401 [RHFS] mg kg^{-1}) in comparison with tyramine (170 [KHCU]–382 [RHFS] mg kg^{-1}). The sum of all biogenic amines was in the range from 255 (RPCU) to 952 (RHFS) mg kg^{-1} at that time (49 days). Miguélez-Arrizado et al. (2006) similarly reported tyramine (average content 140 mg kg^{-1}) and putrescine as the major and the second major amine in Spanish fermented sausages (obtained from the commercial network), and found higher variability in putrescine content (RSD 134%) in comparison with tyramine (RSD 73%). The above values for tyramine in the present experiment is possible to compare with the results of our previous experiment testing the spice mixtures H and P (Komprda et al., 2004): tyramine content after 56 days (21 days of ripening + 35 days of storage) in the H- and P-sausages produced using two different starter cultures was 204 and 18 mg kg^{-1} of dry matter (DM), and 3 and

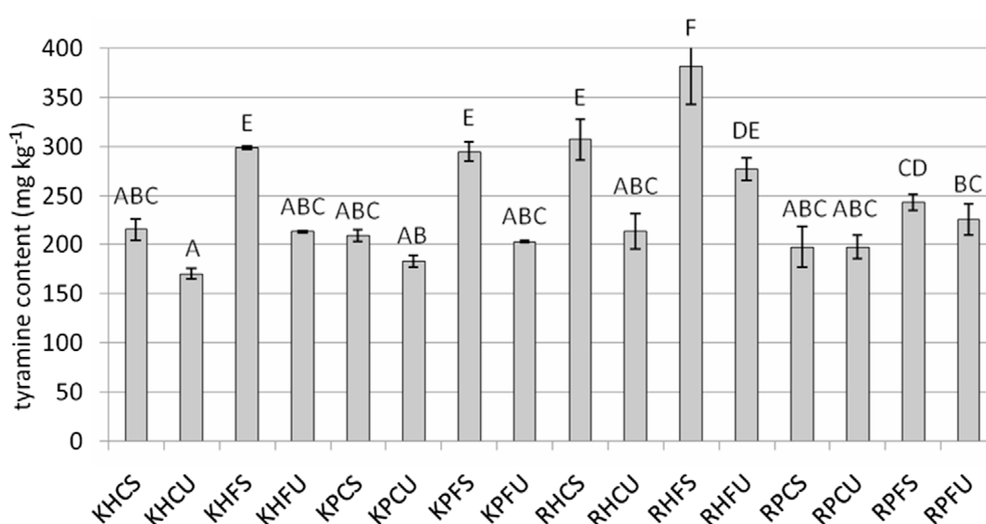

Fig. 8. Tyramine content after 28 days of ripening + 21 days of storage in dry sausages from two producers (K, R), produced in two diameters (thin, T – 4.5 cm; wide, W – 7 cm) using two different spice mixtures (P – spice mix used in the typical Czech dry sausage "paprikáš"; H – spice mix used in the typical Czech dry sausage "herkules") and two different starter cultures (C – *Pediococcus pentosaceus*; F – *Lactobacillus curvatus* + *Staphylococcus carnosus*); A – F: means with different letters differ at $P < 0.05$; $n = 2$.

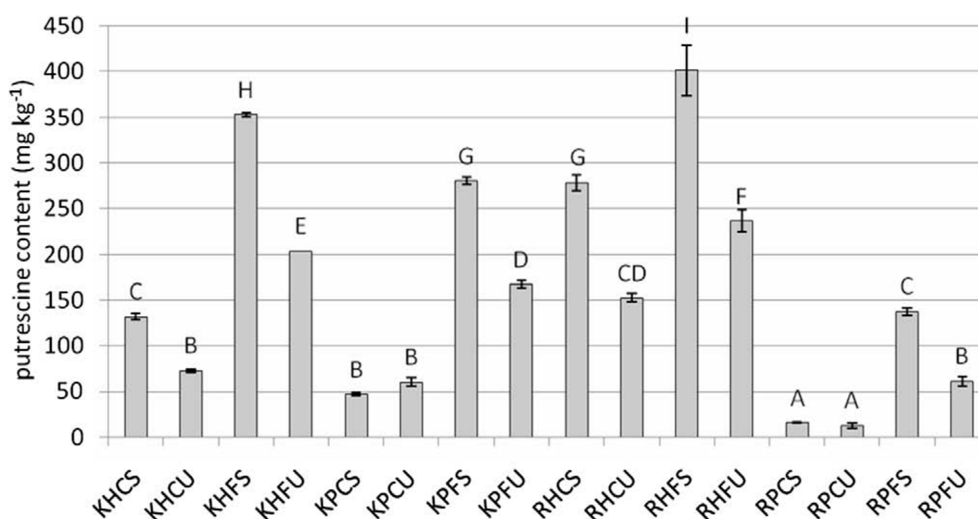

Fig. 9. Putrescine content after 28 days of ripening + 21 days of storage in dry sausages from two producers (K, R), produced in two diameters (thin, T – 4.5 cm; wide, W – 7 cm) using two different spice mixtures (P – spice mix used in the typical Czech dry sausage "paprikáš"; H – spice mix used in the typical Czech dry sausage "herkules") and two different starter cultures (C – *Pediococcus pentosaceus*; F – *Lactobacillus curvatus* + *Staphylococcus carnosus*); A – I: means with different letters differ at $P < 0.05$; $n = 2$.

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2 mg kg^{-1} DM, respectively. Strong effect of both spice and starter culture follows from the quoted results, which is in agreement with the Fig. 6 of the present experiment. Roseiro, Santos, Sol, Silva, and Fernandes (2006) found content of tyramine, putrescine and sum of BA in traditional Portuguese sausages after 40 days of drying/smoking 311, 779 and 2500 mg kg^{-1} DM, the most concentrated BA being cadaverine with 1254 mg kg^{-1} DM. Contrary to the results of Roseiro et al. (2006), cadaverine content after 49 days of ripening/storage was in the range from 2 (RPCU) to 92 (KPFS) in the present experiment, suggesting the good hygienic treatment of the raw materials before sausage manufacture.

As far as the toxicological importance of the above data is concerned, the only BA subjected to legal regulations in European Union is histamine (an upper limit in fish 100 mg kg^{-1}; EC, 2005). This value is not relevant in the present experiment due to the low histamine levels in the analyzed sausages. Regarding tyramine, toxicological limit is difficult to establish due to the wide inter-individual differences in a robustness of the detoxifying system (monoamine oxidase, MAO). Eerola, Roig-Sagués, Lilleberg, and Aalto (1997) reported tyramine toxicity threshold values in a wide range from 100 to 800 mg kg^{-1}. Food migraines were reported in susceptible individuals after ingestion of 100 mg of tyramine, patients treated with a new generation of the MAO-inhibitor drugs can tolerate 50–100 mg tyramine (Latorre-Moratalla et al., 2008). Taking into account the lower value (50 mg) and the sausage portion of 100 g, an upper limit 500 mg kg^{-1} can be considered. As follows from Fig. 8, no sausage in the present experiment would be hazardous food from this viewpoint. Putrescine (the second major BA in the present experiment) is not considered toxic itself, but can enhance the effect of tyramine by interfering with the detoxifying mechanism (Ruiz-Capillas & Jiménez-Colmenero, 2004).

Special attention is currently devoted to a group of polyamines: putrescine, spermidine and spermine (Kalač & Krausová, 2005; putrescine, chemically a diamine, ranks among polyamines as a precursor of spermidine and spermine). In a mammalian (human) organism, polyamines are taken up and used by the fast dividing tissues, which is important e.g. in wound healing. However, from the same reason (fast dividing cells), polyamines are concentrated in tumors. In both cases it is important to know an amount of polyamines in foods. Content of the sum of polyamines (putrescine + spermidine + spermine) after 49 days of ripening/storage varied between 34 (RPCU) and 421 (RHFS) mg kg^{-1}. For a comparison, Eliassen, Reistad, Risøen, and Rønning (2002) reported content of putrescine, spermidine and spermine in wiener sausages 1, 2 and 10 mg kg^{-1}, Hernández-Jover et al. (1997) in dry fermented Spanish sausage "chorizo" in the range 3–416, 2–10 and 14–44 mg kg^{-1}, respectively. Toxicological relevance of polyamines is even harder to evaluate than in the case of tyramine. An amount of polyamines found in the sausage samples in the present experiment cannot be properly assessed before further research elucidates both recommended dietary intake of polyamines (growth and development of the digestive system, wound healing) and the limit whose exceeding would have deleterious effects in cancer patients (Komprda, Burdychová, Dohnal, Cwiková, & Sládková, 2008).

Both producers participating in the present experiment guaranteed the safety and quality of their products until 84 days after ripening is finished (112 days in total). The range of tyramine, putrescine and sum of all biogenic amines content after 112 days of ripening/storage was as follows: 193 (KPCU)–548 (RHFS), 15 (RPCU)–442 (RHFS), and 285 (KPCU)–1149 (RHFS) mg kg^{-1}, respectively. Corresponding values in our previous experiment with similar sausages (Komprda et al., 2004) were 1–359, 1–601 and 18–1029 mg kg^{-1} DM. From this viewpoint, the most favourable combination of factors tested in the present experiment seems to be spice mixture P (containing red pepper) plus starter culture C plus smaller diameter of the sausages of the producer K. On the other pole stood the combination of the spice mixture H plus starter culture F plus larger diameter of the sausages of the producer R. These results are in a full agreement with the generalized data presented in Fig. 6.

4. Conclusions

It follows from the results of the present experiment that despite the proper hygienic treatment of the raw materials for the production of the tested sausages (indicated by low cadaverine content in the products), tyramine and putrescine content in the sausages can nevertheless reach relatively high values.

Biogenic amines content in the sausages can be modified by proper selection of a starter culture (negative amino acid-decarboxylase activity), spicing mixture and casing diameter (thinner sausages are advisable); also a producer (including in-house microbiota) has significant effect on BA production in the sausages. As far as a starter culture is concerned, the declaration of the commercial producer regarding decarboxylase activities can be unreliable.

Contamination of the batter by enterococci (established tyramine producers), probably from the production plant environment, seems inevitable. Moreover, secondary contamination of the sausages after ripening is finished by microorganisms capable of decarboxylating amino acids cannot be excluded. However, the changes in BA content in sausages cannot be accounted for by the counts of groups, genera or species of relevant microorganisms; amino acid-decarboxylase activity is a strain-specific faculty.

At the ripening/storage time interval relevant for consumers, tyramine content in dry fermented sausages seems currently (i.e. after launching on the market of a new generation of the monoamine oxidase-inhibitor drugs) to pose no threat even for risky groups of consumers. On the other hand, based on the current state of knowledge, it is not possible to properly evaluate the content in the sausages of the so called physiological polyamines (putrescine, spermidine, spermine) from the viewpoint of their either positive (wound healing) or negative (uptake by a tumor) effect.

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Publikace VI

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Tyrosine- and histidine-decarboxylase positive lactic acid bacteria and enterococci in dry fermented sausages

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ABSTRACT

Lactic acid bacteria (LAB) and enterococci were isolated immediately after stuffing (day 0), at the end of ripening (28th day) and at the end of storage (112th day) from dry fermented sausages produced by two different producers (K; R) in two diameters (4.5 and 7 cm) using either of two spice mixtures (P; H) and either of two starter cultures (*Pediococcus pentosaceus*, C; *Lactobacillus curvatus* + *Staphylococcus carnosus*, F), resulting in a total of 16 different combinations. Tyrosine-decarboxylase DNA sequence (*tyrdc*) was identified on average in 88% and 44% of enterococci and LAB isolates, respectively at the end of ripening, the corresponding figures regarding histidine-decarboxylase gene sequence (*hisdc*) was 71% and 16%, respectively. *Lactobacillus plantarum*, *L. brevis* and *L. casei*/*paracasei*, and *Enterococcus faecium* and *Enterococcus faecalis* were identified as tyramine/histamine producers in the sausages.
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1. Introduction

In fermented sausages, lactic acid bacteria (LAB), especially enterococci, are usually the main producers of tyramine (Gardini et al., 2008) and histamine, vasoactive biogenic amines (BA), which certain levels (corresponding to ingestion of 50–100 mg; Latorre-Moratalla et al., 2008) can cause hypertension, migraine, brain haemorrhage and heart failure (Til, Falke, Prinsen, & Willems, 1997), and urticaria, hypotension, headache, flushing and abdominal cramps (Stratton, Hutkins, & Taylor, 1991), in susceptible consumers.

Routine (i.e. only genus- or species-aimed) microbiological analysis is not suitable for early detection of particular tyramine- and histamine producers in fermented sausages, because only a fraction of the strains within a given microbial genus or species can decarboxylate tyrosine or histidine (Suzzi & Gardini, 2003). Molecular biology methods (PCR) are currently available to solve the task (Fernández, Linares, del Río, Ladero, & Alvarez, 2007; Coton & Coton, 2005).

Several factors influencing BA content in dry fermented sausages have been considered, most of them summarized in the reviews of Suzzi and Gardini (2003) and Ruiz-Capillas and Jiménez-Colmenero (2004): quality of the raw materials (Eerola, Roig-Sagués, & Hirvi, 1998), starter culture (Gencellep, Kaban, & Kaya, 2007), additives (Bozkurt & Erkmen, 2007), irradiation (Kim et al., 2005), high pressure treatment (de las Rivas et al., 2008), sausage diameter (Bover-Cid, Schoppen, Izquierdo-Pulido, & Vidal-Carou, 1999; Miguélez-Arrizado, Bover-Cid, Latorre-Moratalla, & Vidal-Carou, 2006) and possibly antimicrobials present in the spice mixture used in dry sausage production (Bozkurt, 2007).

The effect of producer, starter culture, spice mix and sausage diameter on BA content and total counts of lactic acid bacteria and enterococci, respectively was evaluated previously (Komprda, Sládková, & Dohnal, 2009). Using the same sausage samples (Komprda et al., 2009), the objectives of the present experiment were: 1) to assess in dry fermented sausages the effect of the four factors on the percentage of tyrosine-decarboxylase and histidine-decarboxylase positive LAB and enterococci isolates; 2) to compare ability of liquid decarboxylating medium and PCR-based identification of tyrosine-decarboxylase and histidine-decarboxylase gene sequences, to detect tyrosine-decarboxylase and histidine-decarboxylase positive LAB and enterococci in the sausages; 3) to perform genus and species identification of the positive isolates.

2. Materials and methods

The overall layout is presented in Fig. 1.

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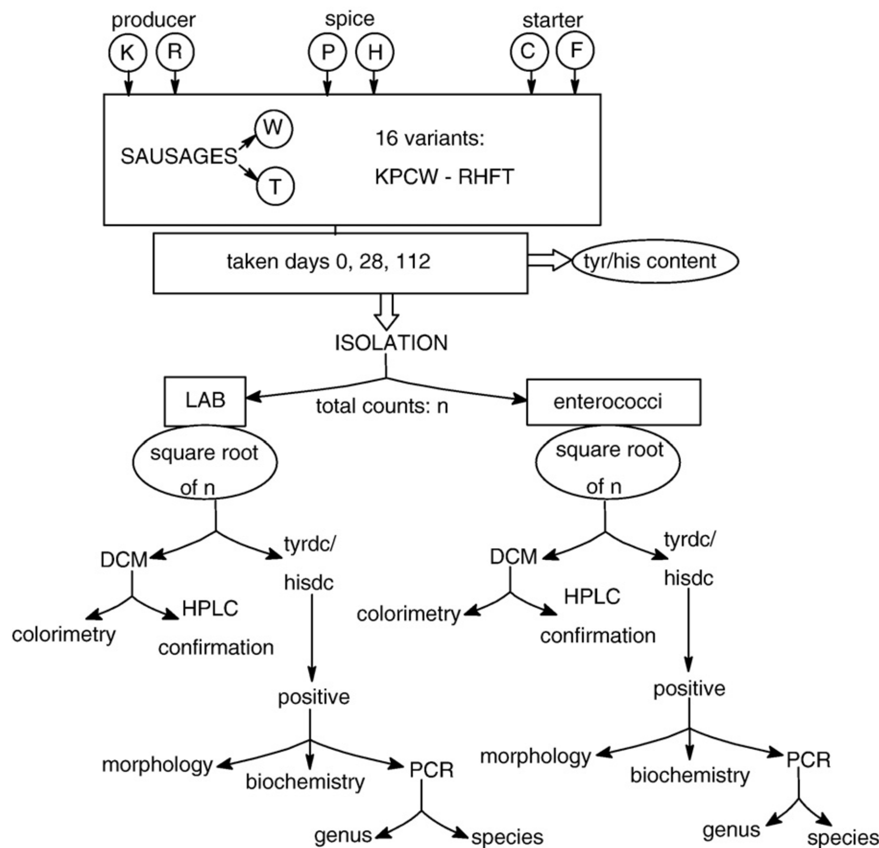

Fig. 1. Experiment layout (P — spice mix typical for Czech dry sausage "paprikáš"; H — spice mix typical for Czech dry sausage "herkules"; C — *Pediococcus pentosaceus*; F — *Lactobacillus curvatus* + *Staphylococcus carnosus*; T — diameter 4.5 cm; W — diameter 7 cm; tyr — tyramine; his — histamine; LAB — lactic acid bacteria; DCM — liquid decarboxylase medium according to Bover-Cid & Holzapfel, 1999).

2.1. Sausage production

The same sausage samples as in our previous paper (Komprda et al., 2009) were used. Two commercial dry sausage producers (designated "K" and "R") participated in the experiment. Two different starter cultures (*Pediococcus pentosaceus*, "C," Christian Hansen, Denmark; *Lactobacillus curvatus* + *Staphylococcus carnosus*, "F," Alfred Willich GmbH, Germany), and two spice mixtures: "P," typical for the Czech dry sausage "paprikáš" (basic components: sweet pepper, sweet pepper oleoresine, chili pepper, black pepper, garlic granules, caraway essential oil, coriander oleoresine, coriander oil); and "H," typical of another Czech dry sausage "herkules" (basic components: black pepper, caraway essential oil, garlic paste, coriander oil) were used. Each producer prepared four 50 kg batches using the same basic raw materials: lean beef meat (4% fat, 19.5% muscle protein, based on wet weight), lean pork (7% fat, 17% net muscle protein, based on wet weight) and pork fat (90% fat, 8% water) in equal parts, nitrite salt mixture (2.5%) and dextrose (1%); and a combination of one spice mixture and one starter culture. Starter culture was mixed to a particular batch as 25 g/50 kg (wet weight), spice mix was added at 850 g/50 kg of the product according to the producers' instructions. Half (25 kg) of chopped and blended ingredients of each batch (vacuum cutter PBV 200-1110; Gebhard, Germany) was stuffed into the edible collagen casing with external diameter 4.5 cm (thin, "T" sausages) and 7 cm (wide, "W" sausages), respectively. Altogether 16 variants — combinations of producer/spice/starter/diameter (KPCT–RHFW) were produced.

All products were ripened under the same temperature/relative humidity (RH)/time conditions (including smoking by cold smoke; chamber smoke house 2080 × 2395 × 2640 mm, two horizontal sections; KWU2 PSS Svidnik, Slovakia), which were: 27 °C/92% RH at day 1; 20 °C/85% RH until day 12; 12 °C/70% RH until day 28 (end of the ripening), and subsequently were stored at 15 °C/70% RH until day 112 (the "best before" date); though 15 °C is neither refrigeration nor ambient temperature, both sausage manufacturers use it routinely for storage of their products. Two 1 kg pieces of the each sausage variant (KPCT–RHFW) were taken at days 0 (stuffing), 28 and 112. Microbiological analysis was performed immediately after refrigerated transportation from the producer to the laboratory; 150 g of the sausage was homogenised in a Moulinex blender (Moulinex, France) and stored in dark-glass powder bottles at −18 °C until biogenic amine analyses. Each type of analysis (microbiological; chemical–biogenic amines; pH) was carried out in two replicates on each sausage.

2.2. Isolation of lactic acid bacteria and enterococci

Immediately after arrival at the laboratory, 10 grams of the sausage was taken aseptically, homogenized in a stomacher with 90 mL of Ringer solution (Noack, Austria) preheated (to facilitate fat homogenization) to 45 °C, and serial decimal dilutions were prepared. Lactic acid bacteria (LAB) were isolated using De Man–Rogosa–Sharpe medium (MRS; Biokar). 1 mL of the diluted solution was inoculated onto MRS agar by the pour-plate method with an MRS overlayer to provide microaerophilic conditions. Plates were incubated for 72 h at 30 °C. Apart from LAB determination as a whole group, enterococci were enumerated separately on Slanetz–Bartley medium enriched with triphenyltetrazolium chloride (TTC; Noack) after 48 h at 37 °C.

2.3. Testing tyrosine- and histidine-decarboxylase activity of isolated bacteria, including the starter cultures

Regarding sausage isolates, a number of LAB and enterococci colonies, equal to the square root of the total countable number

recorded on the Petri dishes were purified (i.e. colonies were repeatedly [3 times] streaked on the media described in Section 2.2). Purified sausage isolates and starter cultures (starter F was tested as a whole culture), were inoculated into the liquid decarboxylase screening medium (DCM) according to Bover-Cid and Holzapfel (1999), containing 1% of tyrosine (di-sodium salt) and histidine (free base). All isolates were inoculated in duplicate in DCM with and without (negative control) tyrosine and histidine and incubated for 4 days at 37 °C. Isolates were assessed as DCM-positive on the basis of color transition from yellow to violet.

2.4. HPLC confirmation of an ability of LAB and enterococci isolated from the sausages, and starter cultures to produce tyramine and histamine in DCM

Samples after incubation in DCM were centrifuged (755 ×*g* for 10 min at 4 °C; Hettich Universal 32R; Hettich, Germany), 1 mL of supernatant was mixed with 1 mL of 0.1 M HCl and 20 μL of internal standard (1,7-diaminoheptane; Sigma-Aldrich), the solution was vortexed on a mini-shaker (MS2 Minishaker, IKA Werke, Germany) and centrifuged again. The supernatant was filtered through a nylon membrane filter (13 mm, 0.45 μm; Chromatography Research Supplies, Addison, USA) and tyramine and histamine (if formed) were determined according to Komprda et al. (2009); briefly: samples were extracted by 5% trichloracetic acid, derivatized with dansyl chloride, separated by gradient elution with H_2O/acetonitrile on a Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, particle size 5 μm) using liquid chromatograph HP 1100 (Agilent Technologies, Wilmington, USA), and detected by a UV/VIS detector at 254 nm. Concentration of tyramine and histamine in the sausages were determined similarly.

2.5. DNA isolation, and PCR screening of the tyrosine-decarboxylase (tyrdc) and histidine-decarboxylase (hisdc) gene fragment

DNA was extracted (including all standard DNA manipulations according to Sambrook & Russel, 2001) from purified colonies of LAB and enterococci isolated from the sausages and the starter cultures. PCR was carried out in a final volume of 25 μL containing approximately 10 ng of genomic DNA, 10 pmol of primers, 1 U *Taq* DNA polymerase and an appropriate amount of Qiagen HotStar Master Mix (Qiagen, Hilden, Germany). The following primers (Coton & Coton, 2005) were used for *tyrdc* and *hisdc* detection, respectively: TD2 (5′-ACATAGTCAACCATRTTGAA-3′)/TD5 (5′-CAAATGGAAGAAGAAGTAGG-3′) and HDC3 (5′-GATGGTATTGTTTCKTATGA-3′)/HDC4 (5′-CAAACACCAGCATCTTC-3′). In each case, DNA was completely denatured by incubation at 94 °C for 15 min and subsequently amplified by 30 cycles of denaturation at 95 °C for 45 s, primer annealing at 52 °C for 45 s, and elongation at 72 °C for 75 s using a thermal cycler PTC-150HB (MJ Research, Waltham, USA). In the last amplification cycle, the samples were incubated at 72 °C for 10 min for complete elongation of the final PCR products. After PCR, amplified DNA fragments were separated by agarose gel electrophoresis (1% agarose; 5 V cm^{-1}; 60 min) in 1× TBE buffer and visualized by ethidium bromide staining.

2.6. Genus and species identification of tyrdc- and hisdc-positive isolates

The original LAB and enterococci colonies isolated from the sausages, in which *tyrdc* and *hisdc* was identified by PCR, were further specified using morphological traits, biochemical assays and PCR-based identification.

The isolates within the genus *Enterococcus* were identified using Gram staining, optical microscopy, growth on Columbia blood agar (Merck, Germany), catalase production, methyl-α-D-glukopyranoside utilization (MGP), pyruvate kinase production (PYU) and ENcoccus test (Pliva-Lachema, Brno, Czech Republic). Primer pairs E_1/E_2 (5′-ATC AAG TAC AGT TAG TCT-3′/3′-ACG ATT CAA AGC TAA CTG-5′; $ddl_{E.\ faecalis}$) and F_1/F_2 (5′-TAG AGA CAT TGA ATA TGC C-3′/3′-TCG AAT GTG CTA CAA TC-5′; $ddl_{E.\ faecium}$) according to Dutka-Malen, Evers, and Courvalin (1995) were used for species identification.

Gram staining, optical microscopy, morphology on the M17 medium (Noack, Austria) and MRS medium, and API-test (BioMerieux, France) were applied within the group of LAB. Presence of the genus *Lactobacillus* (the only identified genus within LAB) was confirmed by the PCR method using primers LbLMA1-rev (5′-CTC AAA ACT AAA CAA AGT TTC-3′) and R16-1 (5′-CTT GTA CAC ACC GCC CGT CA-3′) according to Dubernet, Desmasures, and Guéguen (2002); species identification within the genus *Lactobacillus* was performed using nucleotide sequences of the 16S to 23S spacer regions as described by Dubernet et al. (2002).

2.7. pH

pH was measured in the filtrate of the 60 min water extract of 10 g of the homogenized sample using WTW pH 95 apparatus (Weilheim, Germany).

2.8. Statistical evaluation

All chemical and microbiological traits were measured in duplicate in each sample. Means of these two measurements were used in statistical evaluation. The Statistica 8 package (StatSoft Inc., Tulsa, OK, USA) was used for calculation of the basic statistical characteristics, differences between the sets (one-way classification of the variance-ratio test, including *post hoc* Duncan's test), and for calculation of the correlations between particular traits.

3. Results and discussion

3.1. Tyramine- and histamine-forming LAB and enterococci

Counts of microorganisms and biogenic amine contents in the sausages were presented in the paper of Komprda et al. (2009) using the same sausage samples as in the present experiment. However, in order to make Tables 1–4 more informative, LAB and enterococci counts and tyramine/histamine contents are summarized as follows. At the end of ripening, LAB counts in P, H, K, R, C, F, T and W sausages were 6.7, 6.4, 6.6, 6.5, 6.4, 6.7, 6.5 and 6.6 log cfu g^{-1}; enterococci counts were 2.5, 2.6, 2.8, 2.2, 1.9, 3.0, 2.5 and 2.5 log cfu g^{-1}; tyramine contents were 188, 205, 175, 218, 178, 215, 188 and 205 mg kg^{-1}; and histamine contents 2, 3, 1, 3, 1, 3, 2 and 3 mg kg^{-1}. Corresponding values at the end of storage were as follows: LAB 3.9, 4.2, 3.9, 4.2, 4.1, 3.9, 4.0 and 4.0 log cfu g^{-1}; enterococci 2.6, 2.1, 2.5, 1.8, 1.7, 2.2, 2.0 and 2.3 log cfu g^{-1}; tyramine 267, 325, 274, 318, 260, 332, 239 and 353 mg kg^{-1}; histamine 1, 5, 1, 5, 1, 5, 1 and 5 mg kg^{-1}.

3.1.1. Spices ("P"; "H")

Percentage (from the 70–120 isolates tested within each set of sausages) of the strains of LAB and enterococci, respectively able to form tyramine and histamine did not differ ($P > 0.05$) between P- and H-sausages at the end of ripening (Table 1). However, at the end of storage (Table 2), percentage of the *Enterococcus* isolates both with HPLC-confirmed ability to form histamine in decarboxylating medium and possessing the gene sequence for histidine-decarboxylase was significantly higher in the P-sausages ($P < 0.05$), which demonstrated (in comparison with spice mix H) poor ability of spice mix P to inhibit growth of the decarboxylase-positive microorganisms in the present experiment. Based on the data of Komprda et al. (2004) which showed substantially lower biogenic amine contents in sausages produced using spice mix P in comparison with the H-sausages, the potential of spice mix P to inhibit growth of the decarboxylase-

Table 1
Effect of spice mix, producer, starter culture and sausage diameter on the percentage of strains of lactic acid bacteria and *Enterococcus* spp., with an ability to form tyramine and histamine in dry fermented sausages (% of the positive isolates[1]); end of ripening (28th day).

Microorganisms	Trait	Percentage of the positive isolates (differences between sets within a particular parameter)											
		Spice mix[2]		Difference[10]	Producer		Difference[10]	Starter[3]		Difference[10]	Diameter[4]		Difference[10]
		P	H		K	R		C	F		T	W	
Lactic acid bacteria	DCM[5]	18	19	NS	13	24	*	28	9	*	21	16	NS
	tyr-HPLC[6]	46	37	NS	46	36	NS	46	37	NS	39	44	NS
	tyrdc[7]	46	42	NS	49	38	NS	51	37	NS	40	47	NS
	his-HPLC[8]	4	13	NS	13	4	NS	0	16	NS	13	3	NS
	hisdc[9]	10	22	NS	13	19	NS	6	26	*	29	3	*
Enterococcus spp.	DCM[5]	68	82	NS	73	76	NS	90	60	*	85	65	NS
	tyr-HPLC[6]	78	88	NS	72	94	NS	67	100	*	91	75	NS
	tyrdc[7]	82	94	NS	81	94	NS	75	100	*	94	81	NS
	his-HPLC[8]	68	74	NS	57	85	*	88	55	*	83	59	*
	hisdc[9]	68	74	NS	57	85	*	87	55	*	83	59	*

[1] From the sequence of the decimal dilutions were chosen those with 20–60 countable colonies on an agar plate; square root of this number of colonies was randomly taken for testing; number of all sausage variants within a given parameter irrespective of all other parameters (e.g. all sausage combinations containing spice mix “P”) was 8, each variant was tested in duplicate (altogether 16 sausages), so the number of measurements varied in the range 16x√20–16x√60 (n = 70–120).
[2] P — spice mix used in the typical Czech dry sausage “paprikáš”; H — spice mix used in the typical Czech dry sausage “herkules.”
[3] C — *Pediococcus pentosaceus*; F — *Lactobacillus curvatus* + *Staphylococcus carnosus*.
[4] T — 4.5 cm; W — 7 cm.
[5] Liquid decarboxylase screening medium according to Bover-Cid and Holzapfel (1999) containing 1% of tyrosine (di-sodium salt) and histidine (free base), respectively.
[6] Confirmation of tyramine presence using the HPLC method.
[7] Presence of the tyrosine-decarboxylase gene sequence (primer pair according to Coton & Coton, 2005).
[8] Confirmation of histamine presence using the HPLC method.
[9] Presence of the histidine-decarboxylase gene sequence (primer pair according to Coton & Coton, 2005).
[10] NS — not significant; * — $P<0.05$.

positive microorganisms can be presumed. In this regard, the results of the present experiment cannot explain the published data.

3.1.2. Manufacturers (“R”; “H”)

More ($P<0.05$) *Enterococcus* spp. colonies with an ability to form histamine were isolated from the sausages of producer R compared with the K-sausages at the end of ripening (Table 1). This is contradictory to the tendency ($P>0.05$) of total enterococci counts to be lower in R-sausages (2.21 log cfu g^{-1}) than in the K-sausages (2.83 log cfu g^{-1}) at the end of ripening (the differences were significant immediately after stuffing: 3.86 vs. 4.23 log cfu g^{-1}, $P<0.05$). However, these differences in enterococci counts are not important from the toxicological viewpoint, because histamine concentrations were very low: 3 and 1 mg kg^{-1} in the R- and K-sausages, respectively at the end of ripening.

3.1.3. Starter cultures (“C”; “F”)

In the most cases, percentage of LAB was not influenced by starter culture (Tables 1 and 2); even the difference between the C- and F-sausages as far as confirmation of histamine using the HPLC method at the end of ripening was concerned (Table 1, parameter his-HPLC, C: F = 0:16) was not statistically significant ($P>0.05$) due to the high variability of his-HPLC positive LAB isolated from the F-sausages.

Table 2
Effect of spice mix, producer, starter culture and sausage diameter on the percentage of strains of lactic acid bacteria and *Enterococcus* spp., respectively with an ability to form tyramine and histamine in dry fermented sausages (% of the positive isolates[1]); end of storage (112th day).

Microorganisms	Trait	Percentage of the positive isolates (differences between sets within a particular parameter)											
		Spice mix[2]		Difference[10]	Producer		Difference[10]	Starter[3]		Difference[10]	Diameter[4]		Difference[10]
		P	H		K	R		C	F		T	W	
Lactic acid bacteria	DCM[5]	13	15	NS	9	19	NS	14	13	NS	15	13	NS
	tyr-HPLC[6]	17	31	NS	19	29	NS	32	16	NS	14	35	NS
	tyrdc[7]	30	34	NS	19	45	NS	39	25	NS	26	37	NS
	his-HPLC[8]	3	0	NS	3	0	NS	0	3	NS	0	3	NS
	hisdc[9]	8	22	NS	7	23	NS	10	20	NS	14	17	NS
Enterococcus spp.	DCM[5]	48	21	*	37	31	NS	13	56	*	45	23	NS
	tyr-HPLC[6]	53	48	NS	53	48	NS	13	89	*	57	44	NS
	tyrdc[7]	53	48	NS	53	48	NS	13	89	*	57	44	NS
	his-HPLC[8]	39	11	*	17	32	NS	6	43	*	28	22	NS
	hisdc[9]	39	11	*	17	32	NS	6	43	*	28	22	NS

[1] From the sequence of the decimal dilutions were chosen those with 20–60 countable colonies on an agar plate; square root of this number of colonies was randomly taken for testing; number of all sausage variants within a given parameter irrespective of all other parameters (e.g. all sausage combinations containing spice mix “P”) was 8, each variant was tested in duplicate (altogether 16 sausages), so the number of measurements varied in the range 16x√20–16x√60 (n = 70–120).
[2] P — spice mix used in the typical Czech dry sausage “paprikáš”; H — spice mix used in the typical Czech dry sausage “herkules.”
[3] C — *Pediococcus pentosaceus*; F — *Lactobacillus curvatus* + *Staphylococcus carnosus*.
[4] T — 4.5 cm; W — 7 cm.
[5] Liquid decarboxylase screening medium according to Bover-Cid and Holzapfel (1999) containing 1% of tyrosine (di-sodium salt) and histidine (free base), respectively.
[6] Confirmation of tyramine presence using the HPLC method.
[7] Presence of the tyrosine-decarboxylase gene sequence (primer pair according to Coton & Coton, 2005).
[8] Confirmation of histamine presence using the HPLC method.
[9] Presence of the histidine-decarboxylase gene sequence (primer pair according to Coton & Coton, 2005).
[10] NS — not significant; * — $P<0.05$.

Table 3
Tyramine and histamine-forming lactic acid bacteria present after 28 days of ripening in individual dry fermented sausages from the two producers (K, R), produced in two diameters (thin, T — 4.5 cm; wide, W — 7 cm) using two different spice mixtures (P — spice mix used in the typical Czech dry sausage "paprikáš"; H — spice mix used in the typical Czech dry sausage "herkules") and two different starter cultures (C — *Pediococcus pentosaceus*; F — *Lactobacillus curvatus* + *Staphylococcus carnosus*).

Sample	Percentage of the positive isolates[1]					Identification[7]
	DCM[2]	tyr-HPLC[3]	tyrdc[4]	his-HPLC[5]	hisdc[6]	
KPCT	30	71	71	0	0	*Lactobacillus plantarum*
KPCW	14	100	100	0	0	*L. plantarum; L. brevis*
KPFT	0	0	0	0	0	/
KPFW	12	67	67	0	0	*L. plantarum*
KHCT	16	0	0	0	0	/
KHCW	7	33	60	0	0	*L. plantarum*
KHFT	14	100	100	100	100	*L. brevis*
KHFW	8	0	0	0	0	/
RPCT	58	20	20	0	20	*L. casei/paracasei*
RPCW	11	40	40	0	0	*L. plantarum; L. brevis*
RPFT	13	67	67	33	33	*L. plantarum*
RPFW	12	0	0	25	25	*L. casei/paracasei; L. plantarum*
RHCT	40	50	63	0	25	*L. plantarum; L. brevis*
RHCW	50	50	50	0	0	*L. casei/paracasei*
RHFT	0	0	0	0	0	/
RHFW	15	60	60	0	0	*L. casei/paracasei*
Mean ± SEM	19 ± 4.3	41 ± 8.8	44 ± 8.9	10 ± 6.5	13 ± 6.5	

[1] From the sequence of the decimal dilutions were chosen those with 20–60 countable colonies on an agar plate; square root of this number of colonies was randomly taken for testing; each sample was tested in duplicate (two sausages within each of 16 variants KPCT–RHFW were taken), so the number of measurements varied in the range 2x√20–2x√60 (n = 10–15).
[2] Liquid decarboxylase screening medium according to Bover-Cid and Holzapfel (1999) containing 1% of tyrosine (di-sodium salt) and histidine (free base), respectively.
[3] Confirmation of tyramine presence using the HPLC method.
[4] Presence of the tyrosine-decarboxylase gene sequence (primer pair according to Coton & Coton, 2005).
[5] Confirmation of histamine presence using the HPLC method.
[6] Presence of the histidine-decarboxylase gene sequence (primer pair according to Coton & Coton, 2005).
[7] Gram staining, optical microscopy, morphology on the M17 medium and MRS medium, API-test, PCR (primer pairs according to Dubernet et al., 2002).

On the other hand, starter culture apparently influenced to the greatest extent the ratio of enterococci isolates able to form tyramine and histamine in the sausages. A higher ($P<0.05$) percentage of histamine-positive enterococci isolates was found in the C-sausages at the end of ripening (Table 1). An ability of the starter culture C to form histamine was thus proven. However, *P. pentosaceus* (starter culture C) was not isolated from the C-sausages at the end of ripening and could not therefore influence the results, and secondly, it was histamine-positive enterococci, not LAB, that were present in higher percentage in the C-sausages.

Table 4
Tyramine and histamine-forming enterococci present after 28 days of ripening in individual dry fermented sausages from the two producers (K, R), produced in two diameters (thin, T — 4.5 cm; wide, W — 7 cm) using two different spice mixtures (P — spice mix used in the typical Czech dry sausage "paprikáš"; H — spice mix used in the typical Czech dry sausage "herkules") and two different starter cultures (C — *Pediococcus pentosaceus*; F — *Lactobacillus curvatus* + *Staphylococcus carnosus*).

Sample	Percentage of the positive isolates[1]					Identification[7]
	DCM[2]	tyr-HPLC[3]	tyrdc[4]	his-HPLC[5]	hisdc[6]	
KPCT	43	25	50	100	100	*Enterococcus faecium; E. faecalis*
KPCW	100	50	50	50	50	*E. faecalis*
KPFT	67	100	100	50	50	*E. faecium*
KPFW	64	100	100	33	33	*E. faecium*
KHCT	100	100	100	100	100	Not identified
KHCW	75	0	50	50	50	*E. faecium*
KHFT	100	100	100	33	33	*E. faecium*
KHFW	63	100	100	40	40	*E. faecium*
RPCT	100	100	100	100	100	*E. faecium*
RPCW	100	50	50	100	100	*E. faecium*
RPFT	67	100	100	80	80	*E. faecium*
RPFW	0	100	100	33	33	*E. faecium*
RHCT	100	100	100	100	100	not identified
RHCW	100	100	100	100	100	*E. faecium*
RHFT	50	100	100	100	100	*E. faecium*
RHFW	17	100	100	67	67	*E. faecium*
Mean ± SEM	72 ± 8.0	83 ± 8.2	88 ± 5.6	71 ± 7.2	71 ± 7.2	

[1] From the sequence of the decimal dilutions were chosen those with 20–60 countable colonies on an agar plate; square root of this number of colonies was randomly taken for testing; each sample was tested in duplicate (two sausages within each of the 16 variants KPCT–RHFW were taken), so the number of measurements varied in the range 2x√20–2x√60 (n = 10–15).
[2] Liquid decarboxylase screening medium according to Bover-Cid and Holzapfel (1999) containing 1% of tyrosine (di-sodium salt) and histidine (free base), respectively.
[3] Confirmation of tyramine presence using the HPLC method.
[4] Presence of the tyrosine-decarboxylase gene sequence (primer pair according to Coton & Coton, 2005).
[5] Confirmation of histamine presence using the HPLC method.
[6] Presence of the histidine-decarboxylase gene sequence (primer pair according to Coton & Coton, 2005).
[7] Gram staining, optical microscopy, growth on Columbia blood agar, catalase production test, methyl-α-D-glukopyranoside utilization test, pyruvate kinase production test, ENcoccus test, PCR (primer pairs according to Dutka-Malen et al., 1995).

On the other hand, a higher ($P<0.05$) percentage of tyramine-positive enterococci isolates was found in the F-sausages both at the end of ripening (Table 1) and at the end of storage (Table 2). Again, despite the fact that de las Rivas et al. (2008) identified *L. curvatus* and *S. carnosus* (components of starter F in the present experiment) as main tyramine producers in Spanish dry-cured sausage, "chorizo," only *Lactobacillus plantarum*, *L. brevis* and *L. casei/paracasei* were identified as tyramine producers within the LAB in the present experiment (Table 3), and, as previously mentioned, the isolates in question were not LAB, but enterococci.

Because both sausage producers used starter culture F from the same source (as was true regarding starter C) the numbers presented in Tables 1 and 2 regarding starter culture were calculated as the total means of either starter irrespective of the other factors (producer, spice, diameter), a possible explanation for the differences between F- and C-sausages may be contamination of starter F by adventitious enterococci. This otherwise unlikely conclusion agrees to some extent with the data for cheese of Gelsomino, Vancanneyt, Cogan, Condon, and Swings (2002), who were unable to find the ultimate source of enterococci in their final product.

3.1.4. Diameter ("T"; "W")

An effect of sausage diameter on the percentage of the tyramine and histamine producing LAB and *Enterococcus* spp. isolates was manifested only in the sausages taken at the end of ripening (Table 1): higher percentages ($P<0.05$) of both *hisdc*-positive LAB, and histidine-HPLC-positive and *hisdc*-positive enterococci were isolated from the thinner sausages.

3.1.5. Comparison of the frequencies of amine-forming LAB and enterococci

A higher overall percentage of histamine and tyramine-positive-tested enterococci (the mean value calculated from all positive isolates irrespective of a given testing method was 78%) in comparison with positive-tested LAB (26%) is apparent from Table 1. Bover-Cid and Holzapfel (1999) identified 100% of tested *Enterococcus* strains and 40% of LAB strains as tyramine formers using the screening procedure used in the present study (DCM). This trend found in fermented sausages differs from that seen for fermented cheeses, where Komprda et al. (2008) found that corresponding percentages of tyramine-positive isolates tended to be lower in the case of enterococci compared to LAB.

Similar differences between enterococci and LAB also follow from Table 2, but in this case the percentages of positive isolates were substantially lower for both enterococci and LAB (mean values 37% and 17%, respectively). This trend was in a sense superposed on the general tendency of enterococci counts to decrease between the end of ripening and the end of storage (from 2.52 to 2.15 log cfu g^{-1}, $P>0.05$; based on the total set of sausages irrespective of producer, spice, starter culture and sausage diameter), and the significant decrease of LAB counts between these times (from 6.55 to 4.03 log cfu g^{-1}; $P<0.05$).

Substantially higher percentages of tyramine-positive LAB (both using the HPLC method and *tyrdc*) in comparison with histidine-positive LAB that follow from Tables 1 and 2, agree with de las Rivas et al. (2008), who detected 7.5% of the tested LAB (and staphylococci) strains as tyramine producers in the Spanish dry sausage chorizo, but no histamine producers. Similarly, Pircher, Bauer, and Paulsen (2007) identified only 4% of LAB isolates from fifty retail samples of fermented sausages as strong histamine producers, but 19% as strong tyramine producers.

LAB and enterococci species, respectively able to form tyramine and histamine in the particular sausages (combinations of the tested factors: producer–spice mix–starter culture–sausage diameter; KPCW–RHFS) are presented in Tables 3 and 4. Regarding LAB, only strains within genus *Lactobacillus* were identified as tyramine/histamine producers, namely *L. plantarum* (tyramine and histamine producer in 7 and 3 sausage variants, respectively, Table 4), *L. brevis* (4 and 2 cases, respectively), and *L. casei/paracasei* (3 and 2 sausage variants, respectively). These data only partially coincide with common species representation for (naturally) fermented dry sausages from different European countries (Rantsiou et al., 2005) namely *L. curvatus*, *L. plantarum* and *L. sakei*.

Contrary to lactobacilli, where strains within a given species were rarely producers of both tyramine and histamine in the present experiment, enterococci isolates belonging to the particular species (*Enterococcus faecium*, *Enterococcus faecalis*) formed both amines in each sausage (Table 4). The most active tyramine and histamine formers were strains of *E. faecium* (13 sausage variants). Pircher et al. (2007) identified *E. faecium* and *E. faecalis* as the most prolific strong tyramine producers within *Enterococcus* isolates from fifty retail sausages. As shown by Foulquié Moreno, Sarantinopoulos, Tsakalidou, and Vuyst (2006), *E. faecium* and *E. faecalis* are predominant isolates from pig carcasses, beef and pork cuts, processed meats and sausages.

3.2. Relationships between the traits

Significant ($P<0.05$) correlations between percentages of bacterial isolates testing positive in a given test (decarboxylase screening medium; HPLC confirmation of an ability to form tyramine and histamine in DCM; proof of the DNA sequences for tyrosine-decarboxylase and histidine-decarboxylase), counts of the tested bacteria in the sausages, and tyramine and histamine content in the sausages are presented in Table 5.

A high correlation between HPLC-confirmed ability of LAB/enterococci to form tyramine (histamine) and the presence of *tyrdc* (*hisdc*) is apparent from Table 5. On the other hand, the percentage of isolates possessing the tyrosine (histidine) decarboxylase gene sequences and percentage of isolates in which the genes were actually expressed differed. For example in Table 1, column "P," 82% of the *tyrdc*-positive enterococci isolates (the percentage relates directly to the number of colonies picked from the agar plates, Fig. 1) compared with only 53% of the tyr-HPLC positive enterococci isolates (78% from 68% of DCM-positive isolates; the percentage of tyr-HPLC or his-HPLC relates to the DCM-positive isolates, Fig. 1). The factors influencing expression of *tyrdc/hisdc* were not explicitly examined in the present study, but Gardini et al. (2008) reported reduced *tyrdc* expression in *E. faecalis* EF37 in dry fermented sausages with increasing amounts of sodium chloride and decreasing fermentation temperature; also an effect of glucose concentration was observed.

Lack of relationships between the LAB isolates positive in DCM and the other traits in LAB is seen in Table 5. This is contrary to Bover-Cid and Holzapfel (1999), who reported a good correlation of the decarboxylase screening method with amine-forming capacity using a HPLC procedure. However, the present data agrees with de las Rivas et al. (2008), who found discrepancies between the data obtained in decarboxylating medium (both false-positives and false-negatives) and confirmation of tyramine production by ion-exchange chromatography or the presence of the *tyrdc* gene, regarding tyramine producers isolated from Spanish dry-cured sausage "chorizo." Similarly, due to a high risk of the false-positive results using a decarboxylating broth, Buňková et al. (2009) recommend a combination of PCR/chromatography for detection of bacteria producing tyramine in foods.

At the end of storage, a significant correlation between tyramine content in the sausages and percentage of the PCR-determined *tyrdc*-positive LAB isolates ($r=+0.52$; Table 5) agrees with the results of Fernández et al. (2007) in cheese.

Other interesting data from Table 5 is the large set of negative relationships between percentages of enterococci isolates with a proven ability to form tyramine/histamine (in DCM; by HPLC; possessing *tyrdc/hisdc*) and tyramine/histamine contents in the

Table 5
Correlations between the tested traits; only significant (P<0.05) relationships are presented.

1st variable	2nd variable	Coefficient of correlation (r)			
		End of ripening (day 28)		End of storage (day 112)	
		LAB[1]	*Enterococcus* spp.[1]	LAB[1]	*Enterococcus* spp.[1]
%DCM+[2]	%tyr-HPLC+[3]	/	/	+0.60	/
	%his-HPLC+[4]	/	+0.38	/	+0.64
	%hisdc+[5]	/	+0.38	/	+0.64
	Tyramine[6] [mg kg^{-1}]	/	−0.39	/	−0.67
	Histamine[7] [mg kg^{-1}]	/	/	−0.40	−0.47
%tyr-HPLC+[3]	%tyrdc+[8]	+0.98	+0.95	+0.89	+0.98
	Tyramine[6] [mg kg^{-1}]	/	+0.51	/	/
	Enterococcus spp.[9] [log cfu g^{-1}]	/	/	−0.48	−0.82
%tyrdc+[8]	Tyramine[6] [mg kg^{-1}]	/	+0.52	+0.52	/
	LAB[10] [log cfu g^{-1}]	/	/	+0.55	/
	Enterococcus spp.[9] [log cfu g^{-1}]	/	/	−0.56	−0.82
%his-HPLC+[4]	%hisdc+[5]	+0.84	+0.98	/	+0.98
	Enterococcus spp.[9] [log cfu g^{-1}]	/	−0.40	+0.57	−0.56
%hisdc+[6]	*Enterococcus* spp.[9] [log cfu g^{-1}]	/	−0.40	/	−0.56

[1] Lactic acid bacteria (LAB) and *Enterococcus* spp. (EC) isolates, respectively tested positive in a given test (from the sequence of the decimal dilutions were chosen those with 20–60 countable colonies on an agar plate; square root of this number of colonies was randomly taken for testing; there were 16 sausage variants [KPCT–RHFW], each variant was taken in duplicate three times (days 0, 28, and 112), altogether 96 sausages were analyzed; number of measurements varied in the range 96x√20–96x√60, i.e. n = 480–670.
[2] Percentage of LAB and EC isolates, respectively tested positive in the decarboxylase screening medium (DCM) according to Bover-Cid, and Holzapfel (1999).
[3] Percentage of the LAB and EC isolates, respectively with HPLC-confirmed ability to form tyramine in DCM.
[4] Percentage of the LAB and EC isolates, respectively with HPLC-confirmed ability to form histamine in DCM.
[5] Percentage of the LAB and EC isolates, respectively possessing DNA sequences for histidine-decarboxylase (primer pair according to Coton & Coton, 2005).
[6] Tyramine content in the sausage samples, n = 16.
[7] Histamine content in the sausage samples, n = 16.
[8] Percentage of the LAB and EC isolates, respectively possessing DNA sequences for tyrosine-decarboxylase (primer pair according to Coton & Coton, 2005).
[9] *Enterococcus* spp. counts in the sausage samples, n = 16.
[10] Lactic acid bacteria counts in the sausage samples, n = 16.

sausages, and enterococci counts in the sausages. In other words, the less enterococci in the sausages, more of their isolates were able to form tyramine and histamine. This may be indicative of different optimal growth conditions for decarboxylase-positive enterococci isolates compared to other enterococci strains. Though this hypothesis was not tested it is in agreement with Pereira, Matos, San Romão, and Barreto Crespo (2009), who concluded, using *E. faecium* E17, that the decarboxylation pathway (producing proton motive force due to the membrane potential resulting from electrogenic transport of tyrosine in exchange for tyramine, and the pH gradient formed due to proton consumption in the decarboxylation reaction) gives the strain a competitive advantage in nutrient-depleted conditions and acidic environments, which contributes to higher counts in fermented food products.

No relationship (P>0.05) between LAB counts and pH was found, but a relatively strong positive correlation between pH and enterococci counts (r = 0.41; P<0.01) was found in the set of all sausages irrespective of a producer, spice mix, starter culture and sausage diameter. On the other hand, the relationship between pH and tyramine content was negative (r = −0.56; P<0.01); this corresponds with Miguélez-Arrizado et al. (2006), who reported that more acidic sausages are more intensively fermented and therefore higher biogenic amine levels can be expected.

4. Conclusions

A hypothesis regarding a presumed ability of the spice mix P to inhibit in sausages the growth of decarboxylase-positive microorganisms was not confirmed: the percentage of decarboxylase-positive enterococci isolated from the P-sausages was higher (P<0.05) compared to the H-sausages.

On average, substantially higher percentages of enterococci isolates were able to form tyramine/histamine in the sausages compared to lactic acid bacteria as a whole group.

Total enterococci counts isolated from the sausages were negatively correlated with percentages of DCM-positive, and *tyrdc*- or *hisdc*-positive enterococci isolates, which may be indicative of different abilities of decarboxylase-positive enterococci to grow in dry fermented sausages compared to other enterococci strains.

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Publikace VII

LABORATORNÍ PŘÍSTROJE A POSTUPY

VYUŽITÍ HPLC KE STANOVENÍ PRODUKTU EXPRESE GENU PRO MIKROBIÁLNÍ TYROSINDEKARBOXYLASU

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Klíčová slova: tyrosindekarboxylasa, biogenní aminy, tyramin, HPLC, PCR

Úvod

Tyrosindekarboxylasa je mikrobiální enzym ze třídy lyas, který katalyzuje přeměnu volné aminokyseliny tyrosinu v potravinovém substrátu na toxický biogenní amin tyramin. Mezi nejdůležitější druhy mikroorganismů s výraznou produkcí tyrosindekarboxylasy patří rody *Bacillus, Citrobacter, Clostridium, Escherichia, Klebsiella, Proteus, Pseudomonas, Salmonella* a *Shigella,* ale také mléčné bakterie rodu *Lactobacillus, Pediococcus a Streptococcus* a mnoho příslušníků čeledi *Enterobacteriaceae*[1].

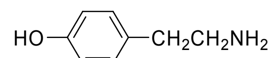
Enzym tyrosindekarboxylasa je v mikrobiálním genomu kódován specifickou DNA sekvencí, genem pro tyrosindekarboxylasu (*tyr*DC). Tato DNA sekvence je součástí genového klastru, který je kromě genu pro tyrosindekarboxylasu složen z genů pro tyrosyl-tRNA synthetasu (*tyr*RS), genu pro tyrosin permeasu (*tyr*P) a genu pro Na+/H+ antiporter (*nha*C). Molekulární detekce potenciálních producentů tyrosindekarboxylasy spočívá v identifikaci specifické *tyr*DC sekvence v genomu jednotlivých mikroorganismů.

Exprese genu pro *tyr*DC je řízena promotorem, umístěným proti směru jeho transkripce. Produkt exprese, enzym tyrosindekarboxylasa, může být nepřímo detegován stanovením obsahu tyraminu ve vzorku, v němž je přítomen mikroorganismus s dekarboxylasovou aktivitou. Pro přesné stanovení je využíváno optimální živné médium pro konkrétní mikroorganismus s tyrosindekarboxylasovou aktivitou s přesně definovaným množstvím tyrosinu, substrátu pro enzymovou katalytickou přeměnu tyrosinu na biogenní amin tyramin. Při stanovení tyraminu je využívána řada separačních analytických metod, nejčastěji vysokotlaká kapalinová chromatografie (HPLC), v některých případech je nutné použít micelární elektrokinetickou kapilární chromatografii. Vzhledem k absenci chromoforu je nutné stanovovaný analyt před vlastní detekcí derivatizovat, což je možné uskutečnit buď před nástřikem na kolonu nebo až po jeho výstupu z kolony. Nejčastěji používanými derivatizačními činidly jsou dansylchlorid (5-dimethylaminonaftalen-1-sulfonylchlorid) a *o*-ftaldialdehyd[1].

Tyramin (*p*-hydroxyfenylethylamin) je aromatický biogenní amin (obr. 1), který je ve výživě člověka důležitý jako zdroj dusíku a prekurzor pro syntézu hormonů, alkaloidů, nukleových kyselin a bílkovin[2].

Ačkoli je tyramin nepostradatelný pro zajištění řady velmi důležitých životních funkcí, může konzumace potraviny obsahující vysoké koncentrace této látky vyvolat toxický účinek, nejčastěji vazoaktivní, konkrétně vazokonstrikční[3]. Toxické dávky tyraminu je obtížné stanovit, velmi totiž záleží na individuálních rozdílech mezi lidmi a na přítomnosti různých biogenních aminů v potravině (potenciace). Pro tyramin je toxická dávka asi 20–80 mg kg^{-1} potraviny.

Zatím v žádné zemi neurčuje legislativa výrobcům deklarovat obsah tyraminu na obalu. Takovýto údaj by jistě ocenili především jedinci trpící migrénami, dietologové a alergici. Pro výrobce by však tato povinnost byla značně obtížná, což vyplývá z velkého kolísání obsahu tyraminu v rámci určitého druhu potraviny. Výzkum proto vyvíjí nejen metody pro stanovení tyraminu v potravinách, ale i jednoduché, rychlé a levné metody pro odhalení příčin jeho produkce.



Obr. 1. **Tyramin (*p*-hydroxyfenylethylamin)**

Experimentální část

Bakteriální kmeny

Bakteriální buňky *Enterococcus faecalis* CNRZ 238 (cit.[4]) byly kultivovány na médiu s kanamycinem, eskulinem a azidem sodným (Merck, Německo) při 37 °C. Kultura byla použita jako pozitivní kontrola pro PCR a po kultivaci v dekarboxylačním médiu s tyrosinem i jako pozitivní kontrola pro HPLC analýzu.

Startovací kultury určené pro výrobu fermentovaných tepelně neopracovaných trvanlivých salámů (BioCarna, Německo) byly kultivovány na PCA, MRS a M17 agaru

Tabulka I
Použité startovací kultury pro výrobu fermentovaných tepelně neopracovaných masných výrobků, výrobce BioCarna

Označení startovací kultury výrobcem	Složení startovacích kultur (bakteriální druhy)	Detetekce DNA sekvencí pro tyrosindekarboxylasu	HPLC detekce tyraminu [mg l^{-1} média]
PPX	*Pediococcus pentosaceus*	–[a]	–[c]
	Staphylococcus xylosus	–	–
SG1	*Staphylococcus carnosus*	–	–
	Streptomyces griseus	–	–
SC1	*Staphylococcus carnosus*	–	–
	Lactobacillus curvatus	+[b]	0,45 ± 0,01
PLS	*Pediococcus acidilactici*	–	–
	Staphylococcus carnosus	–	–
	Lactobacillus plantarum	–	–
CXK	*Staphylococcus carnosus*	–	–
	Staphylococcus xylosus	–	–
	Lactobacillus sakei	+	0,40 ± 0,01
PPLX	*Pediococcus pentosaceus*	–	–
	Lactobacillus plantarum	–	–
	Staphylococcus xylosus	–	–
LG1	*Staphylococcus carnosus*	–	–
	Lactobacillus plantarum	–	–
	Streptomyces griseus	–	–

[a] Sekvence nedetegována, [b] sekvence detegována, [c] tyramin nedetegován

(Merck, Německo). Pro rozlišení a identifikaci jednotlivých mikrobiálních druhů byly použity komerční API testy (Biomerieux, Francie), katalasa test a Gramovo barvení. Složení použitých startovacích kultur je uvedeno v tabulce I.

Izolace DNA a PCR screening

Lyze buněk, izolace DNA z bakteriálních kultur, její purifikace a způsob kontroly koncentrace a čistoty byly provedeny dle autorů[5,6]. Pro amplifikaci specifické DNA sekvence kódující bakteriální tyrosindekarboxylasu byla použita dvojice specifických oligonukleotidových primerů TD2/TD5 (cit.[7]). Gen pro tyrosindekarboxylasu byl amplifikován pomocí polymerázové řetězové reakce (PCR). PCR byla provedena v termocykleru PTC 130 (MJ Research, Waltham, MA, USA) v celkovém objemu 25 μl a obsahovala 10 ng purifikované DNA, 10 pmol každého primeru TD2/TD5, 1 U HotStar *Taq* DNA polymerasy a příslušné množství optimalizované směsi specifických komponent pro PCR (HotStar Master Mix, Qiagen, Hilden, Německo). Templátové DNA byly nejprve denaturovány inkubací při 95 °C 15 min. DNA byla amplifikována ve 30 cyklech (denaturace při 95 °C 45 s, hybridizace primerů při 52 °C po dobu 45 s a syntéza komplementárního DNA řetězce při 72 °C 75 s). V posledním amplifikačním cyklu byla teplota 72 °C prodloužena na 10 min, a to pro kompletní syntetizování finálního PCR produktu. PCR produkty byly vizualizovány pomocí agarosové gelové elektroforézy na přístroji Easy, model B1 (Owl Scientific, USA) a vizualizovány na UV transluminátoru (EB-20E Ultralum, USA) po barvení ethidium bromidem (0,5 μg ml^{-1}). Jako pozitivní kontrola PCR byla použita DNA purifikovaná z buněk *Enterococcus faecalis* CNRZ 238, jako negativní kontrola PCR byly použity komponenty PCR bez DNA.

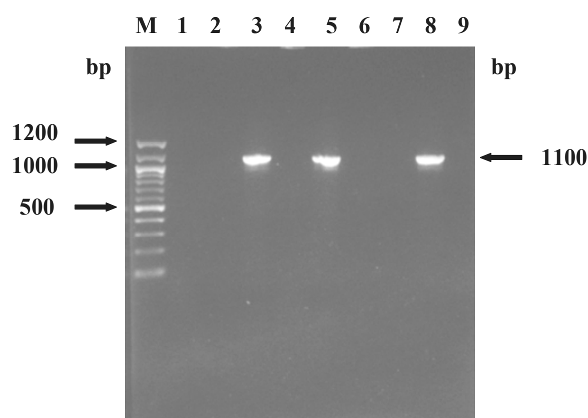
Stanovení tyraminu

Bakteriální kmeny byly kultivovány v dekarboxylačním médiu[8] s tyrosinem (10 g l^{-1} média) při 37 °C 48 h. Mikrobiální produkce tyraminu byla potvrzena analýzou HPLC a vzorky byly připraveny modifikovanou metodou[9]. Kultivační média byla po kultivaci centrifugována při 3000 otáčkách/min po dobu 10 min a při teplotě 4 °C na centrifuze Hettich Universal 32R, Německo. Supernatant (1 ml) byl smíchán s 1 ml 0,1 M kyseliny chlorovodíkové a 20 μl interního standardu 1,7-diaminoheptanu (Sigma-Aldrich, Německo). Směs byla promíchána (MS2 Minishaker, IKA Werke, Germany) a znovu centrifugována. Supernatant byl filtrován přes 0,45 μm nylonovou membránu. Filtrát byl derivatizován *o*-ftalaldehydem v borátovém pufru (pH 9,5) (Sigma-Aldrich, Německo) za přítomnosti 2-sulfonylethan-1-olu (Merck, Německo). Stej-

ným způsobem byl zpracován i standard tyraminu (Sigma-Aldrich, Německo).

Vzorky byly podrobeny chromatografické analýze použitím Agilent HP 1100 systému (Agilent, Německo), který byl složen z vakuové odplyňovací jednotky (G1322A), kvarterní pumpy (G1311A), automatického dávkovače (G1313A) a fluorescenčního detektoru (G1321A). Pro analýzu byla dále použita Zorbax XDB C8 kolona (4,6 × 150 mm, velikost částic 5 μm) s předkolonou Meta Guard Inertsil C18 (4,6 × 30 μm, velikost částic 5 μm) a metoda vyvinutá v naší laboratoři[9]. Ke gradientové eluci tyraminu byla použita mobilní fáze složená z 0,1 M acetátového pufru, A (pH 5,8) a acetonitrilu, ACN (Merck, Německo) (čas 0–27 min: A 60–23 %, ACN 40–77 %) při průtokové rychlosti 0,6 ml min^{-1}.

Kvalitativně byl tyramin ve vzorcích stanoven porovnáním retenčních časů jednotlivých vzorků a retenčního času standardu. Kvantitativní zastoupení tyraminu bylo vypočteno z plochy specifického píku a využitím kalibrační křivky.



Obr.2. **PCR detekce *tyr*DC pozitivních startovacích kultur; M: 100 bp ladder (New England Biolabs), běh č. 1–7: startovací kultury (sestupné pořadí tab. I), běh č. 8: pozitivní kontrola – *E. faecalis* CNRZ 238, běh č. 9: negativní kontrola (komponenty PCR bez DNA)**

Výsledky a diskuse

Většina odborníků se domnívá, že startovací kultury mohou zvýšit obsah biogenních aminů ve finálním výrobku. Z hlediska prevence vzniku biogenních aminů v potravinách je tedy nutné volit takové mikroorganismy, které netvoří biogenní aminy. Zároveň by startovací kultura měla být schopná potlačit konkurenční mikroflóru tvořící biogenní aminy[10,11]. Pro odhalení potenciálních producentů biogenních aminů musí být definovány vhodné skríningové metody. V této práci byla optimalizována metoda pro typizaci mikroorganismů produkujících tyramin. Tato metoda může být použita nejen pro skríning startovacích kultur používaných pro výrobu fermentovaných potravin, ale i pro odhalení jakýchkoli jiných mikroorganismů tvořících tyramin, např. z řad kontaminující a doprovodné mikroflóry.

Při nepřímé detekci biogenního aminu tyraminu byly využity metody molekulární biologie v kombinaci s analytickými metodami. Specifita polymerázové řetězové reakce (PCR) pro detekci specifické sekvence genu pro tyrosindekarboxylasu byla ověřena použitím purifikované DNA buněk *Enterococcus faecalis* CNRZ 238, u nichž byla prokázána[7] produkce tyrosindekarboxylasy. V reakci byl amplifikován PCR produkt o předpokládané velikosti (1100 bp).

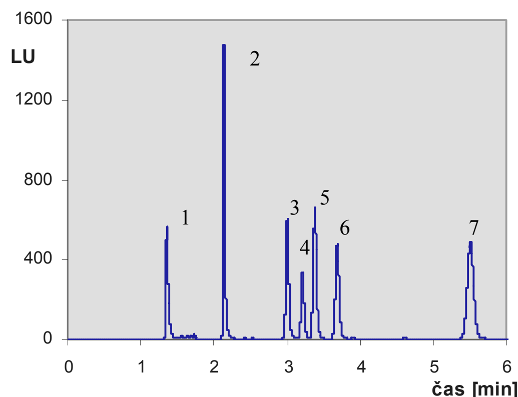
Po ověření specificity a funkčnosti byla PCR použita pro skríning startovacích kultur používaných pro výrobu fermentovaných tepelně neopracovaných trvanlivých salámů. Přehled analyzovaných startovacích kultur uvádí tabulka I.

Z čistých kultur jednotlivých bakteriálních druhů byla izolována a purifikována DNA, která byla použita jako DNA matrice (10 ng) pro PCR na detekci genu pro tyrosindekarboxylasu. Postup detekce genu pro tyrosindekarboxylasu byl stejný jako u kontrolního kmene. Specifické PCR produkty byly amplifikovány s použitím DNA bakterií druhu *Lactobacillus sake* a *Lactobacillus curvatus*. Přítomnost specifických PCR produktů v těchto kulturách je uvedena na obr. 2.

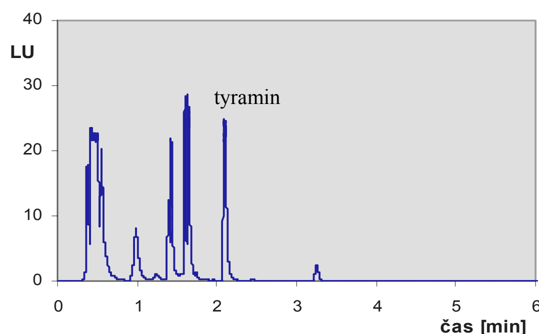
Exprese specifické sekvence DNA byla ověřena analytickým stanovením produktu exprese této sekvence – tyraminu, a to v médiu s přesně definovaným množstvím aminokyseliny tyrosinu (10 g l^{-1} média). Kvalitativně a kvantitativně byl tyramin stanoven metodou vysoce účinné kapalinové chromatografie (HPLC).

V obou vzorcích médií po kultivaci prověřovaných bakteriálních kultur byla potvrzena přítomnost tyraminu. Vzhledem ke skutečnosti, že detekční limit tyraminu je 2,90 ± 0,01 ng ml^{-1}, lze s jistotou prohlásit, že u sledovaných kultur došlo při kultivaci v médiu s tyrosinem k expresi sledovaného genu, tedy k produkci bakteriální tyrosindekarboxylasy. Stanovená množství tyraminu se vzájemně podstatně nelišila (0,40 ± 0,01 mg l^{-1} média pro *Lactobacillus sake* a 0,45 ± 0,01 mg l^{-1} média pro *Lactobacillus curvatus*). To však může být ovlivněno standardním složením kultivačního média, konkrétně uniformní dostupností substrátu. Je známo, že se dekarboxylasová aktivita mikroorganismů může značně lišit. V rámci určitého mikrobiálního druhu dokonce existují kmeny, které se mohou touto aktivitou lišit až o tři řády[12]. V této práci uvedená hypotéza potvrzena nebyla.

Startovací kultury obsahující blíže nespecifikované kmeny dvou bakteriálních druhů *Lactobacillus sake* a *Lactobacillus curvatus* byly identifikovány jako potenciální producenti tyrosindekarboxylasy a mohou se ve fermentovaných trvanlivých salámech účastnit tvorby biogenního aminu tyraminu.



Obr. 3. **Chromatogram standardu biogenních aminů (c = 0,1 mg ml^{-1});** 1: histamin, 2: tyramin, 3: tryptamin, 4: putrescin, 5: 2-fenylethylamin, 6: kadaverin, 7: 1,7-diaminoheptan; LU – luminiscence



Obr. 4. **Chromatogram vzorku živné půdy s pozitivním nálezem tyraminu;** LU – luminiscence

Vzhledem k ochraně konzumenta před nežádoucími vlastnostmi tyraminu, který mohou za vhodných podmínek startovací kultury SC1 a CXK (tabulka I) obsahující druhy *Lactobacillus sake* a *Lactobacillus curvatus* tvořit, je třeba použití těchto startovacích bakteriálních kultur pro výrobu potravin důkladně zvážit.

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R. Burdychová and V. Dohnal *(Department of Food Technology, Mendel University of Agriculture and Forestry, Brno, Czech Republic):* **The Use of HPLC for Determination of Expression Product of Microbial Tyrosine Decarboxylase**

The aim of this study was verification of the presence or absence of specific DNA sequences coding for tyrosine decarboxylase in the starter cultures used in the fermented sausages production and confirmation of the tyrosine decarboxylase gene expression by rapid HPLC analysis affording the expression product – tyramine.

Genomic DNA from microorganisms used as starter cultures for production of fermented sausages was extracted with phenol. The DNA was used as a template for PCR identification in which a set of oligonucleotide primers based on tyrosine decarboxylase gene sequence was used. Two strains with high tyrosine decarboxylase activity were detected (*Lactobacillus curvatus* and *Lactobacillus sake)*. The tyrosine decarboxylase gene expression of these two strains was analyzed by an optimized rapid HPLC method, which confirmed the presence of high concentrations of tyramine. The results show suitability of the described PCR and HPLC methods of screening starter cultures for the presence and expression of the above gene.

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REVIEW ARTICLE

Biological degradation of aflatoxins

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Abstract
Aflatoxins are cancerogenic compounds produced predominantly by certain strains of the *Aspergillus* genus. The ideal solution for minimization of health risk that aflatoxins pose is the prevention of foods and feeds contamination. Unfortunately, these contaminants can never be completely removed, and on that account, many studies have been carried out to explore an effective process of their detoxification to a threshold level. Biological decontamination seems to be attractive because it works under mild, environmentally friendly conditions. This review is focused on the biological detoxification of aflatoxins, especially aflatoxin B_1, by microorganisms. There are briefly mentioned aflatoxin metabolic pathways in the human and animal body. Microorganisms such as soil or water bacteria, fungi, and protozoa and specific enzymes isolated from microbial systems can degrade aflatoxin group members with varied efficiency to less- or nontoxic products. Some aflatoxin-producing fungi from *Aspergillus* species have the capability to degrade their own synthesized mycotoxins. Yeasts and lactic acid bacteria work as biological adsorbents that prevent aflatoxin's transfer to the intestinal tract of humans and animals. Aflatoxin B_1 absorbed into the organism could be metabolized by significantly different pathways. They lead to the production of the relatively nontoxic compounds, on the one hand, or to highly toxic active forms on the other hand.

Keywords: *Aflatoxin; metabolism; biodegradation; decontamination; bacteria*

Introduction

Aflatoxins are a group of highly toxic secondary metabolites produced mainly by *Aspergillus* species fungi. *A. flavus* produces only aflatoxins B, while *A. parasiticus* produces both aflatoxins B and G. Aflatoxins can be found mainly in cereals, oilseeds, tree nuts, spices, and milk. Among 18 different types of aflatoxins, such as B_1, B_2, G_1, G_2, P, Q, M_1, M_2, B_{2a}, etc., were identified. The most commonly occurring ones in fungi cultures are aflatoxins B_1, B_2, G_1, and G_2, then aflatoxins M_1 and M_2 in milk.

Naturally occurring aflatoxins and aflatoxin B_1 (AFB_1) are classified by the International Agency for Research on Cancer (IARC) as group 1 carcinogens ("carcinogenic to humans") (IARC, 2002). The AFB_1 metabolite, 8,9-epoxide, forms DNA adducts primary with N^7 of guanine (Cullen and Newberne, 1994). The toxic effect on living organisms is reviewed in a World Health Organization report (WHO, 1998). The toxicity of aflatoxin decreases in order B_1, G_1, B_2, and G_2. When AFB_1 and aflatoxin B_2 (AFB_2) are consumed, they are metabolized to aflatoxin M_1 (AFM_1), respective M_2 (AFM_2), and distributed in tissues and biological fluids and milk (Zarba et al., 1992).

Biodegradation of aflatoxins, using microorganisms or enzymes, is one of the well-known strategies for the management of aflatoxins in foods and feeds. The methods of biodegradation are being actively studied and can be a highly promising choice, since it is efficient, specific, and

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environmentally friendly to reduce or eliminate the possible contaminations of aflatoxins in foods and feeds.

Soil bacteria

Many bacteria in soil are able to degrade aflatoxins. *Flavobacterium aurantiacum* NRRL B-184, a kind of bacteria from soils and water, showed a very high capability of detoxifing aflatoxins in feeds and foods (Ciegler et al., 1966a). The aflatoxin-contaminated substance and *F. aurantiacum* NRRL B-184 were mixed together and incubated at 28°C for 12 hours, all of the aflatoxin G was removed, as well as a part of aflatoxin B, which was diminished. Ducking assays implied detoxification of aflatoxin solutions by *F. aurantiacum* NRRL B-184 was complete, with no new toxic products being formed. Lillehoj showed that this bacterium can remove AFM_1 from milk in 1971 (Shapira, 2004). Later, it was observed that the radioactively labeled ^{14}C-AFB_1 is partially metabolized and partially adsorbed to *F. aurantiacum* cells (Line et al., 1994). D'Souza and Brackett (1998, 2000, 2001) have been monitoring the effects of cations and several chemical compounds on AFB_1 degradation by these bacteria. For example, Cu^{2+}, Mn^{2+}, and Zn^{2+} lower the reduction capacity of *F. aurantiacum*. This confirms the influence of the enzymes in the degradation process. More applications of *F. aurantiacum* for decontamination of food/feed were discussed in a review (Bata and Lásztity, 1999).

Other microorganisms were also tested for their possible ability to degradate aflatoxins. The strain *Nocardia asteroides* reduces AFB_1 by biotransformation to another fluorescent product (Arai et al., 1967), and *Corynebacterium rubrum* is able to detoxify aflatoxin as well (Shapira, 2004).

In another work, *Mycobacterium fluoranthenivorans* sp. nov. DSM44556^T isolated from soils of a former coal gas plant, which was polluted with polycyclic aromatic hydrocarbons, was found to be capable of degrading AFB_1 as a single carbon source (Hormisch et al., 2004). The AFB_1 concentration was reduced to amounts of 70–80% of the initial concentration within 36 hours, and no AFB_1 was detectable after 72 hours. In addition, the cell-free extracts of *M. fluoranthenivorans* sp. nov. DSM44556^T degraded AFB_1 more efficiently (Teniola et al., 2005). More than 90% of the initial amount of AFB_1 was degraded at 30°C within 4 hours, and no AFB_1 was detected after 8 hours.

Teniola et al. (2005) investigated *Rhodococcus erythropolis* isolated from polycyclic aromatic hydrocarbon (PAH) soils for AFB_1 degradation activity. Dramatic reduction of AFB_1 was observed during incubation in the presence of *R. erythropolis* cells. Then, 17% residual AFB_1 was left after 48 hours and only 3–6% was detectable after 72 hours. In addition, this work team also found AFB_1 was effectively degraded by extracellular extracts from *R. erythropolis* (only 32% residual AFB_1 was detectable after 72 hours) (Alberts et al., 2006). Results indicated AFB_1 was most likely metabolized to degradation products with chemical properties different from that of AFB_1, because the equipments they utilized could not reveal the formation of any breakdown products.

The high degradation rate and wide temperature range for degradation by both *R. erythropolis* and *M. fluoranthenivorans* sp. nov. DSM44556^T indicate a potential and promising application for the degradation of AFB_1 in the foods and feeds process.

Fungi

Fungi cannot only produce aflatoxins, but they are able to degrade them as well. Four fungal strains, *Aspergillus niger*, *Eurotium herbariorum*, a *Rhizopus sp.*, and nonaflatoxin (AF)-producing *A. flavus*, are able to convert AFB_1 to aflatoxicol (AFL) by reducing the cyclopentenone carbonyl of AFB_1. These fungi could convert AFB_1 to aflatoxicol-A (AFL-A), then AFL-A was converted to aflatoxicol-B (AFL-B) by the actions of medium components or organic acids produced from the fungi. In addition, the interconversion between AFL-A and AFL-B was observed to have occurred independently of fungal metabolic activity (Nakazato et al., 1990). Fungi *A. niger* was capable of converting AFL to AFB_1; afterward, AFB_1 could be converted further to AFB_{2a}. However, the sum of AFL and AFB_1 was found to be decreased with time, which suggested both AFB_1 and AFL were further metabolized to unknown substances by the fungi (see Figure 1).

AFB_1 and AFG_1 production and degradation during fungi cultivation in liquid media by *A. flavus* strains (NRRL 2999, 3000, A-13570, and A-13367; also called M 001) was investigated (Ciegler et al., 1966b). The production was observed for the first 72 hours of growing and depended on aeration and mycelia dispersion. After this time, the lowering of aflatoxin concentrations was observed. The degradation was not dependent on carbohydrate concentration. On the other hand, the fragmentation of mycelia of previously aflatoxin nondegrading fungi (*A. flavus* NRRL 2999 and M 001) induced their ability to degrade aflatoxins. The aflatoxin reduction can be prevented by fermentation at low temperatures and a low agitation rate. Research made by Doyle and Marth (1978) proved that *A. parasiticus* NRRL 2999 and NRRL 3000 actively degraded aflatoxins. *A. flavus* NRRL 3353 was less active, and *A. flavus* NRRL 482 and *A. parasiticus* NRRL 3315 degraded minimal amounts of aflatoxins. Those aspergilli producing the greatest amounts of aflatoxins also degraded aflatoxins the most rapidly. In the presence of asparagines, *Aspergillus candidus* converts AFB_1 to aflatoxin D (Lafont and Lafont, 1974).

Fungi *Penicillium raistrickii NRRL* 2038 is able to transform AFB_1 to a new compound that is similar to AFB_2 (Ciegler et al., 1966a). Kusumaningtyas et al. (2006) found another fungi, *Rhizopus oligosporus*, was able to inhibit synthesis or to degrade AFB_1 when

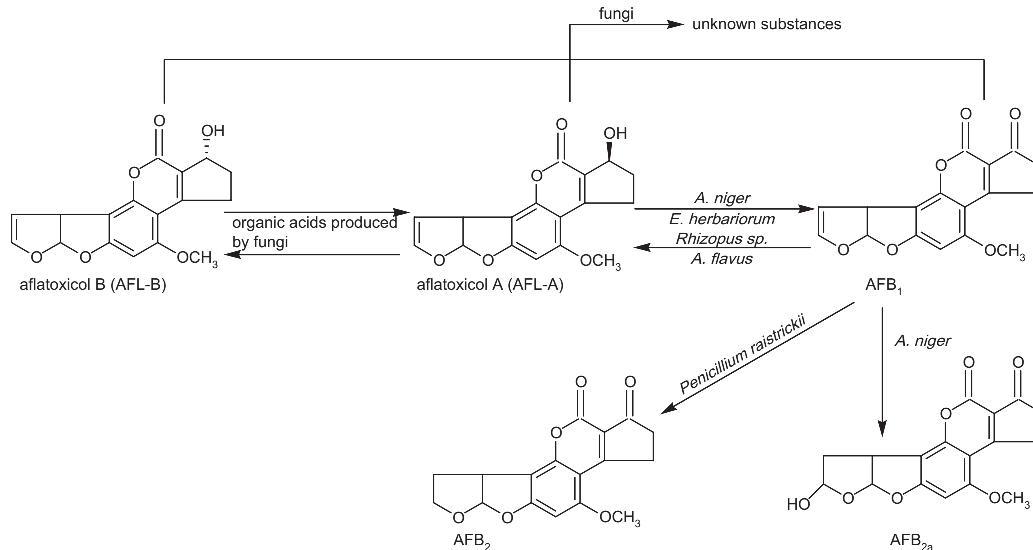

Figure 1. Metabolic pathways of aflatoxin B_1 by fungi.

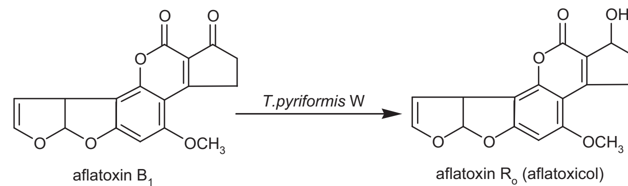

Figure 2. Degradation of AFB_1 by protozoa.

cultured together with AFB_1-producing fungi *A. flavus*. *R. oligosporus* had the best degrading ability on Day 5. Fungi *R. arrhizus*, *R. stolonifer* NRRL 1477, and *A. arrhizus* NRRL 2585 transform AFB_1 to aflatoxicol (AFL), trivially named as aflatoxin R_o (AFR_o) (Cole et al., 1972). Incubation time longer than 3 days resulted in isomerization to AFR_o. A short review about other aflatoxin (AFG_1) degradation by *Rhizopus* species was published by the same work team (Cole and Kirksez, 1971).

Metabolization of AFB_1 to AFR_o was also reported for *D. dendroides* NRRL 2575, *Mucor alterans* NRRL 3358, *M. griseocyanus* NRRL 3359, *Absidia repens* NRRL 1336, and *Helminthosporium sativum* NRRL 3356 (Detroy and Hesseltine, 1969). Longer times of incubation led to the formation of new, unidentified blue-fluorescent product.

Protozoa

Cells of the protozoon *Tetrahymena pyriformis* W have the ability to degradate pure AFB_1 to another bright-blue fluorescent product and decrease the AFB_1 concentration to 25% in 30 hours. The course of detoxification of AFG_1 by this strain was different. During 10 hours, the concentration decreased to 80% and was constant for the next 20 hours (Teunisssion and Robertson, 1967). One year later, it was concluded by Robertson et al. (1970) that *T. pyriformis* W reduced the carbonyl in the cyclopentane ring of AFB_1 to a hydroxyl group (Figure 2). The reduced aflatoxin appears to be identical to AFR_o.

Digestive tract microorganisms

Rumen bacteria have been reported to degradate aflatoxins. Kiessling et al. (1984) investigated the metabolism of AFB_1 by intact rumen fluid, rumen protozoa, and rumen bacteria *in vitro*. They found that AFB_1 was not degraded by rumen microorganisms. A slight decrease in the amount of AFB_1 occurred within 30 minutes; however, after this time, no further reduction was evident.

In another work, Westlake et al. (1987) studied four strains of *Butyrivibrio fibrisolvens* (CE46, CE51, CE52, and CE56), one of the predominant rumen bacteria. Results showed that there was no significant degradation of AFB_1. The researchers proposed that microbial activity was solely responsible for aflatoxin's degradation within the rumen. The efficiency of the rumen metabolism was demonstrated by the fact that only 2–5% of AFB_1 fed to cows reached the intestine (Engel and Hagemeister, 1978; Karlovsky, 1999).

AFB_1-contaminated corn mixed with poultry litter was incubated, and mycotoxin was destroyed by feces microorganisms in several weeks (Jones et al., 1996).

Yeasts and lactic acid bacteria

The mechanism of reducing aflatoxins by yeasts and lactic acid bacteria is due to their adhesion to cell-wall components. Blanco et al. (1993), Jesperson et al. (1994), and Wiseman and Marth (1983) in (Westby et al., 1997) show no effect of lactic acid bacteria. However, many other researchers report very efficient aflatoxin reductions associated with fermentation.

Yeasts *Saccharomyces cerevisiae* have capability to bind AFB_1. Further investigation showed that components of yeast cell wall, called oligomannanes after their chemical modification, esterification, were able to bind up to 95% of AFB_1 (Devegowda et al., 1996). In addition, they are more efficient than commonly used adsorbent HSCAS (hydrated sodium calcium aluminium silicate) (Mahesh and Devegowda, 1996). The addition of 0.05% of esterified glucomannans to the basal diet resulted to improved performance in broilers (Kumprecht et al., 1977; Aravind et al., 2003).

Significant decreasing of AFB_1 concentration during brewing process in the work of Chu et al. (1975) is probably caused by sorption of mycotoxin on *S. cerevisiae* yeasts. This hypothesis is supported by other researchers (Shetty et al, 2007; Celyk et al., 2003). A 19% reduction of spiked AFB_1 during dough fermentation in breadmaking was reported (El-Banna and Scott, 1983).

The addition of yeast cell walls into feed can lead to a decrease of aflatoxin toxic effect in broilers (Stanley et al., 1993; Santin et al., 2003).

AFB_1 was detoxified into AFB_{2a} during yogurt (Megalla and Hafez, 1982) and dairy product (Megalla and Mohran, 1984) fermentation.

Peltonen et al. (2001) investigated the ability of 12 *Lactobacillus*, 5 *Bifidobacterium*, and 3 *Lactococcus* bacteria strains to bind AFB_1. These bacteria are commonly used in the food industry as a starter culture in the production of fermented milk and meat products, such as yogurt, cheese, salami, etc. During the last few years, the selected strains of bifidobacteria and lactobacilli are rising in use in combination with prebiotics as probiotic cultures applied in renewing of intestinal microflora.

El-Nezami et al. (1998) studied the ability of selected *Lactobacillus* strains to remove AFB_1 from the cultivation media. The probiotic strains *L. rhamnosus* GG and *L. rhamnosus* strain LC-705 showed significant ability to remove toxin from media (around 80% of added amount). The binding of AFB_1 to *L. rhamnosus* GG decreases its subsequent adhesion capability to Caco-2 cells, which means that these bacteria may reduce the accumulation of aflatoxins in the intestine via increased excretion of an aflatoxin-bacteria complex (Kankaankpää et al., 2000).

Enzymes

Specific enzymes that are capable of degrading aflatoxins have been purified from microbial systems. The detoxification by specific enzymes avoids the drawback of using the microorganism, which may, in addition to its degradation activity, change flavor or impair the nutritional value and acceptability of the product (Shapira, 2004).

A novel aflatoxin degradation enzyme had been isolated and purified from *Pleurotus ostreatus* by Motomura et al. (2003). AFB_1 was treated with culture supernatants from 19 mushroom strains. The supernatant from *P. ostreatus* showed aflatoxin-degradation activity, whereas other strains had no or only weak activity. In addition, the novel enzyme showed the best aflatoxin-degradation activity at 25°C with a pH of 4.0–5.0. Fluorescence measurements suggested that the specific enzyme cleaved the lactone ring of aflatoxin, although the degradation products of aflatoxin were not investigated clearly.

In another work (Liu et al., 2001), an enzyme named aflatoxin-detoxifizyme (ADTZ), which exhibited detoxification activity on AFB_1, was isolated and purified from *Armillariella tabescens* (E-20). AFB_1 seemed to be degradated into difuran ring-opening AFB_1, which was less toxic than AFB_1, and the optimum activity for the enzyme was at 35°C with a pH of 6.8. This work team had already isolated a multienzyme that was able to degradate AFB_1 from *A. tabescens* in the earlier time (Liu et al., 1998). In addition, they gave a proposed pathway of the degradation of AFB_1 by the multienzyme: AFB_1 was first transformed to AFB_1-epoxide, followed by hydrolysis of the epoxide to give the dihydrodiol. Then, the difuran ring would open in the subsequent hydrolysis step (Figure 3).

Metabolism of aflatoxins in animals and humans

Generally, there is a great diversity in the metabolism of aflatoxins among different animal species or even, in some cases, individual animals. There are four metabolic pathways of AFB_1: O-dealkylation to AFP_1, ketoreduction to AFL, epoxidation to AFB_1-8,9-epoxide (highly toxic, mutagenic, and carcinogenic), and hydroxylation to AFM_1 (highly toxic), AFP_1, AFQ_1, or AFB_{2a} (all relatively nontoxic). Thus, the main reactions in aflatoxins metabolism are hydroxylation, oxidation, and demethylation.

Most of the metabolites of AFB_1 are able to be transformed to further metabolites. AFQ_1 is able to be transformed to AFH_1 in liver. For AFB_1-8,9-epoxide, there exists three further metabolic pathways in animals and humans: hydrolysis to form AFB_1-8,9-dihydrodiol, conjugation with to AFB_1-8,9-dihydro-8-(N^7-guanyl)-3-hydroxy (AFB_1-N^7-Gua), and conjugation with soluble nucleophilic molecules, such as glutathione (Yiannikouris and Jouany, 2002).

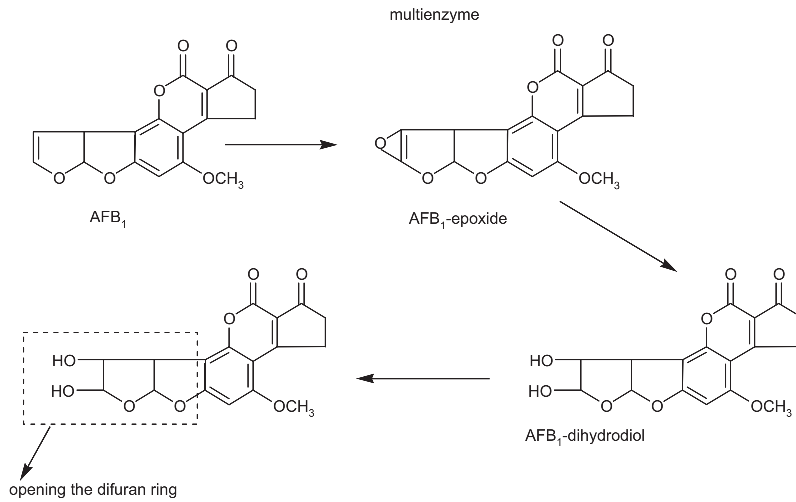

Figure 3. Proposed pathway of degradation of AFB$_1$ by *Armillariella tabescens*.

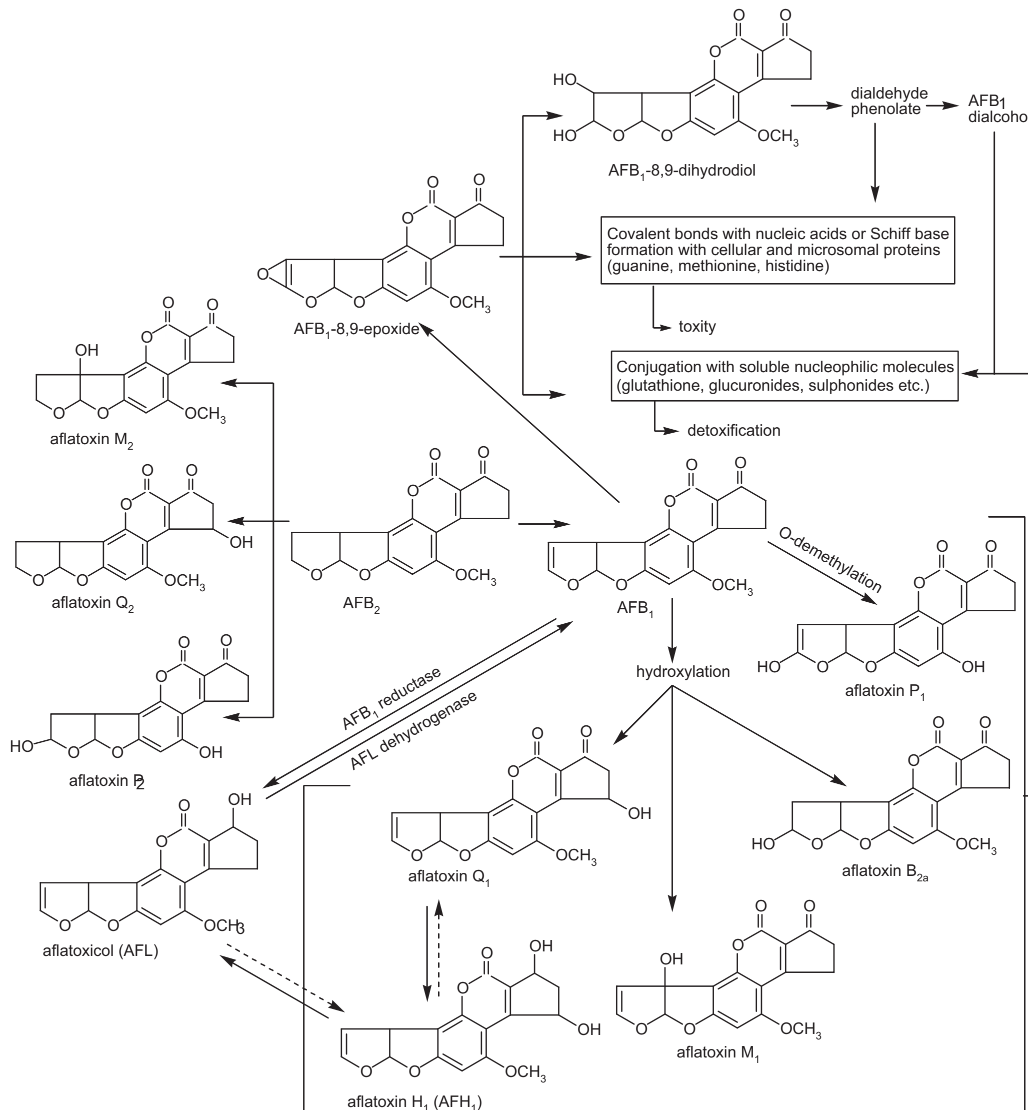

Figure 4. Metabolic pathways of aflatoxin B$_1$ in animals (adapted from Yiannikouris and Jouany (2002)).

Various forms of P-450 serve different biotransformation capacities, depending on the animal species. AFB_1 is transformed to AFM_1, AFQ_1, and AFB_1-8,9-epoxide by mixed-function mono-oxygenase in rat liver. In addition, Yoshizawa et al. (1982) found AFM_1 was strictly mediated by cytochrome P-448, while AFQ_1 was catalyzed by both P-450 and P-448 in rat liver. Cytochrome P-450-dependent polysubstrate mono-oxygenase system, especially CYP3A4 and CYP1A2, are the major isoforms involved in human liver. However, in distinct contrast, lipoxygenase (LOX) and prostaglandin H synthase (PHS) were found to be the main enzymes of the biotransformation of AFB_1 in human lung, whereas P-450 played a relatively minor role (Donnelly et al., 1996).

Numerous researchers have investigated the metabolism of aflatoxins *in vitro* and *in vivo*. In an investigation by Roebuck and Wogan (1977), AFL was found to be the main metabolite of AFB_1 in duck liver, whereas AFB_1 was able to be converted to AFQ_1 and AFP_1 by monkey and human liver. In addition, AFB_1 was able to be converted to AFM_1 and AFM_{2a} in all species (duck, rat, mouse, and monkey) except humans. *In vitro* metabolism of AFB_2 by animal and human liver was also studied by Roebuck et al. (1978). Duck liver had a much higher level of activity than the tissues from other species. The significant pathway in duck liver was 2,3-desaturation to form AFB_1. AFL was also found in duck liver, as well as AFB_1, AFM_1, and AFM_2. However, AFB_1 was only transformed to AFQ_2 and AFP_2 by rat, mouse, and human liver. Different with ducks, a peptide (or amino acid) conjugate of AFB_{2a} and a glucuronide conjugate of AFM_1 were the main metabolites of AFB_1 in chicken liver *in vivo* (Chipley et al., 1974).

In another work (Salhab and Edwards, 1977), *in vitro* metabolism of AFL by liver preparations from animals and humans was investigated. AFL was metabolized to AFB_1, AFQ_1, AFH_1, AFP_1, AFM_1, and AFM_{2a} by liver S12 fractions from monkey, dog, rat, mouse, and human. Human and hamster preparations were most active in the metabolic pathway from AFL to AFB_1, whereas rabbit and trout had the best ability to convert AFB_1 to AFL. However, whether AFH_1 was produced directly form AFL or via the AFB_1 and AFQ_1 intermediates was not identified (metabolic pathways in animals can be found in Figure 4).

Conclusion

There have been many studies of aflatoxin degradation carried out in laboratory conditions, but no biological system exists to be used in the full commercial sphere currently. Interesting results have been obtained by *Flavobacterium aurantiacum* application. This soil bacterium is supposed to be removing aflatoxins B, G, and M_1 from substrate. The use of *Rhodococcus erythropolis* and *Mycobacterium fluoranthenivorans* sp. nov. DSM44556^T seems to be a promising opportunity for the degradation of AFB_1 in the foods and feeds process. Studies suggest that certain fungi, including *Aspergillus* or *Rhizopus* sp., convert aflatoxin B_1 to less toxic aflatoxicol, aflatoxin B_2, or B_{2a}. Enzymes isolated from *Pleurotus ostreatus* and *Armillariella tabescens* showed aflatoxin-degradationactivity as well. *Saccharomyces cerevisiae* and *Lactobacillus spp.* can reduce aflatoxin amount due to binding of toxin on its cell walls. Isolated rumen bacteria do not significantly reduce aflatoxin amount, as they seem to be able to metabolize toxins in bovine ruminal fluid.

The fate of aflatoxin B_1 differs in human and animal organisms and among other species as well. There are four metabolic pathways of AFB_1: O-dealkylation, ketoreduction, epoxidation, and hydroxylation. These reactions lead to the creation of highly toxic aflatoxin B_1-8,9-epoxide and aflatoxin M_1 or relatively nontoxic forms, such as AFP_1, AFQ_1, or AFB_{2a}.

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***Declaration of interest*:** The authors report no conflicts of interest.

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Publikace IX

Metabolic Pathways of T-2 Toxin

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Abstract: Among the naturally-occurring trichothecenes found in food and feed, T-2 toxin is the most potent and toxic mycotoxin. After ingestion of T-2 toxin into the organism, it is processed and eliminated. Some metabolites of this trichothecene are equally toxic or slightly more toxic than T-2 itself, and therefore, the metabolic fate of T-2 toxin has been of great concern. The main reactions in trichothecene metabolism are hydrolysis, hydroxylation and deep oxidation. Typical metabolites of T-2 toxin in an organism are HT-2 toxin, T-2-triol, T-2-tetraol, 3'-hydroxy-T-2, and 3'-hydroxy-HT-2 toxin. There are significant differences in the metabolic pathways of T-2 toxin between ruminants and non-ruminants. Ruminants have been more resistant to the adverse effects of T-2 toxin due to microbial degradation within rumen microorganisms. Some plant species are resistant to T-2 toxin, while others are capable of its intake and metabolisation.

Keywords: T-2 toxin, trichotecene, metabolic pathways, biodegradation, *Fusarium.*

INTRODUCTION

Mycotoxins are the most commonly-occurring toxins of fungi origin in cereals. Generally, these are defined as toxic secondary metabolites that are formed during the growth of certain fungi, some of which are very toxic to man and farm animals. They are present in all parts of the food chain and have also been implicated in a variety of mycotoxicoses. One of these very toxic compounds is trichothecene mycotoxin T-2 toxin, known also as fusariotoxin T2, insariotoxin, NSC 138780, etc. It is produced by various species of *Fusarium*, and is found occurring in various agricultural products such as corn, barley, oats, wheat, sorghum, and peanuts.

In general, trichothecenes absorbed by the alimentary tract are evenly distributed amongst many various tissues and organs, with weak accumulation in specific organs only [1]. Yoshizawa reported the transmission of T-2 toxin into even eggs and milk [2]. Toxic effects of T-2 toxin may include the disruption of membrane transport and function, suppression of the immune response, and abnormal blood function.

Metabolism and defense mechanisms are important factors in understanding mycotoxin toxicity in specific species or individual animals. Specificity of such mechanisms are well demonstrated in the significant difference between ruminants and non-ruminants in handling mycotoxins. Ruminants have generally been more resistant to the adverse effects of mycotoxins [3].

BIODEGRADATION

Plant Metabolism

The trichothecene mycotoxins are toxic for many plants and animals, while some species are resistant to them. In addition, certain plants have the ability of T-2 toxin intake and metabolisation.

Species of *Baccharis* found in Brazil and neighboring countries are relatively tolerant to T-2 toxin (500 ppm T-2 in solution), and they are able to transform trichothecenes to other toxic metabolites, likely roridins and verrucarins, found in these species. *Baccharis coridifolia* stores verrucarins and roridins (Fig. **1**) in the seeds of its female plants. The variation in composition of these toxins has been observed by Rizzo et al [4]. All Argentinian *B. coridifolia* plants contain roridins A and E and verrucarins A and J; some of these plants produce also roridin D or H. The Brazilian plants contain roridins, but do not contain any verrucarins. A higher content of roridins can be found in in *B. artemisioides* than in *B. coridifolia* species.

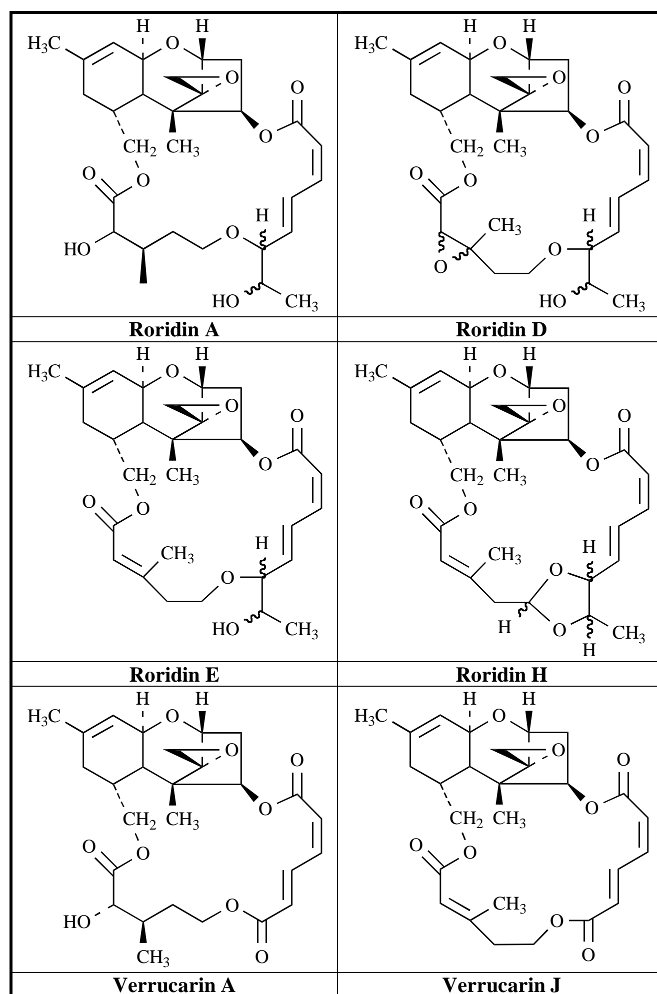

Fig. (1). Chemical structures of selected verrucarins and roridins.

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However, the *B. megapotamica* can hydroxylate the A ring of macrocyclic trichothecenes. The hybrids resulted from the crosses *B. sarothroides* x *B. pilularis* (FS1), *B. sarothroides* (FS2) and *B. megapotamica* were tested for their tolerance and ability to metabolise the T-2 toxin [5] (Fig. **2**). While the control groups of such crosses did not produce any trichothecene metabolites, the twigs and leaves of FS2 at 6 and 21 hours after exposure to T-2 toxin contained T-2 toxin, HT-2 and T-2-tetraol. In addition, the FS2 hybrid showed the highest tolerance to the T-2 in comparison with two other species tested. The FS1 hybrid also contained all of these toxins, and additionally contained 3'-hydroxy-HT-2 (sometimes called TC-3). The fastest conversion of T-2 toxin was observed by the leaves of *B. megapotamica*, where within 24 hours T-2 toxin was metabolized mainly to hydroxylation products 3'-hydroxy-HT-2, 3'-hydroxy-T-2 (called TC-1) and partially to HT-2. After 48 hours only a small amount of T-2-tetraol was detected. The metabolism of *Baccharis* species is similar to that found in chickens [6], cows [7] and rats [8].

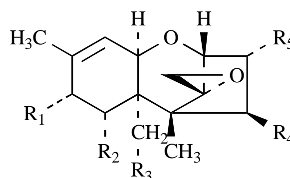
Soil and Freshwater Bacteria

The possible role of soil bacteria in trichothecene biotransformation was first suggested by Ueno et al [9]. Aerobic cultures of a *Curtobacterium* species strain 114-2 isolated from soil was found to be capable of degrading T-2 toxin as a single carbon and energy source to HT-2 toxin T-2-triol and finally to T-2-tetraol and an unknown by-product. Addition of Mn^{2+} and Mg^{2+} ions lead to the decrease of HT-2 toxin and the increase of T-2-triol. These ions probably play the role of a catalysator in HT-2 hydrolysis. The distribution of enzymes in fractions was determined. Enzymes with T-2 toxin hydrolase activity were found in whole cells, cell-free soluble fraction (after sonification of the whole-cell suspension) and culture filtrate. Cell-free soluble fraction was not capable of degrading T-2-triol, which can support the idea that the T-2-triol hydrolyzing system is not present in soluble fraction.

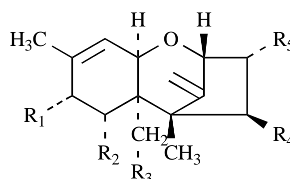
Beeton and Bull [10] screened 20 bacterial communities isolated from soils and waters with 4 different geographical origins (Antarctica, England, Germany, and Thailand) for T-2 toxin degradation activity. Seventeen of these cultures were able to transform T-2 to HT-2 and T-2-triol, respectively, and in certain cases degradation pathways involved the conversion of T-2 toxin to neosolaniol and then probably to 4-deacetylneosolaniol. The biodegradation of both pathways sometimes resulted in T-2-tetraol. Bacteria that degrade T-2 toxin and grow on acetate (*Arthrobacter-Brevibacterium* sp., *Pseudomonas* sp.), isovalerate (*Pseudomonas* sp.), or both isovalerate and acetate (*Pseudomonas* sp.) were distinguished from isolates.

Fungi

The regioselective acetyl-conjugation at the C-3 position of T-2 toxin, respectively its derivatives by mycelia of several fungi that produce 12,13-epoxytrichothecenes, is another possible means of T-2 toxin metabolism. Fungi *Fusarium graminearum* ATCC 28114



	R1	R2	R3	R4	R5
T-2	$OC(O)OCH_2CH(CH_3)_2$	H	$OCOCH_3$	$OCOCH_3$	OH
HT-2	$OC(O)OCH_2CH(CH_3)_2$	H	$OCOCH_3$	OH	OH
T-2-triol	$OC(O)OCH_2CH(CH_3)_2$	H	OH	OH	OH
T-2-tetraol	OH	H	OH	OH	OH
3'-hydroxy-T-2	$OC(O)OCH_2C(OH)(CH_3)_2$	H	$OCOCH_3$	$OCOCH_3$	OH
3'-hydroxy-HT-2	$OC(O)OCH_2C(OH)(CH_3)_2$	H	$OCOCH_3$	OH	OH
neosolaniol	OH	H	$OCOCH_3$	$OCOCH_3$	OH
4-deacetylneosolaniol	OH	H	$OCOCH_3$	OH	OH
3-acetyl-HT-2	$OC(O)OCH_2CH(CH_3)_2$	H	$OCOCH_3$	OH	$OCOCH_3$
3'-hydroxy-T-2-triol	$OC(O)OCH_2C(OH)(CH_3)_2$	H	OH	OH	OH
3',7-dihydroxy-T-2	$OC(O)OCH_2C(OH)(CH_3)_2$	OH	$OCOCH_3$	$OCOCH_3$	OH
3',7-dihydroxy-HT-2	$OC(O)OCH_2C(OH)(CH_3)_2$	OH	$OCOCH_3$	OH	OH



	R1	R2	R3	R4	R5
Deepoxy-HT-2	$OC(O)OCH_2CH(CH_3)_2$	H	$OCOCH_3$	OH	OH
Deepoxy-T-2-triol	$OC(O)OCH_2CH(CH_3)_2$	H	OH	OH	OH
Deepoxy-3'-hydroxy-T-2-triol	$OC(O)OCH_2C(OH)(CH_3)_2$	H	OH	OH	OH
Deepoxy-3'-hydroxy-HT-2	$OC(O)OCH_2C(OH)(CH_3)_2$	H	$OCOCH_3$	OH	OH

Fig. (2). Chemical structures of selected trichothecenes and metabolites.

and *Calonectria nivalis* ATCC 26559 degrade T-2 toxin via 3α-acetylation and 4β-hydrolysis, *F. graminearum* 0588, while *F. nivale* degrades T-2 toxin only via 3α-acetylation, *F. sporotrichoides* ATCC 26553 by 4β- respective 8α-hydrolysis [11]. In addition, the formation of partial acetylated HT-2 toxin was observed on C-3 which originates from the biotransformation of T-2 toxin. The possibility of 3-acetyl HT-2 toxin formation from T-2 toxin through acetyl T-2 was excluded, because the C-4 ester linkage of acetyl T-2 toxin was not hydrolyzed preferentially [12].

Fusarium nivale and *F. solani* fungi are able to convert T-2 acetate into T-2 toxin by eliminating the C-3 acetyl group and then probably hydrolyze C-4 ester (deacetylation) to yield HT-2 toxin [12]. The stability of C-15 ester group against biological elimination originates probably from the steric effect of large substituents at the 7α position. Therefore, hydrolysis at the C-4 ester bond is preferred.

Saccharomyces cerevisae

There is no known report about metabolism of T-2 toxin in yeast. The reduction of free (unbounded) T-2 toxin in beer during the brewing process is due to its adsorption on malt polysaccharides or proteins and beta-D-glucomannans of *S. cerevisae* cell walls.

Animals

The trichothecenes are relatively soluble in water and body fluids and generally accumulate very weakly in animal tissues. Slight accumulation was found in lymphoid organs. For example, 72 hours after T-2 toxin administration with a 0.15 mg/kg per day dosage, there remained only 5 % non-metabolized T-2 toxin. With a four-times higher dose, the toxin was not eliminated, probably due to the saturation of the enzymatic system [1].

The main reactions in trichothecene metabolism are hydrolysis, hydroxylation and deep oxidation. Typical metabolites of T-2 toxin in an organism are HT-2 toxin (hydrolysis), T-2-triol (hydrolysis), T-2-tetraol (hydrolysis), 3'-hydroxy-T-2 (oxidation), 3'-hydroxy-HT-2 toxin (oxidation), deepoxy-3'-hydroxy-T-2-triol, deepoxy-3'-hydroxy-HT-2 (hydrolysis), 3'-hydroxy-T-2-triol and dihydroxy-HT-2 toxin, neosolaniol (hydrolysis), 3'7-dihydroxy-T-2 (oxidation), and 3',7-dihydroxy-HT-2 toxin (oxidation). Degradation products, such as HT-2 toxin or neosolaniol, are excreted as glucuronide conjugates. Almost the entire amount of T-2 toxin intake is biotransformed in the organism [13]. The half-life of T-2 toxin in plasma is less then 20 minutes, due to its rapid deacetylation to HT-2 toxin and in small portions to T-2-triol [14].

The first step in T-2 toxin transformation is its deacetylation to HT-2 toxin. T-2 toxin is hydroxylated by CYP450 esterases of intestinal and hepatic microsomes in rats, guinea-pigs, mice, pigs, and cows. This activity was observed partially in chickens, but was not detected in rabbits [15]. Ohta [16] reported that esterase activity was found mainly in the microsomes of the livers, kidneys, and spleens of laboratory animals.

The excretion, transmission, and metabolism of T-2 toxin have been studied *in vivo*, for example, by radioactivity measurement after the intoxication of animals with tritiumlabeled T-2 toxin [6,17-19] or by isolation of T-2 and its metabolites from urine and feces [20].

RUMINANTS

Generally, ruminants are less susceptible to trichothecene mycotoxicoses than monogastric animals. Gastric bacteria play an important role in toxin degradation. The bacteria *Selenomonas ru*

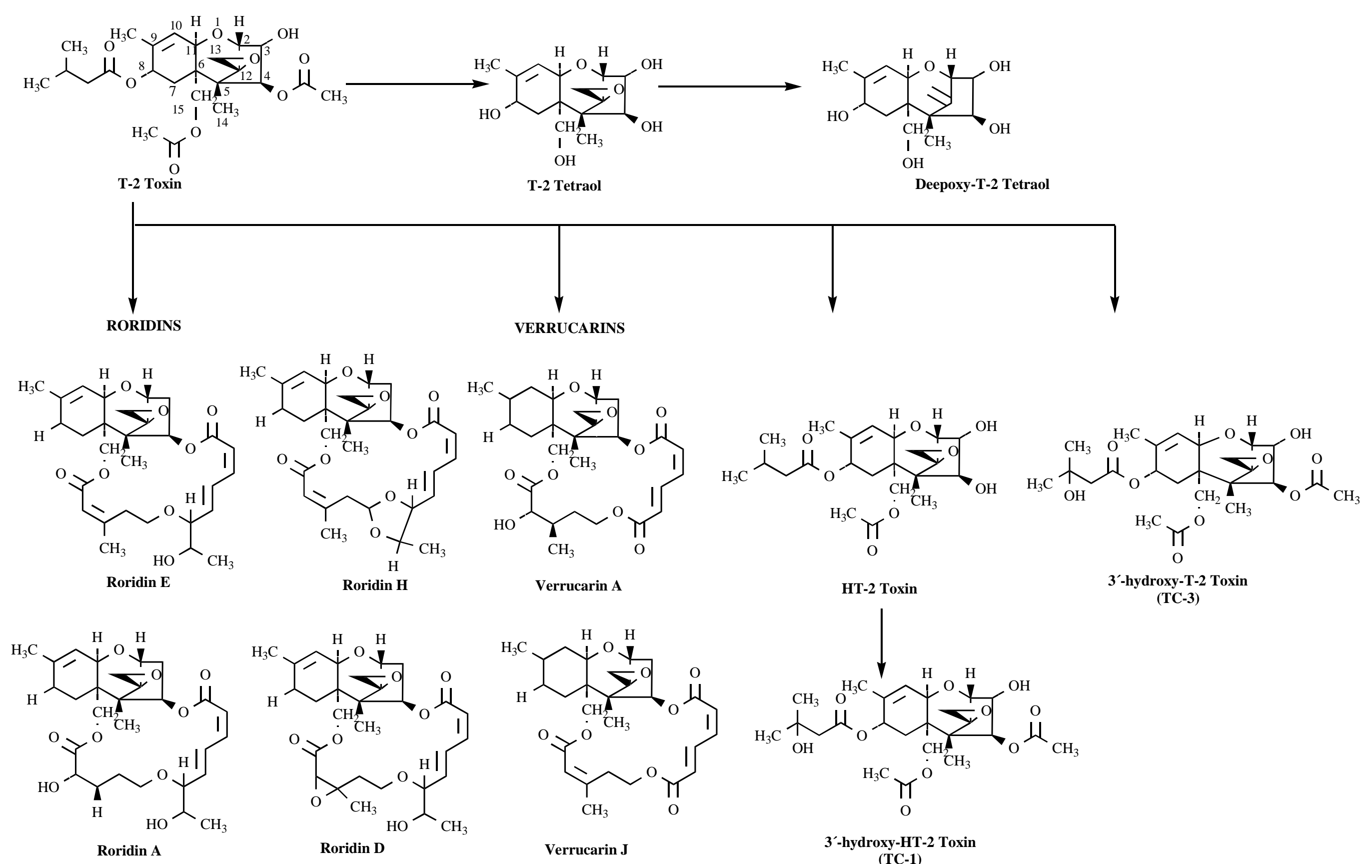


Fig. (3). Metabolic pathways of T-2 toxin in *Baccharis* sp. plants.

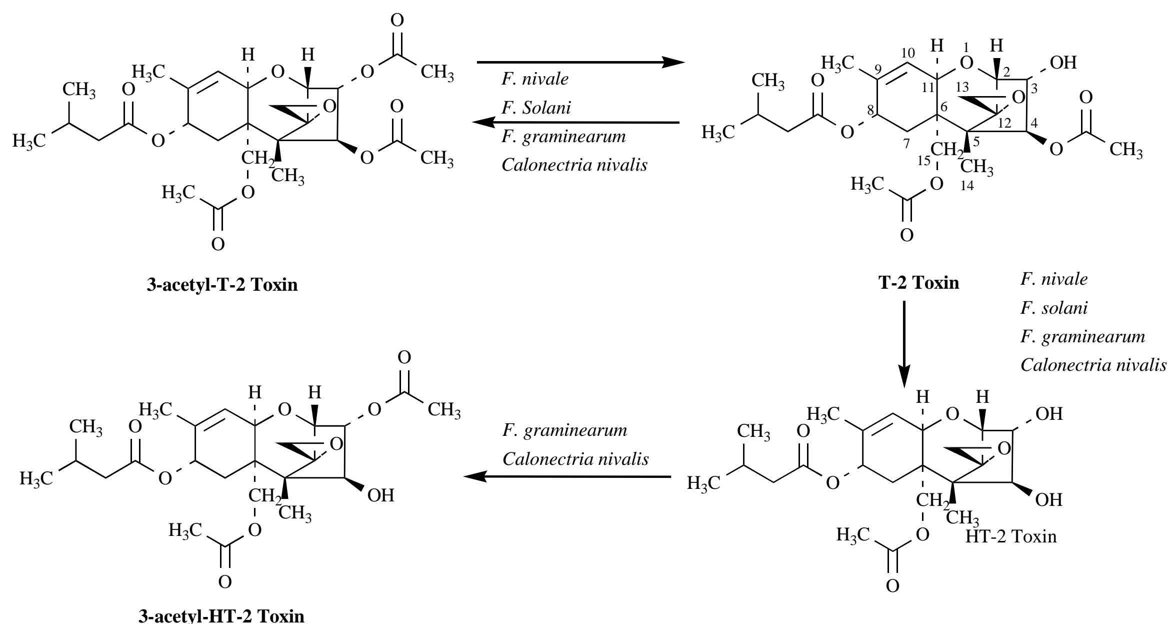


Fig. (4). Metabolic pathways of T-2 toxin in fungi.

T-2 Toxin

ACETATE WAY
Butyrivibrio fibrisolvens
Selenomodos ruminatium
Curtobacterium sp.
Soil bacteria *(Arthhrobacter-Brevibacterium* sp.,
Pseudomonos sp.

ISOVALERATE WAY
Butyrivibrio fibrisolvens
Soil bacteria *(Pseudomonas* sp.)

Neosolaniol (NEO)

Butyrivibrio fibrisolvens
S. ruminatium
Curtobacterium sp.
Soil bacteria

HT-2 Toxin

Soil bacteria

Soil bacteria

Curtobacterium sp.

Intestinal bacteria

4-deacetyl-NEO

T-2 Tetraol

T-2-Tetraol

Curtobacterium sp.

Deepoxy-HT-2 toxin

Deepoxy-T-2 Tetraol

Fig. (5). Metabolic pathways of T-2 toxin in bacterias.

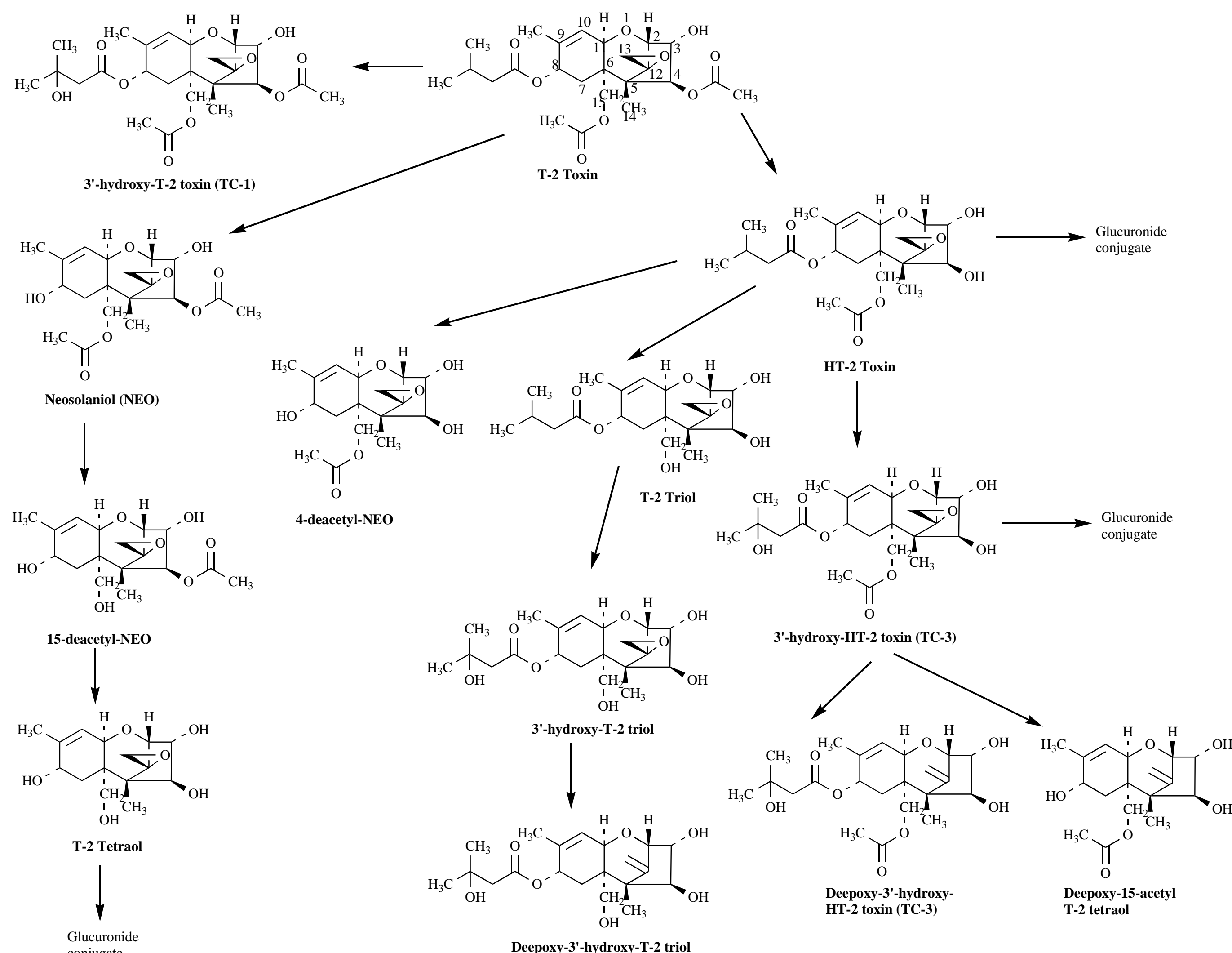


Fig. (6). Metabolic pathways of T-2 toxin in animals.

minatium and *Anaerovibrio lipolytica* isolated from rumen fluid are tolerable to a low concentration of T-2 toxin (10 µg/ml), while the growth rate of *Butyrivibrio fibrisolvens* was not affected significantly at 1 mg/ml. Strains *B. fibrisolvens* CE6 and CE51 and *S. ruminatium* showed an increase in growth rate in the presence of T-2 toxin. It may indicate that these bacteria assimilate T-2 toxin as an energy and carbon source [21]. The metabolic pathways of these bacteria differ in enzymatic systems.

Enzymic hydrolysis of T-2-toxin at C-4 followed by C-15 led to T-2-triol, while cleavage of isovaleryl group on C-8 would lead to neosolaniol. In the case of *B. fibrisolvens* the enzymatic system is localized in the cell membrane. *B. fibrisolvens* CE51 degrade T-2 toxin through HT-2 toxin and T-2-triol to neosolaniol, whereas *A. lipolytica* and *S. ruminatium* degrade T-2 toxin to HT-2 toxin and T-2-triol only. In addition, *B. fibrisolvens* CE51 also degrades the acetyl T-2 into T-2 toxin.

In another work [22], the filtrate of rumen fluid from sheep or cows after feeding was tested for mycotoxin degradation. T-2 toxin was degraded with rumen microorganisms, but the protozoa were more active than the bacteria. Differences were not observed in the metabolism of T-2 toxin between the rumen fluid from sheep and cattle. T-2 toxin was degraded to HT-2 toxin. The kinetics of biotransformation was similar for both kinds of rumen fluid.

Bacteria from intestinal microflora produces deepoxy-HT-2 toxin. Intestinal cells are probably able to metabolize T-2 toxin to HT-2 toxin, which can be found in blood after T-2 toxin administration to animals [13].

Swanson [23] incubated T-2 *in vitro* for 12, 24 and 48 hours with rumen microorganisms obtained from a fistulated dairy cow. T-2 toxin was completely biotransformed to the products HT-2, T-2 triol and two new metabolites identified as 15-acetoxy-3α,4β-dihydroxy-8α-(3-methylbutyryloxy)trichothec-9,12-diene (deepoxy-HT-2) and 3α,4β,15-trihydroxy-8α-(3-methylbutyryloxy)trichothec-9,12-diene (deepoxy-T-2 triol).

NON-RUMINANTS

Biotransformation of T-2 toxin in intestinal epithelium, kidney and liver involves two phases. The first phase consists of oxidation by enzymes in microsomal cytochromes P450, such as flavin oxygenases and alcohol dehydrogenases, hydrolysis by non-specific esterases and amidases, and reduction by epoxide-hydrolases. The second phase is a conjugating reaction with the goal of decreasing the toxicity of metabolites and increasing the solubility in water, facilitating their excretion, and thus, protecting the organism. The major means of conjugation is glucuronation by glucuronosyltransferases.

Degradation of T-2 toxin by chicken intestinal microbes goes through HT-2 toxin to deepoxy-HT-2 toxin. Direct deep oxidation of T-2 toxin was not observed. The isovalerate moiety is resistant against microbial cleavage [14].

CONCLUSION

Metabolism of T-2 toxin is a very complex process differing in fungi, plants, animals, and microorganisms. In *Baccharis sp*. plants it is partially degraded and used for the synthesis of roridins and verrucarins toxins. Fungi are able to acetylate it and produce its acetylated metabolites. In bacteria there are two main pathways – through hydrolysis at C4 and C15 to T-2-triol or by cleavage of the isovaleryl group on C8 to neosolaniol and finally to T-2 tetraol. In animals, deep oxidation products are observed. Their metabolisation involves mainly hydrolysis and oxidation. In the second phase, the conjugation of metabolites with glucuronic acid are formed. The summaries can be found in Fig. (**3**-**6**).

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REVIEW ARTICLE

Metabolic pathways of trichothecenes

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Abstract
Trichothecenes are a group of mycotoxins mainly produced by the fungi of *Fusarium* genus. Consumers are particularly concerned over the toxicity and food safety of trichothecenes and their metabolites from food-producing animals. The metabolism of T-2 toxin, deoxynivalenol (DON), nivalenol (NIV), fusarenon-X (FX), diacetoxyscirpenol (DAS), 3-acetyldeoxy-nivalenol (3-aDON), and 15-acetyldeoxynivalenol (15-aDON) in rodents, swine, ruminants, poultry, and humans are reviewed in this article. Metabolic pathways of these mycotoxins are very different. The major metabolic pathways of T-2 toxin in animals are hydrolysis, hydroxylation, de-epoxidation, and conjugation. After being transformed to HT-2 toxin, it undergoes further hydroxylation at C-3' to yield 3'-hydroxy-HT-2 toxin, which is considered as an activation pathway, whereas transformation from T-2 to T-2 tetraol is an inactivation pathway in animals. The typical metabolites of T-2 toxin in animals are HT-2 toxin, T-2 triol, T-2 tetraol, neosolaniol (NEO), 3'-hydroxy-HT-2, and 3'-hydroxy-T-2, whereas HT-2 toxin is the main metabolite in humans. De-epoxidation is an important pathway for detoxification in animals. De-epoxy products, DOM-1, and de-epoxy-NIV are the main metabolites of DON and NIV in most animals, respectively. However, the two metabolites are not found in humans. Deacetyl can occur rapidly on the acetyl derivatives, 3-aDON, 15-aDON, and FX. DAS is metabolized in animals to 15-monoacetoxyscirpenol (15-MAS) via C-4 deacetylation and then transformed to scirpentriol (SCP) via C-15 deacetylation. Finally, the epoxy is lost, yielding de-epoxy-SCP. De-epoxy-15-MAS is also the main metabolite of DAS. 15-MAS is the main metabolite in human skin. The review on the metabolism of trichothecenes will help one to well understand the fate of these toxins' future in animals and humans, as well as provide basic information for the risk assessment of them for food safety.

Keywords: *Trichothecenes; metabolic pathways; biodegradation;* Fusarium*; animals; human*

Introduction

Trichothecenes are a large group of structurally related mycotoxins mainly produced by the fungi of *Fusarium* genus. Other species, such as *Stachybotrys*, *Myrothecium*, *Cephalosporium*, *Verticimonsporium*, and *Trichotheciu*, are the minor species producing trichothecenes. The mycotoxins are commonly found in cereals, particularly in wheat, barley, oats, and maize (Conkova et al., 2003; Eriksen and Petterson, 2004; D'Mello et al., 1999).

Trichothecenes in the class of sesquiterpenoids contain an olefinic group, an epoxide, and variable numbers of hydroxyl and acetoxy groups. Depending on their functional groups, trichothecenes have been classified into A, B, C, and D groups (Ueno, 1977) (Figure 1).

Members of group A do not contain carbonyl on C-8. The examples are represented by T-2 toxin, HT-2 toxin, and diacetoxyscirpenol. Hydrolysis of ester groups leads to the formation of a basic trichothecene moiety with one to five hydroxyl groups. Group B differs from group A by the presence of a carbonyl group on C-8. Group C members, such as crotocine, have another epoxy group between the C-7 and C-8 or C-8 and C-9 positions, respectively. Compounds in group D, such

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as satratoxin G, include a macrocyclic ring between C-4 and C-15.

Groups A and B are more concerned by people (WHO, 1990; Sudakin, 2003). Despite the identification of over 150 members of trichothecenes, data about their natural occurrence in foods are mostly limited to T-2 toxin, diacetoxyscirpenol (DAS), nivalenol (NIV), and deoxynivalenol (DON) due to their high toxicity and prevalent occurrence (Pittet, 1998; Voyksner et al., 1987; Yoshizawa et al., 1980b). DON may be the most commonly occurring trichothecene in nature. T-2 toxin does not occur as much as DON, but its toxicity is higher than that of DON. In addition, the acetyl derivatives of trichothecenes, 3-acetyldeoxy-nivalenol (3-aDON), 15-acetyldeoxynivalenol (15-aDON), and fusarenon-X (FX), co-occur regularly all over the world.

Trichothecenes initiate a wide range of toxic effects on farm animals and humans (Table 1). Hemorrhaging, diarrhea, skin lesion, emesis, feed refusal, weight loss, leucopenia, immunosuppression, oxidative stress, radiomimetic injury to tissues, and death are the clinical signs (Eriksen and Pettersson, 2004; Glavits et al., 1983; Glavits and Vanyi, 1988; Beasley et al., 1986; Sokolovic et al., 2008; Borutova et al., 2008). Alimentary toxic aleukia (ATA), a typical disease for human, was found to be associated primarily with the ingestion of moldy cereal infected with T-2 toxin (Joffe, 1974, 1978). *In vitro*, T-2 toxin and DON are strongly cytotoxic for murine and human hematopoietic progenitors (Hymery et al., 2006). Proliferation and immunoglobulin production in human lymphocytes can be inhibited by trichothecenes, as well (Bondy et al., 1991). There has been high attention paid to the understanding of toxic effects of trichothecenes on domestic animals or humans. Risk assessment and management for trichothecenes in human food have been made by the European Union (SCF, 1999, 2000, 2001, 2002) and JECFA (JECFA, 2001a, 2001b). Most of the trichothecene

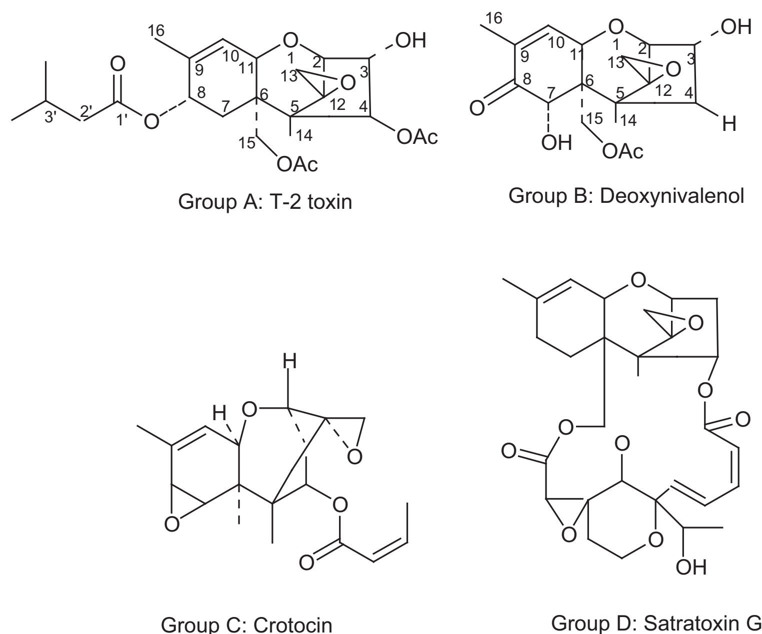

Figure 1. Chemical structures of trichothecenes (examples of groups A–D).

Table 1. Adverse effects of individual trichothecenes in animals.

Compound	Effects	References
T-2 toxin	Feed refusal; weight loss; decreases red blood cell count; reduces leucocyte count; reproductive disorders; increases mortality of piglets after birth, reduces plasma glucose in piglets, pathological changes in liver and stomach, increases infection rate; alimentary toxic aleukia (ATA); induces apoptosis in the thymus and spleen; inhibits the synthesis of DNA and RNA	D'Mello et al., 1999; Rafai et al., 1995a, 1995b; Vanyi et al., 1991; Conkova et al., 2003; Doi et al., 2008
DON	Food refusal; vomiting; digestive disorders; weight loss; decreases levels of serum protein; oxidative stress and blood phagocytic activity in broilers; cytotoxic effect on human primary hepatocytes	Conkova et al., 2003; Borutova et al., 2008; Konigs et al., 2008
NIV, FX	Gastrointestinal erosions; nephropathy; reduction of feed intake; cytotoxicity	Conkova et al., 2003; D'Mello et al. 1999; Fornelli et al., 2004
DAS	Reduces feed intake and weight gain; oral lesions; gastrointestinal lesions; diarrhea	Ademoyero and Hamilton, 1991; Kubena et al., 1997; Galhardo et al., 1997

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mycotoxins are transformed to less toxic products after metabolism in animals. However, some metabolites are more toxic than the parents. So, in order to assess the toxicity of trichothecenes to human health, it is urgently needed to elucidate the metabolic pathways of the mycotoxins in animals and humans.

Metabolism of trichothecenes has been studied in many laboratories. Some of the mycotoxins have extensive metabolites in animals, such as T-2 toxin and DAS. However, the metabolism of most trichothecenes in animals and humans remains unclear. There is a high possibility of transmission of trichothecenes and their metabolites into edible tissues of farm animals. Consequently, metabolism studies of the mycotoxins in animals could provide important information for evaluating and controlling human exposure to residues of trichothecenes in foods of animal origin. To date, reviews have been made only on the metabolism of T-2 toxin in animals (Yagen and Bialer, 1993; Cavret and Lecoeur, 2006; Dohnal et al., 2008), but the metabolism of T-2 toxin in human and other trichothecenes (DON, 3-aDON, 15-aDON, NIV, FX, and DAS) in animals and human have not not covered yet. In order to have a scientific and comprehensive understanding on the transformation of trichothecenes in animals and humans, metabolic pathways of trichothecenes mainly including T-2 toxin, DON, 3-aDON, 15-aDON, NIV, FX, and DAS, in rodents, swine, poultry, ruminants, and humans are reviewed in this article, which gives a full-scale illustration about the transformation of trichothecenes, and further sums up the defects and trends of metabolism study of trichothecenes. This work will provide guidance for further study on these mycotoxins and also will improve the risk assessment of trichothecenes on human health.

T-2 toxin

T-2 toxin is one of the most acutely toxic among trichothecenes. It is produced by *F. acuminatum*, *F. equiseti*, *F. poae*, and *F. sporotrichoides* (Moos, 2002). *F. sporotrichoides* is probably associated with ATA in the former Soviet Union, where T-2 toxin was produced on overwintered cereals at temperatures as low as –2°C. Similarly, as in the case of other fusarial toxins, the toxic effect of T-2 toxin can be potentiated by other trichothecenes.

Rodents

The study on the metabolism of T-2 toxin in livers of rabbits, rats, guinea pigs, and mice *in vitro* showed that T-2 toxin was selectively hydrolyzed at C-4, giving rise to HT-2 toxin as the only metabolite (Ohta et al., 1977). The capacity of hepatic microsomes to convert T-2 toxin into HT-2 toxin was the highest in rabbits, which was about 80 times higher than that of rats. Nonspecific carboxyesterase [EC 3.1.1.1] of microsomal origin participated in the selective hydrolysis of T-2 toxin was concluded, since the enzymatic hydrolysis of T-2 toxin was inhibited by eserine and diisopropylfluorophosphate. Subsequent work (Ohta et al., 1978) confirmed this conclusion. Further, the substitution at C-3 and C-8 of T-2 toxin was found to play an important role in the enzymatic hydrolysis of the C-4 acetyl residue.

Two unknown metabolites, designated as TMR-1 and -2, were found in the *in vitro* metabolism of T-2 toxin in rats (Yoshizawa et al., 1980a), in addition to HT-2 and T-2 tetraol in the S-9 fraction of rat-liver homogenates. TMR-1 was characterized as 4-deacetylneosolaniol (15-acetoxy-tetraol) by gas chromatography–mass spectrometry (GC-MS). Since the same metabolites were also obtained from HT-2 toxin used as a substrate, a proposed metabolic pathway was concluded: T-2 toxin was hydrolyzed preferentially at the C-4 position to give HT-2 toxin, which was then metabolized to T-2 tetraol via 4-deacetylneosolaniol (15-acetyl-tetraol) (Figure 2). A trace amount of neosolaniol transformed from T-2 toxin by rat intestinal strips was also observed. Although TMR-2 was not identified in this investigation, it was identified as 8-acetyl-T-2 tetraol, an isomer of TMR-1 (Visconti and Mirocha, 1985) (Figure 3).

After 3'-hydroxy-T-2 and 3'-hydroxy-HT-2 toxins were identified as new main metabolites of T-2 toxin in the urine of a lactating cow (Yoshizawa et al., 1982), the two metabolites were found in mice-liver homogenates, as well (Yoshizawa et al., 1984). Moreover, the conclusion that a cytochrome P-450 could catalyze the hydroxylation at the C-3' position of T-2 and HT-2 toxins was given from their results. In order to investigate the further metabolites of 3'-hydroxy-HT-2 toxin, the *in vivo* metabolism of 3'-hydroxy-HT-2 toxin and T-2 tetraol in male Wistar rats was carried out (Yoshizawa et al., 1985). Four metabolites having a trichothec-9,12-diene nucleus were newly found in the excreta. They were confirmed as deepoxy-3'-hydroxy-HT-2 toxin, deepoxy-3'-hydroxy-T-2 triol, deepoxy-15-acetyl-T-2 tetraol, and deepoxy T-2 tetraol on the basis of GC-MS and nuclear magnetic resonance (NMR) spectroscopy. The metabolites were also identified in other *in vivo* studies (Pfeiffer et al., 1988; Swanson et al., 1988). This indicates that deepoxidation is an important *in vivo* metabolic pathway for T-2 toxin in rats. In addition, route and time were the two important factors for T-2 toxin metabolism *in vivo*, but not dose (Pfeiffer et al., 1988).

In vitro isolated perfused rat livers were used to study the metabolism and clearance of T-2 toxin (Pace, 1986). [^{3}H]T-2 toxin was delivered under constant perfusing flow (8 mL/min, 33.9 μg T-2 toxin/min) in a single-pass

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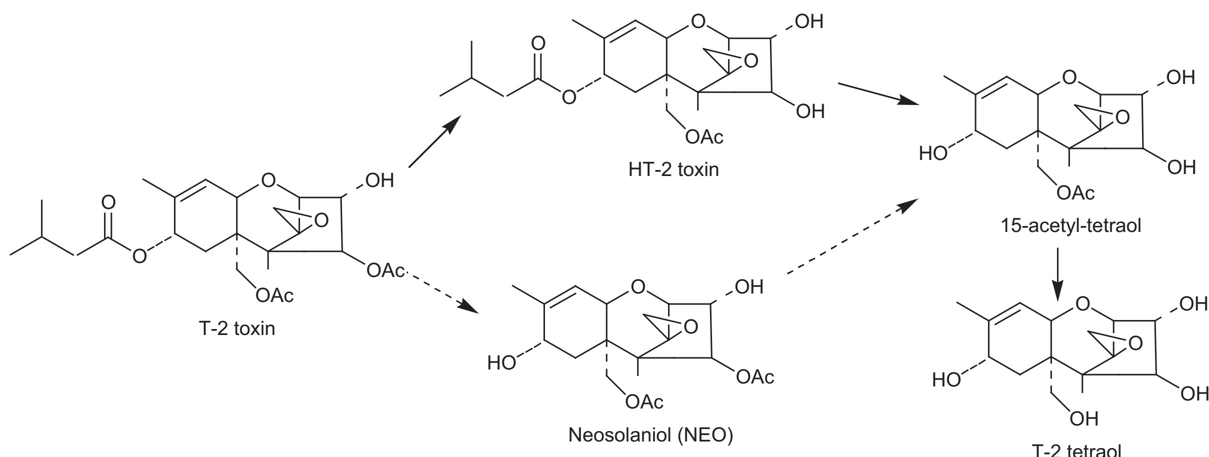

Figure 2. Proposed metabolic pathways for the *in vitro* metabolism of T-2 toxin in rats. Solid arrows indicate major pathways in liver and intestines, and dotted arrows indicate minor pathways in intestines (Yoshizawa et al., 1980a).

experiment. Besides 3′-hydroxy-HT-2 toxin, 3′-hydroxy-T-2 triol, 4-deacetylneosolaniol, and T-2 tetraol, three glucuronide conjugates of HT-2, 3′-hydroxy-HT-2, and T-2 tetraol, were first identified in rat livers. Two minor metabolites of T-2 toxin, TMP-1 and TMP-2, in perfused bile were also found, but their structures were not identified. The perfusion model was highlighted to have potential as a tool for the isolation of minor metabolites for structural analysis by the investigator.

In another work (Conrady-Lorck, 1988), T-2 toxin metabolism was investigated in rats by using the method of the vascular autoperfused jejunal loop *in situ*. HT-2 toxin was the main metabolite, whereas 3′-hydroxy-HT-2, 3′-hydroxy-T-2, T-2 tetraol, and 4-deacetylneosolaniol were found, as well. However, in contrast to the previous work of Pace (1986), any glucuronide or sulfate conjugates were not detected. It seems that T-2 toxin is only able to be metabolized to glucuronide conjugates in the liver and bile, but not in the intestine.

In vitro metabolism of T-2 toxin in different animals (e.g., rats, mice, rabbits, and chickens) were investigated (Knupp et al., 1987a). Detailed metabolism diversity was given in their study. HT-2 toxin was found to be the major metabolites in microsomal preparations from both phenobarbital (PB)-induced and control mice, rats, and rabbits, whereas 3′-hydroxy-T-2 toxin was the predominant metabolite in PB-treated chickens. Only the rabbit microsomal system was capable of producing a significant amount of 4-deacetylneosolaniol (>1.0%). In addition, two new unidentified T-2 metabolites, RLM-2 and -3, were detected in rats, mice, and chickens. RLM-3 was identified as 4′-hydroxy-T-2 through GC-MS and ^{1}H- and ^{13}H-NMR (Knupp et al., 1987b). Moreover, the metabolite of 4′-hydroxy-T-2 was shown to be deacylated at the C-4 position to yield 4′-hydroxy-HT-2 when incubated with rat hepatic S-9 preparations. From the rat skin toxicity bioassay with 4′-hydroxy-T-2 (Knupp et al., 1987b), this new metabolite was found to be more toxic than 3′-hydroxy-T-2 and nearly equal in dermal toxicity to T-2 toxin. This indicated that hydroxylation of T-2 toxin at the C-4′ position was not a detoxification reaction. No *in vivo* data pertaining to 4′-hydroxy-T-2 are available to date; thus, additional experiments to elucidate the fate of 4′-hydroxy-T-2 and 4′-hydroxy-HT-2 *in vivo* are necessary to determine the toxicological significance of the two new metabolites.

In vitro metabolic pathways of T-2 toxin in liver could be modified in the presence of esterase inhibitors. In the system of S-9 fraction from the livers of rats and pigs added with different esterase inhibiters, NaF, *p*-hydroxymercuribenzoate, phenylmethylsulfonyl fluoride, eserine sulfate, diisopropylfluorop-hate, and diethyl *p*-nitrophenyl phosphate, the metabolism of T-2 toxin was completely shifted to the hydroxylation at the C-3′ position (Wei and Chu, 1985). Diethyl p-nitrophenyl phosphate was found to be the most potent in the presence of 10^{-4} mol/L diethyl *p*-nitrophenyl phosphate and 3′-hydroxy-T-2 toxin to be the only metabolite. Similar results were obtained when other T-2-related metabolites were tested in their study

In summary, rodents, including rats, mice, rabbits, and guinea pigs, have the strong metabolic activity of T-2 toxin. Both liver and intestine are able to metabolize it. HT-2 toxin is its main metabolite, especially in rabbits. Besides HT-2 toxin, 3′-hydroxy-T-2, 3′-hydroxy-HT-2, neosolaniol, 4-deacetylneosolaniol, 8-acetoxy-T-2 tetraol, 3′-hydroxy-T-2 triol, 15-deacetylneosolaniol, T-2 tetraol, 4′-hydroxy-T-2, 4′-hydroxy-HT-2, deepoxy-3′-hydroxy-HT-2 toxin, deepoxy-3′-hydroxy-T-2 triol, deepoxy-15-acetyl-T-2 tetraol, deepoxy T-2 tetraol, glucuronide conjugates of HT-2, 3′-hydroxy-HT-2, and T-2 tetraol are also the metabolites in rodents. Nonspecific carboxyesterase [EC 3.1.1.1] of microsomal origin participates in the selective hydrolysis of T-2 toxin, whereas

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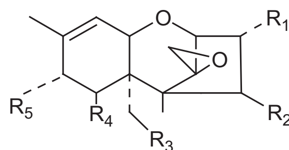
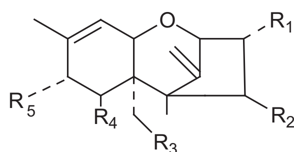


Compound	R_1	R_2	R_3	R_4	R_5
T-2	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
HT-2	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
T-2 triol	OH	OH	OH	H	$OCOCH_2CH(CH_3)_2$
T-2 tetraol	OH	OH	OH	H	OH
3'-hydroxy-T-2	OH	OAc	OAc	H	$OCOCH_2C(OH)(CH_3)_2$
3'-hydroxy-HT-2	OH	OH	OAc	H	$OCOCH_2C(OH)(CH_3)_2$
3'-hydroxy- T-2 triol	OH	OH	OH	H	$OCOCH_2C(OH)(CH_3)_2$
3',7-dihydroxy-HT-2	OH	OH	OAc	OH	$OCOCH_2CH(CH_3)_2$
4'-hydroxy-T-2	OH	OAc	OAc	H	$OCOCH_2CH(CH_3CH_2OH)$
4'-hydroxy-HT-2	OH	OH	OAc	H	
3-acetyl-T-2	OAc	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
3-acetyl-HT-2	OAc	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
Neosolaniol (NEO)	OH	OAc	OAc	H	OH
15-deacetyl-NEO	OH	OAc	OH	H	OH
8-acetyl-T-2 tetraol	OH	OH	OH	H	OAc
15-acetyl-T-2 tetraol	OH	OH	OAc	H	OH



Compound	R_1	R_2	R_3	R_4	R_5
Deepoxy-HT-2	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
Deepoxy-T-2 triol	OH	OH	OH	H	$OCOCH_2CH(CH_3)_2$
Deepoxy-T-2 tetraol	OH	OH	OH	H	OH
3'-hydroxy-T-2	OH	OAc	OAc	H	$OCOCH_2C(OH)(CH_3)_2$
Deepoxy-3'-hydroxy-HT-2	OH	OH	OAc	H	$OCOCH_2C(OH)(CH_3)_2$
Deepoxy-3'-hydroxy-T-2 triol	OH	OH	OH	H	$OCOCH_2C(OH)(CH_3)_2$
Deepoxy-15-acetyl-T-2 tetraol	OH	OH	OAc	H	OH

Figure 3. Chemical structures of T-2 toxin and metabolites in animals.

cytochrome P-450 can catalyze the hydroxylation at the C-3' position of T-2 and HT-2 toxins.

Swine

Metabolite profiles of T-2 toxin in the bile and urine of swine were investigated after the intravenous (i.v.) administration of $[^3H]$T-2 toxin at 0.15 mg/kg of body weight (Corley et al., 1985). Urine was collected hourly for 4 hours, and bile was collected after the animals were killed at 4 hours. 3'-hydroxy-HT-2 and T-2 triol were identified as the major unconjugated metabolites in both bile and urine, whereas glucuronide conjugates were determined with glucuronidase for T-2 toxin, 3'-hydroxy-T-2, neosolaniol, HT-2, 3'-hydroxy-HT-2, T-2 triol, 4-deacetylneosolaniol, and T-2 tetraol. Conjugated metabolites represented an average of 77 and 63% of the recovered radiolabel in bile and urine, respectively. It was concluded that glucuronidation played an important role in the metabolism of T-2 toxin in intravascularly dosed swine. The actual location of the glucuronic acid moiety was unknown. T-2 toxin, however, has only one hydroxyl group located at the C-3 position. Consequently, similar to DAS (Roush

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et al., 1985), the assumption that glucuronide conjugation was likely to occur at the C-3 position for T-2 toxin was tentatively put forward by the investigators. In this study, three unknown metabolites (PM-1–PM-3) were not identified, which were account for 2–8% of the total radioactivity.

Metabolism of T-2 toxin in plasma and tissues of swine were studied (Corley et al., 1986). Following reversed-phase high-performance liquid chromatography (HPLC) radiochromatography separation, 21 metabolites were identified in tissues and the gastrointestinal tract at concentrations, ranging from less than 0.01 to 67.56 ng/g. Approximately 55% of the extractable radioactivity in tissues and the gastrointestinal tract corresponded to T-2 toxin, HT-2, deepoxy-HT-2, T-2 triol, deepoxy-T-2 triol, 3'-hydroxy-T-2, 3'-hydroxy-HT-2, T-2 tetraol, and deepoxy-T-2 tetraol. Only one metabolite (PM-XV), that represented an additional 27% of the extractable radioactivity, was not identified.

In summary, glucuronide-conjugated products were found to be the main metabolites in the urine of swine. The glucuronides were HT-2, 3'-hydroxy-T-2, 3'-hydroxy-HT-2, and T-2 toxins. The major free metabolites in urine were 3'-hydroxy-T-2 and T-2 triol. In total, 21 metabolites were found in tissues and the gastrointestinal tract. Among them, HT-2, 3'-hydroxy-T-2, and 3'-hydroxy-HT-2 were the major ones. Deepoxy metabolites, deepoxy-HT-2, deepoxy-T-2 triol, and deepoxy-T-2 tetraol were also found in swine (Figure 3).

Ruminants

The metabolism of T-2 toxin in cattle was investigated much more clearly than other ruminants. After daily oral administration of unlabeled T-2 toxin to a lactating Jersey cow for 3 consecutive days, [^{3}H]T-2 toxin was administered orally on day 4 (Yoshizawa et al., 1981). In addition to HT-2, neosolaniol, 4-deacetylneosolaniol, and three major unknown metabolites (designated TC-1, TC-3, and TC-6) were detected. Within the first 24 hours, the three metabolites accounted for 30–40% of the extractable radioactivity in urine, 60–70% in milk, and 50–60% in plasma. However, TC-1 was not found in feces. TC-1 and -3 were identified as 3'-hydroxy-T-2 and 3'-hydroxy-HT-2, respectively (Yoshizawa et al., 1982). As for TC-6, based on MS, it was identified as 3'-hydroxy-7-hydroxy-HT-2 toxin (Pawlosky and Mirocha, 1984).

Another new metabolite, iso-TC-1, was found in cow urine (Visconti et al., 1985). It was identified as 3,15-diacetoxy-4-hydroxy-8-(3-methyl-3'-hydroxy-butryloxy)-12,13-epoxytric- hothec-9-ene, an isomer of TC-1 (3'-hydroxy-T-2). The hydroxyl and acetoxy groups at the C-3 and -4 positions in iso-TC-1, respectively, were reversed. This metabolite was one of the main products of T-2 toxin metabolism in cow. It was much more abundant than TC-1. TC-1 was only present as a minor metabolite.

Deepoxy-T-2 tetraol was identified as another metabolite of T-2 toxin in cow (Chatterjee et al., 1986). The metabolite was found in the blood up to 24 hours and in the urine up to 48 hours after orally administered T-2 toxin to a Holstein cow. This metabolite was found in rat excreta, as well (Yoshizawa et al., 1985). The presence of this metabolite in the blood and urine of animals suggests the possibility that it can be used for monitoring T-2 intoxication on farm animals.

T-2 toxin underwent both acetylation and deacetylation during incubation with bovine rumen fluid in a shaken water bath in the dark at 39°C (Munger et al., 1987). Acetyl T-2, acetyl-HT-2, and HT-2 toxins were detected, as well. Both acetylation and deacetylation were preferred at C-3 versus -4. Further, there were interconversions among acetyl T-2, acetyl-HT-2, T-2, and HT-2 toxins. HT-2 toxin could be acetylated to acetyl-HT-2, T-2, and acetyl-T-2 toxins, while acetyl-HT-2 could be converted to HT-2 toxin and less acetyl T-2 toxin. T-2 triol and T-2 tetraol were not detected, which indicated the ester linkages at C-8 and -15 were apparently stable in rumen fluid.

In summary, TC-1 (3'-hydroxy-T-2 toxin), iso-TC-1 (3-acetyl-3'-hydroxy-HT-2 toxin), TC-3 (3'-hydroxy-HT-2 toxin), deepoxy-T-2 tetraol, TC-6 (3'-hydroxy-7-hydroxy-HT-2 toxin), acetyl T-2, and acetyl-HT-2 are the typical metabolites in bovines. HT-2, neosolaniol, 4-deacetylneosolaniol, 3-acetyl-3'hydroxy-HT-2 toxin, 3'-hydroxy-HT-2, deepoxy-T-2 tetraol, and 3'-hydroxy-7-hydroxy-HT-2 toxin are the main metabolites detected in urine and rumen fluid of bovines. Interconversions between acetylation and deacetylation in the metabolism of T-2 toxin can occur in bovines.

Poultry

^{3}H-labeled T-2 toxin was administered orally in a single dose of 1.6 mg/kg of body weight to 47-day-old broiler chickens (Yoshizawa et al., 1980b). The excreta were collected and analyzed. In addition to neosolaniol, HT-2 toxin, deacetyl-HT-2 (T-2 triol), T-2 tetraol, eight new unidentified metabolites (TB-1–TB-8) were detected through GC-MS and thin-layer chromatography (TLC). Among them, TB-3, -4, -5, -6, and -8 were the major metabolites, the concentrations of which were estimated as 12.25, 5.31, 4.94, 2.09, and 25%, respectively. Known derivatives, such as HT-2 toxin, neosolaniol, T-2 tetraol, and deacetyl-HT-2 (T-2 triol), became minor metabolites. One of the metabolites (TB-6) was identified as 4-deacetylneosolaniol in the study.

In chicken excreta and tissue, 3'-hydroxy-HT-2 toxin was found as a major metabolite (Visconti and Mirocha, 1985). T-2 toxin, 3'-hydroxy-T-2 toxin, 3-acetoxy-3'-

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hydroxy-HT-2 toxin, HT-2 toxin, 4-acetoxy-T-2 tetraol (TMR-1), 8-acetoxy-T-2 tetraol (TMR-2), 15-acetoxy-T-2 tetraol, T-2 triol, and T-2 tetraol were detected in the excreta, as well. TB-1 and -2 were identified as the same compound, 3'-hydroxy-T-2 toxin, although a small contribution of its isomer (3-acetoxy-3'-hydroxy-HT-2 toxin or iso-TC-1) should be considered. TB-3 was identified as 3'-hydroxy-HT-2 toxin. TB-4 and -5 were tentatively identified as monoacetylated T-2 tetraol isomers, in particular, to 8-acetoxy and 15-acetoxy-T-2 tetraol. TB-7 and -8 were not identified, though they could be the glucuronic-acid adducts of some trichothecenes. TB-6, identified as 4-deacetylneosolaniol (Yoshizawa et al., 1980a), was not detected in this study. It was assumed that TB-6 was retained by the Sep-Pak cartridge they utilized. Neosolaniol was not found, either. In distinct contrast with the metabolites in cows, TC-1 (3'-hydroxy-T-2 toxin), rather than iso-TC-1 (3-acetoxy-3'-hydroxy-HT-2 toxin), was found to be the main metabolite in chicken excreta. Further studies are needed to identify the structure of the unknown T-2 tetraol monoacetate isomer and the other very polar compounds present in the excreta.

Recently, several uncharacterized metabolites (T2T-U1, T2T-U2, and T2-U1) in chicken intestinal microbes were observed. However, they were not identified (Young et al., 2007).

In summary, T-2 toxin is able to be metabolized to an extensive range of products, especially in the intestine, such as HT-2 toxin, 3'-hydroxy-HT-2 toxin, 3'-hydroxy-HT-2 toxin, 3'-hydroxy-T-2 toxin, 3-acetoxy-3'-hydroxy-HT-2 toxin, 4-acetoxy-T-2 tetraol, 8-acetoxy-T-2 tetraol, 15-acetoxy-T-2 tetraol, T-2 triol, and T-2 tetraol. However, some of the metabolites are not identified yet. Most investigations are just concerned with the intestine, similar to the status of T-2 toxin metabolism in other animals. T-2 toxin could be metabolized in chicken liver. However, few researchers studied the metabolism of T-2 toxin in liver, to date. Consequently, further studies are needed for the metabolism of T-2 toxin in chickens.

Human

After the dermal surface of the skin was bathed by phosphate-buffered saline (PBS) with antibiotics and amphotericin-B (PBSA) to reduce bacterial and fungal growth, the epidermal surface was dosed with [^{3}H] T-2 toxin dissolved in either methanol or dimethyl sulfoxide (DMSO) and incubated in the receptor fluid (PBSA) for 48 hours. HT-2 toxin (71%), T-2 tetraol, and four unknowns (6.3%) were detected in receptor fluids (PBSA) and skin extracts by liquid scintillation (LSC) techniques (Kemppainen et al., 1986a, 1986b). The four unknowns were tentatively identified as 3'-hydroxy-T-2, 3'-hydroxy-HT-2, 3'-hydroxy-T-2 triol, and acetyl-T-2 toxin. Since the quantities of these unknowns were too small, they had not been positively identified by GC-MS. Compared with methanol, DMSO enhanced the penetration of T-2 toxin in skin.

In order to determine which animal species was the best model for evaluating the metabolism and percutaneous penetration in human skin, the metabolism of T-2 toxin in humans, rabbits, guinea pigs and rat skin was carried out (Kemppainen et al., 1987). In addition to T-2 triol and T-2 tetraol, HT-2 was found as the major metabolite in human skin. Moreover, the rabbit provided the best approximation of human skin, both in metabolic pathway and penetration kinetics.

Confluent cells of human fibroblasts were exposed to [^{3}H]T-2 toxin (0.01 μg/mL) for 1 hour at 37°C (Trusal, 1986). In contrast to human skin, the metabolism of T-2 toxin was found to be much more limited in human fibroblasts. Only HT-2 was identified, based on comigration with known standards.

In summary, because of the extreme toxicity of T-2 toxin, it is not ethical for the study of metabolism in living humans. Therefore, *in vitro* methods were used to obtain the information. HT-2 toxin was found to be the main metabolite in human skin and fibroblasts. T-2 triol, T-2 tetraol, and tentatively identified metabolites, 3'-hydroxy-T-2, 3'-hydroxy-HT-2, 3'-hydroxy-T-2 triol, and actyl-T-2 toxin, were also found in human skin. The rabbit was the best model for the *in vitro* metabolism of T-2 toxin in human skin. The minor metabolic pathway (hydroxylation of T-2) is an activation reaction, since 3'-hydroxy-T-2 is found to be more toxic than T-2 toxin (Yoshizawa et al., 1984); therefore, it will be more toxic for the body when T-2 toxin is transformed in humans. Further studies on the metabolism of T-2 toxin in humans are urgently needed for reasons of human health. The proposed metabolic pathway of T-2 toxin in humans and animals can be found in Figures 4 and Figure 5, respectively.

Deoxynivalenol (DON)

DON, also called vomitoxin or Rd-toxin, is one of the most commonly occurring trichothecene mycotoxins. It is usually found on cereals and produced primarily by two pathogens: *F. graminearum* and *F. culmorum*. The presence of DON in cereals is reported from South America, Canada, European Union countries, etc. (Moos, 2002). DON has lower acute toxicity, in comparison with T-2 toxin, but its importance is by reason of its high incidence.

Rodents

Yoshizawa et al. (1983) first reported the metabolic pathways of DON in rats. The deepoxy metabolite of

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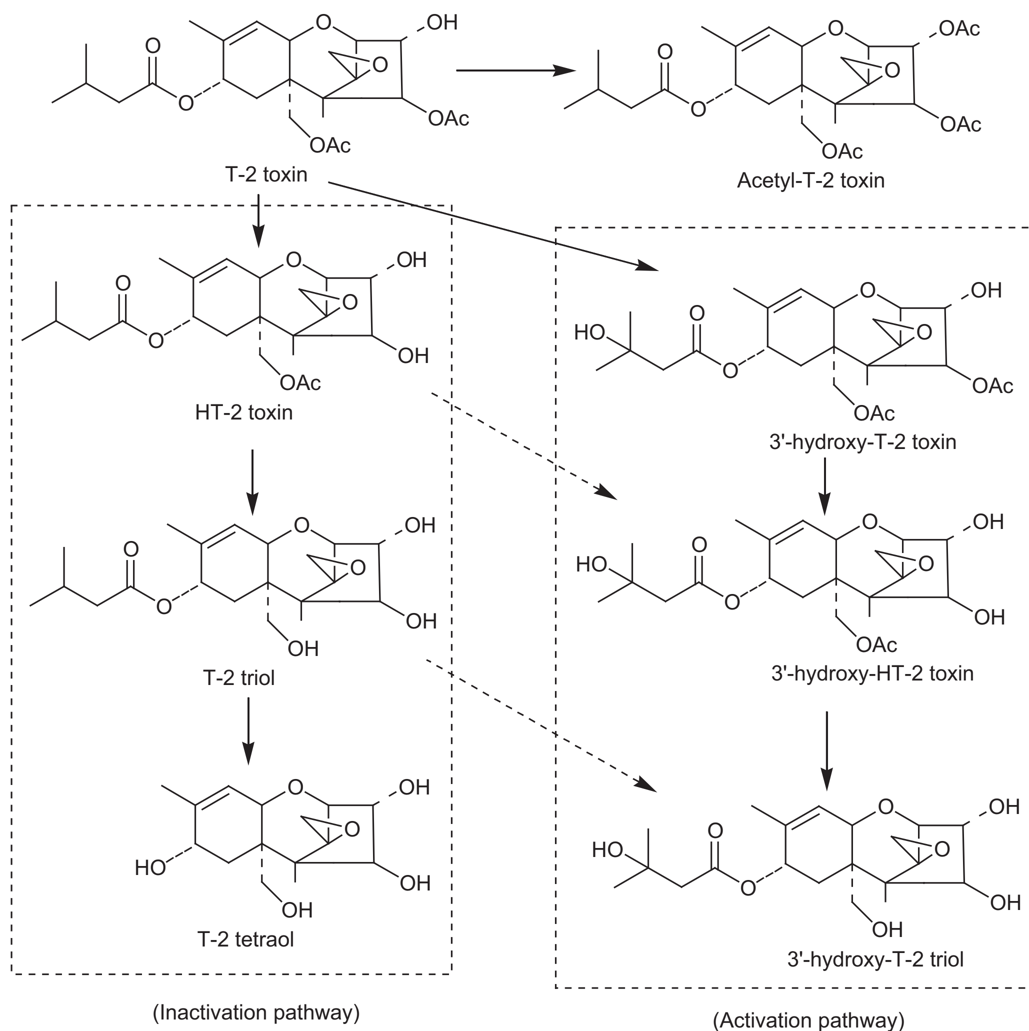

Figure 4. Proposed metabolic pathways of T-2 toxin in humans.

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DON was identified by GC-MS in the urine and feces of male Wistar rats given oral doses of deoxynivalenol at 8–11 mg/kg body weight and was designated as DOM-1. Compared with corresponding ions in the TMS (trimethylsilyl ethers) of DON, DOM-1 was found to have lost 16 mass units (i.e., one oxygen atom). Moreover, a trichothec-9,12-diene skeleton was found. Based on the data in the study, 3α,7α,15-trihydroxy-trichothec-9,12-dien-8-one was proposed for the chemical structure of the newly found metabolite, DOM-1. It was concluded that the transformation of DON in rats underwent a direct deoxygenation (i.e., deepoxidation) at an oxide ring to form a double bond.

Deepoxy metabolite of DON in rats was confirmed by Lake et al. (Lake et al., 1987). Following the administration of a single oral 10-mg/kg dose of [^{14}C]DON to male PVG rats, metabolites in urine and feces were determined by GC-MS. DOM-1 was detected in urine and feces, which represented 10 and 13% of the radioactivity in urine and feces, respectively.

The presence of DOM-1 in rat excreta was the result of microbial metabolism in the gut (Worrell et al., 1989). When DON was administered to male Sprague-Dawley rats treated with antibiotics (e.g., bacitracin sulphate, neomycin sulphate, and tetracycline hydrochloride), there was very little excretion of radioactivity as the deepoxy metabolite in feces or urine. Moreover, when the incubation of DON with a strictly anaerobic preparation of gut contents was performed, the progressive appearance of DOM-1 was detected during a 24-hour incubation period. DON was incubated with liver homogenate as well. However, any formation of DOM-1 was not found, which demonstrated that DON could not be metabolized by rat liver, but by microorganisms in the gut.

Metabolisms of DON and DOM-1 in rat hepatic microsomes were investigated (Cote et al., 1987). In the incubation test, no enzymatic degradation of either DON or DOM-1 occurred, which was closely in agreement with the findings of Worrell et al. (1989). In the NADPH-

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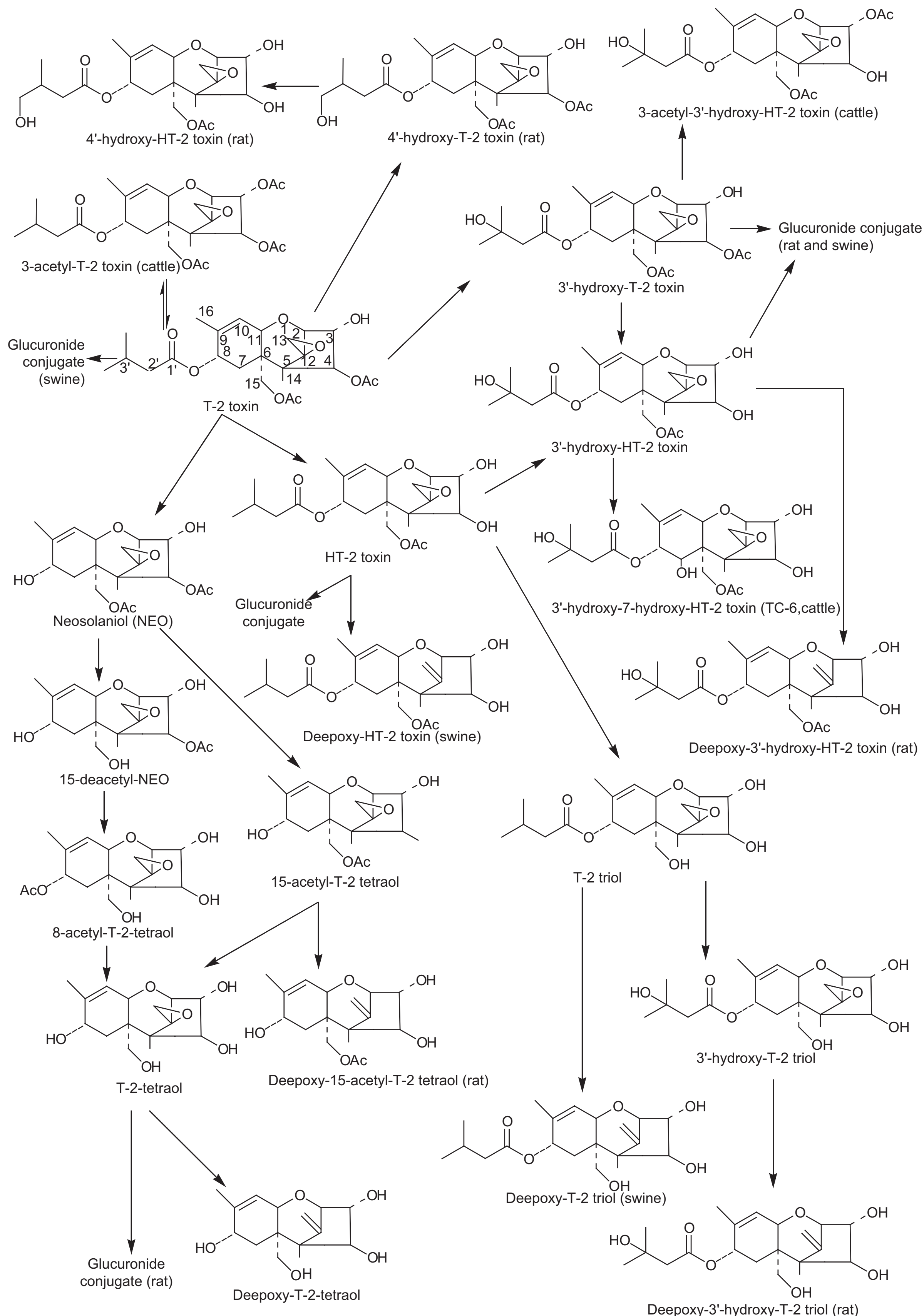

Figure 5. Proposed metabolic pathways of T-2 toxin in animals.

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oxidase experiment, no cytochrome P-450–dependent metabolism of DON or DOM-1 was observed, and no glucuronide conjugates were found when DON or DOM-1 were incubated with UDPGA and liver microsomes from either rats or pigs. Based on these data, it was concluded that DON was neither bioactivated to a more toxic reactive intermediate nor oxidized to a less toxic product by the hepatic mix-function oxidase system in the rat. In the rat and pig, hepatic glucuronidation of DON could not occur.

In summary, DOM-1 was found to be the only metabolite of DON in rats. No glucuronide conjugates were found in feces and urine. However, it cannot be concluded that no glucuronides are formed in the gut, since the β-glucuronidase that is present may have hydrolyzed any glucuronides that are formed. Researchers found that DOM-1 in rat excreta was the result of microbial metabolism in the gut, but not liver metabolism.

Swine

Blood, urine, bile, and feces were collected from pigs over 24 hours after they were given [^{14}C]DON intragastrically at a dose of 0.6 mg/kg of body weight or i.v. at a dose of 0.3 mg/kg of body weight (Prelusky et al., 1988). In distinct contrast to other species, little metabolism was detected through GC-MS analysis. About 95% of the administered dose was recovered as unchanged DON, and the actual glucuronide conjugate of DON was not found, although a glucuronide-conjugate metabolite was found with the treatment of urine and bile samples with β-glucuronidase (Prelusky et al., 1987). The limited metabolism of DON in swine demonstrates that pigs are more vulnerable to DON toxicity.

DON was incubated anaerobically with the suspensions of intestinal contents (e.g., duodenum, jejunum, cecum, colon, and rectum) of porcine origin for 24 hours at 37°C (Kollarczik et al., 1994). The only metabolite, DOM-1, was found and identified by GC-MS. The caudal segments (e.g., cecum, colon, and rectum) of the gut, particularly the colon content showed stronger metabolic activity, whereas the microorganisms of the cranial segments exhibited no transforming activity. Further, that biotransformation of DON to DOM-1 resulted in a significant loss of cytotoxic activity was proven by their MTT cell-culture test.

Castrated male pigs were adapted to a diet containing DON (402 mg/kg) over a period of 7 days (Danicke et al., 2004). Metabolites were analyzed in serum and digesta from consecutive segments of the digestive tract. No DOM-1 was detected in serum and only traces in the stomach and small intestine, whereas notable amounts of the metabolite were found in the distal segments of the gut, which agreed closely with the *in vitro* study (Kollarczik et al., 1994). Developing fetuses could also be exposed to DON and DOM-1 when the sows were fed a *Fusarium* toxin–contaminated diet (Danicke et al., 2007).

Absorption, metabolism, and excretion of 3-aDON in pigs were studied (Eriksen et al., 2003; Eriksen and Pettersson, 2003). Deacetyl metabolite, DON, was detected in plasma as soon as 20 minutes after the start of feeding. Moreover, a significant part of DON in plasma was in a glucuronide-conjugated form (42 ± 7%). DOM-1 was found in feces, which constituted 52 ± 15% of the total amount of 3-aDON-metabolites detected in feces; the remaining part in feces was DON. However, no free or conjungated 3-aDON or deepoxide metabolites of DON were found in urine and plasma. No deepoxide of 3-aDON with an intact acetyl side chain (deepoxy-3-aDON) was found in any sample, which was concordant with previous studies (Eriksen et al., 2002).

Recently, DOM-1 in the bile and kidney of pigs was found in the *in vivo* test, as well (Doll et al., 2008). Moreover, the carryover factor of DON and DOM-1 for the kidney was found to be higher than for muscle and liver.

In summary, pigs are more vulnerable to DON toxicity, duo to their limited metabolic activity. DOM-1 and glucuronide-conjugated DON are the metabolites in pigs. DON can be metabolized in the gut, especially in the caudal segments. The acetylated form of the toxin is deacetylated and then transformed to DOM-1 *in vivo*. Deepoxides could not be found in the urine and plasma of pigs. However, in rats, the presence of deepoxides in plasma and urine was a result of the rat being coprophagous, ingesting deepoxides present in the feces (Eriksen et al., 2003; Eriksen and Pettersson, 2003). It is noticed that the deepoxydation of DON, which primarily occurs in the hindgut, probably does not contribute much to a detoxification in pigs (Danicke et al., 2004).

Ruminants

DON was incubated *in vitro* with rumen fluid for 24 hours (King et al., 1984). A single product was identified as 3α,7α,15-trihydroxy-trichothec-9,12-dien-8-one (DOM-1) by infrared (IR) spectra, ultraviolet (UV) detection, MS, and NMR. 3-aDON was transformed to the deacetylated product, DON, during the incubation. DOM-1 was found with *in vitro* rumen incubation in other studies, as well (Cote et al., 1986a; He et al., 1992). However, this transformation was not found in the *in vitro* incubation with rumen fluid from sheep (Kiessling et al., 1984).

The finds that cattle are able to transform DON to DOM-1 was confirmed by the *in vivo* trial (Yoshizawa et al., 1986). Lactating Holstein cows were fed twice a day for 5 consecutive days with a ration spiked with naturally DON-contaminated corn. Milk, excreta, and

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blood samples were detected by GC-MS. DOM-1 was clearly identified in urine, plasma, and milk. Data in the investigation indicated that DON orally administered was deepoxidized, and DOM-1, as a free metabolite, was transmitted into the cow's milk through the blood circulation. Thus, DOM-1 appears to be an appropriate diagnostic index for estimating the intake of DON in some animals, including the ruminants.

Following a single bolus oral dose of 920 mg DON to 2 dairy cows (Prelusky et al., 1984), conjugated DON accounted for 24–46% of the total levels present in serum. Free and conjugated DON were also present in cow's milk, but only extremely low amounts (less than 4 ng/mL) were detected. Consequently, the conclusion of no transmission of DON to milk following oral administration to dairy cows was proposed.

No unconjugated DON was detected in any milk sample, following the feeding of DON contaminated corn to 3 dairy cows for 5 days (Cote et al., 1986b). This confirmed the findings of Prelusky et al. (1984). However, DOM-1 was detected in the milk of all three cows during the 5-day period of feeding. Unconjugated DOM-1 was also found in urine and feces. Based on an increase in the recovery of the deepoxy metabolite after treatment with β-glucuronidase, that conjugated DOM-1 would also be present in milk was deduced.

Three metabolites, glucuronide-conjugated DON, DOM-1, and glucuronide-conjungated DOM-1, were found to be in the urine of male sheep following either i.v. or oral administration of the toxin at levels of 0.5 and 5.0 mg/kg of body weight, respectively (Prelusky et al., 1986). Only trace unconjugated DOM-1 were detected in bile, whereas conjugated DOM-1 was identified as only a minor metabolite in plasma, which was consistent with the observation of the earlier works (Prelusky et al., 1985). Although glucuronic acid conjugated both DON and DOM-1, they were present in considerably greater amounts in urine (21.5%) and bile (3.5%), respectively. Approximately 33% of the administered dose remained unaccounted for. Consequently, the remainder of the dose may be attributed to other unknown metabolites.

Metabolic fate of DON in lactating sheep, using ^{14}C-labeled DON to examine the metabolic pattern of DON in lactating sheep by total radioactivity determination, was studied (Prelusky et al., 1987). Seven metabolites were detected in urine by a combination of radioisotopic counting and chromatographic detection techniques. In addition to DON-glucuronide, DON-sulfate, DOM-1, and DOM-l-glucuronide, and another three unknown metabolites were detected. These metabolites were excreted essentially in the urine (91%) and, to a lesser extent, in the bile (6%).

In summary, there is an extensive metabolism of DON in ruminants. DOM-1, DOM-l-glucuronide, DON-glucuronide, and DON-sulfate are the major metabolites identified, to date. However, some metabolites remain unclear. The metabolite, DOM-1, is able to be transmitted to milk. Because of the bacteria and protozoa in rumen fluid, DON and its metabolites are excreted efficiently in ruminants, with most being eliminated by urinary excretion in the conjugated form.

Poultry

In vitro biotransformation of DON in the contents of the large intestines of chickens was investigated (He et al., 1992). DOM-1 was identified by GC-MS. The inhibition of DON transformation by low pH was observed, which may be due to either the inactivation of the microorganisms in the acidic conditions or by specific inhibitory effects on the deepoxidation process. In addition, the energy dependence of biotransformation was studied. Sodium azide, at 0.1% (wt/vol), was added in the medium, and the deepoxidation reaction was found to be completely blocked. The epoxy-reductase activity may depend on either the electron transport or the energy supply in the bacterial cells.

In vitro metabolism of DON by transformation from the chicken hindgut was also suggested by Lun et al. (1988). *In vitro* incubation of DON in media having large intestinal contents (e.g., cecal and colonic) led to a high rate of reduction in DON concentration; however, the mechanism was not identified.

Recently, *in vitro* biodegradation of DON, 3-aDON, and 15-aDON by chicken intestinal microbes were studied (Young et al., 2007). DOM-1 was the metabolite of DON. DON and DOM-1 were the metabolites of 3-aDON. As for 15-aDON, deepoxy-15-aDON, DON, and DOM-1 were the metabolites. 15-aDON was the only one of three trichothecenes to show a deepoxidation product with the acetyl group still intact.

In summary, DON can be transformed to DOM-1 in the hindgut of chickens. Low pH could inhibit DON transformation. 3-aDON can be metabolized to DON and DOM-1. In contrast to DON and 3-aDON, 15-aDON has the direct deepoxidation product, deepoxy-15-aDON. However, little data are available for the *in vivo* metabolism of DON in chickens, to date. Consequently, more work is needed on the metabolism of DON in chickens, particularly through *in vivo* investigation. The metabolic pathway of DON in animals can be found in Figure 6.

Humans

Samples of human feces were incubated under anaerobic conditions for 48 hours with 3-aDON (Eriksen et al., 2003; Eriksen and Pettersson, 2003). 3-aDON was deacetylated to DON during incubation. In contrast to what has been reported from experiments with

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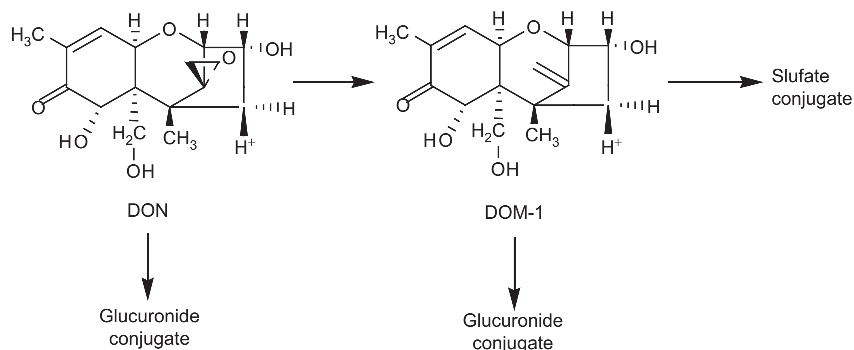

Figure 6. Metabolism of DON in animals.

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rats, mice, and pigs, no deepoxidated metabolites were detected in the fecal incubates. It suggests that humans in this study may lack the relative microflora for a key detoxification of DON.

Metabolism of DON in human proximal tubule cells and lung fibroblasts in primary culture was investigated by utilizing liquid chromatography (LC)-MS (Konigs et al., 2007). Due to the recovery rate being close to 100%, and the negative results of MS experiments, it was concluded that DON was metabolized neither by proximal tubule cells nor by lung fibroblasts.

Nivalenol (NIV) and fusarenon-X (FX)

NIV and FX belong to group B trichothecenes. Both NIV and FX are produced by *Fusarium* genus and often co-occur with DON. They are commonly reported from Europe in the ears of cereals affected by *Fusarium* head blight. Their presence is attributed mainly to *F. graminearum* and *F. culmorum*. They can be also produced by *F. poae* and *F. cerealis*. The toxic effect of these mycotoxins can also be potentiated with more toxic trichothecenes, such as T-2 toxin or DON.

Rodents

Onji et al. (1989) first studied the *in vivo* metabolism of NIV in rats. Each of 5 male Wistar rats were orally administered NIV (5 mg/kg of body weight) 12 times at 2- or 3-day intervals. Urine and feces were collected daily for 39 days. A metabolite isolated from rat feces was identified as 3,4,7,15-tetrahydroxytrichothec-8,12-dien-8-one, or namely, deepoxy-NIV, on the basis of MS and ^{1}H- and ^{13}C-NMR spectroscopy. Moreover, deepoxy-NIV was found excreted predominantly in feces rather than in urine, the excretion was 24 hour later than that of NIV.

Both *in vivo* and *in vitro* metabolisms of NIV and FX in female ICR mice were investigated by utilizing ^{3}H-NIV and ^{3}H-FX (Poapolathep et al., 2003). During *in vivo* study, a large proportion of ^{3}H-FX was found to be excreted as ^{3}H-NIV in urine and feces (Figure 7). ^{3}H-NIV was mostly excreted in the unchanged form, except for an unknown metabolite. However, in distinct contrast to the findings of Onji et al. (1989), deepoxy-NIV was not found in their study. In addition, when ^{3}H-FX was incubated with homogenates of various tissues, such as liver, kidney, small intestine, thymus, spleen, plasma, and red blood cells, the tissues were able to metabolize ^{3}H-FX to ^{3}H-NIV. The highest activity was in the liver, followed by the kidney. Consistent with this, the same conversation of FX was observed in the studies with *in vitro* liver microsomes of rat and rabbit (Ohta et al., 1978). FX was excreted mainly into urine, whereas NIV was excreted mainly into feces in mice. In subsequent studies (Poapolathep et al., 2004a, 2004b), NIV was found to be transferred in unchanged form to fetal or suckling mice via placenta or milk, respectively, while FX did so after being metabolized to NIV in maternal body.

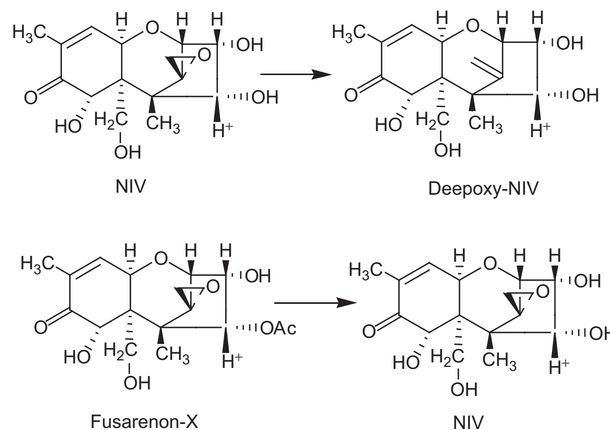

Figure 7. Metabolic pathways of nivalenol (NIV) and fusarenon-X (FX) in animals.

In summary, NIV is able to be transformed to deepoxy-NIV in rats and is excreted mainly though feces. FX can be metabolized to NIV via deacetylation in mice, excreted mainly in urine. The liver and kidney are the organs responsible for the FX-to-NIV conversion.

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Swine

After NIV was fed to pigs with doses of 0.05 mg/kg of body weight, twice-daily for a week, no metabolites were found in plasma, urine, and feces, either as glucuronic acid or sulphate conjugates or as deepoxy-NIV (Hedman and Pettersson, 1997). When the time of feeding NIV was prolonged for 3 weeks in order to investigate if there was a time-dependent effect of exposure to NIV on the ability to form deepoxy-NIV, all pigs were able to metabolize NIV to deepoxy-NIV (Hedman et al., 1997). When NIV was incubated at 37°C with the feces collected from the pigs exposed to NIV for 48 hours, 99.1 ± 1.3% of NIV was transformed to deepoxy-NIV. However, no such metabolite was detected in incubations with feces collected prior to the exposure period.

Deepoxy-NIV was also found in the anaerobic incubation with pig feces collected at different pig farms (Eriksen et al., 2002). No deepoxidation ability was found in samples of feces or ileum content from pigs at the start of the feeding trial or during the first 2 weeks when the pigs were fed uncontaminated feed. However, after the pigs were exposed to feces from pigs known to have the deepoxidation ability for 1 week, the deepoxidation ability was found in fecal and ileal incubates from 4 of the 5 pigs. A conclusion that the deepoxidation ability was able to be transferred between pigs in a stock was proposed.

In summary, NIV is able to be transformed to deepoxy-NIV in pigs exposed to NIV for a long period. The deepoxidation ability may be transferred between pigs in a stock.

Ruminants

Data available for ruminants are limited. Ruminal fluid was taken 2 hours after feeding from a fistulated cow (Swedish Red and White) and filtered through a coarse net. After being incubated for 48 hours at 37°C, 78–82% of NIV was deepoxidated to deepoxy-NIV (Hedman and Pettersson, 1997).

Poultry

FX was administered orally at a dosage of 2.2 mg/kg of body weight to 4-week-old broiler chickens and ducks, and NIV and its metabolite in plasma and excreta were determined by GC-MS (Poapolathep et al., 2008). NIV appeared in the plasma of both broilers and ducks administered at 10 minutes, indicating that FX was absorbed and metabolized very rapidly, presumably in the liver and kidney. In addition, to study the tissue capable of the conversion of FX to NIV in broilers and ducks, FX was incubated with liver and kidney postmitochondrial fractions, red blood cells, and plasma. The FX-to-NIV conversion was noted clearly in the liver and kidney, with the highest activity being in the liver of ducks (98.95%), but in the kidney of broiler chickens (94.39%). The FX-to-NIV conversion by broiler liver was 70.12%, and that by duck kidney was 94.32%. Data from the *in vitro* incubations demonstrated that the liver and kidney were capable of FX-to-NIV conversion.

Feces were collected after 3 weeks of feeding 2.5 or 5 mg/kg NIV to broiler chickens (Cobb). Deepoxy-NIV was not detected in any samples of feces. However, an unidentified metabolite was found in all feces samples, except for 1 bird fed 5 mg/kg NIV, which was tentatively identified as an acetylated metabolite of NIV (Hedman and Pettersson, 1997).

Humans

Human feces were collected within 1 minute after defecation from 10 volunteers (5 men and 5 women, 25–55 years). After anaerobic incubation for 48 hours at 37°C, no deepoxide of NIV was detected by GC-MS (Eriksen and Pettersson, 2003).

Diacetoxyscirpenol (DAS)

DAS belongs to group A trichothecenes. It is a potent mycotoxin produced by certain *Fusarium* strains. It is one of the most important contaminants of agricultural products, together with other trichothecenes.

Rodents

A metabolite of DAS was found after DAS was incubated in the rat- and rabbit-liver microsomes, which was identified as 15-monoacetoxyscirpenol (15-MAS) by GC-MS and NMR (Ohta et al., 1978). The microsomal nonspecific carboxyesterase from rat and rabbit liver played a key role of hydrolyzing DAS to 15-MAS.

Three metabolites were found when DAS was incubated with uridine 5'-diphospho-glucuronic acid (UDPGA; 12 mmol/L), β-naphthoflavone–induced hepatic microsomes from male Long-Evans rats, $MgCl_2$, and K_2HPO_4 at 37°C. They were identified as glucuronide-DAS, 15-MAS, and 4-MAS (Roush et al., 1985).

Male Wistar rats were orally administered DAS (2.8 mg/kg of body weight) three times at 7-day intervals. Urine and feces were collected daily for 21 days (Sakamoto et al., 1986). In addition to 15-MAS, and scirpentriol (SCP), which were already identified in rats and pigs (Bauer et al., 1985), another two new products, DRM-1 and -2, were found in urine and feces. DRM-1 and -2 were identified as 15-acetoxy-3α,4β-dihydroxytrichothec-9,12-diene (deepoxy-15-MAS) and 3α,4β,15-trihydroxytrichothec-9,12-diene (deepoxy-

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SCP), respectively. 15-MAS, SCP, deepoxy-15-MAS, and deepoxy-SCP could be found in urine. However, only deepoxy-15-MAS and deepoxy-SCP were in feces. The four metabolites, 15-MAS, SCP, deepoxy-15-MAS, and deepoxy-SCP, were also detected when DAS was incubated in the feces of rats (Swanson et al., 1988).

In summary, DAS can be transformed to glucuronide-DAS, 15-MAS, 4-MAS, SCP, deepoxy-15-MAS, and deepoxy-SCP *in vivo* and *in vitro* studies. There should be deepoxy-4-MAS in the metabolites, although it has not been isolated yet. Metabolism of DAS can occur in both rat liver and intestinal tract. Metabolic pathways of DAS can be found in Figure 8.

Swine

Following orally administered DAS (2 mg/kg of body weight) to female pigs, DAS and its two metabolites were detected in blood serum extracts by GC-MS (Bauer et al., 1985). Based on the corresponding standards, they were characterized as 15-MAS and SCP, which supposed that DAS is deacetylated in a stepwise manner, first at C-4 and then at C-15.

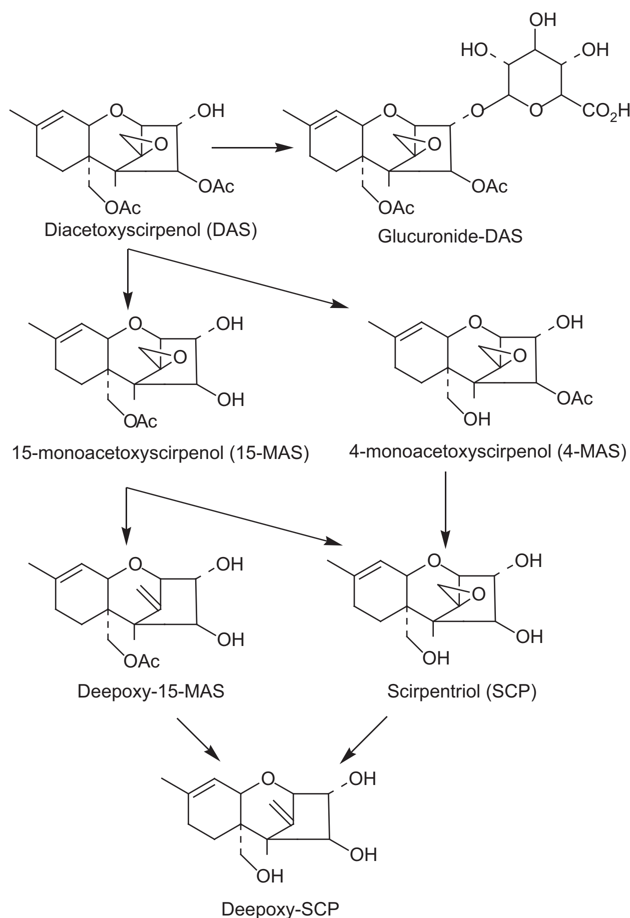

Figure 8. Metabolic pathways of diacetoxyscirpenol (DAS) in animals.

Similar to rats, four metabolites were found in the anaerobic incubations of feces of pigs (Swanson et al., 1988). In addition to 15-MAS and SCP, microorganisms obtained from pigs completely biotransformed DAS, primarily to another two deacylated deepoxidation products, deepoxy-15-MAS and deepoxy-SCP.

Ruminants

When DAS was incubated with rumen fluid (cattle and sheep) for 3 hours, only one metabolite, 15-MAS, was detected; the protozoa were more active than the bacteria. Only minor differences were observed in the rate of DAS metabolism between rumen fluids from sheep and cattle (Kiessling et al., 1984).

DAS was extensively biotransformed when incubated with bovine rumen fluid from a fistulated dairy cow for 12, 24, and 48 hours (Swanson et al., 1987). Four products were detected, including 15-MAS, SCP, deepoxy-15-MAS, and deepoxy-SCP. No parent DAS was observed at any of the three incubation time periods.

Data of *in vivo* metabolism of DAS in ruminants are not available.

Poultry

Feces microflora from chickens, in contrast to other species (e.g., rats, pigs, and cattle), were not able to produce the deexpoxide group in DAS, but could only be transformed to deacylation products, MAS and SCP. Similar to poultry, horses and dogs were found to lack the deepoxy ability, as well (Swanson et al., 1988).

Humans

After the dermal side of the skin was bathed by PBS with antibiotics and amphotericin-B (PBSA) to reduce bacterial and fungal growth, the epidermal surface was dosed with [^{3}H]-DAS dissolved in either methanol or DMSO and incubated in the receptor fluid (PBSA) for 48 hours (Kemppainen et al., 1986b). 15-MAS was identified during the penetration of DAS through excised human skin. However, the metabolism of [^{3}H]-DAS in receptor was minimal, which was consistent with the hypothesis that metabolism of [^{3}H]-DAS by enzymes leaching out of the skin into the receptor fluid was minimal.

Conclusions

Metabolism of T-2 toxin can notably happen in the liver, but also in the digestive tract and, more particularly, in the rumen for ruminants. More than 20 metabolites of T-2 toxin have been identified. A diversity of metabolic pathways of this mycotoxin has been found. The

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major metabolic pathways of T-2 toxin concerned are hydrolysis, hydroxylation, deepoxidation, and conjugation. T-2 toxin is hydrolyzed preferentially at the C-4 position to give HT-2 toxin. HT-2 toxin is then metabolized in two ways, one of which is the hydroxylation at C-3′ to yield 3′-hydroxy-HT-2 toxin in the liver, which is excreted in both free and glucuronide form. The isomer of 3′-hydroxy-HT-2 toxin, 4′-hydroxy-T-2, a more toxic metabolite, is also found *in vitro*. 3′-hydroxy-HT-2 has been found in many species, including mice, rats, rabbits, cows, swine, chickens, and even in humans. This metabolite is found to be more toxic than the parent T-2 toxin, so this is an active metabolic pathway. In contrast, the second way is an inactivation pathway. This portion of HT-2 toxin is further hydrolyzed to T-2 tetraol via 4-deacetylneosolaniol and T-2 triol, which is more water soluble and less toxic than T-2 and HT-2 toxins. Cytochrome P-450 can catalyze the hydroxylation at the C-3′ position of T-2 and HT-2 toxins.

Deepoxidation is an important *in vivo* metabolic pathway for T-2 toxin in rodents, ruminants, and swine. Moreover, deepoxidation is suggested as an important metabolic detoxification for animals. Interconversions between acetylation and deacetylation reactions in the metabolism of T-2 toxin could occur in bovine. HT-2 toxin is the main metabolite in human skin and fibroblasts.

DON is able to be transformed to deepoxy metabolite, DOM-1, in rodents, swine, chickens, and ruminants. However, this metabolite cannot be found in humans. 3-aDON can be deacetylated to DON by humans. 15-aDON has the direct deepoxidation product, deepoxy-15-aDON, but not for 3-aDON. Conjugated products, DON-glucuronide, DON-sulfate, and DOM-l-glucuronide, can be found in ruminants. DON cannot be metabolized by the liver, but by microorganisms in the hindgut of animals.

NIV can be metabolized to deepoxy-NIV by rats, swine, ruminants, and excreted from feces. However, chickens and humans do not have this ability. The deepoxidation ability of NIV may be transferred between pigs in a stock.

FX is quickly metabolized to NIV via deacetylation in mice, chickens, and ducks. Liver and kidney are the organs responsible for the FX-to-NIV conversion.

DAS is first metabolized to 15-MAS via C-4 deacetylation, which is then transformed to SCP via C-15 deacetylation. Finally, the epoxidic group is lost to yield deepoxy-SCP. Deepoxy-MAS was also found. Glucuronide-DAS and 4-MAS are found in rats. Chickens lack the deepoxidation ability and produce only 15-MAS and SCP. DAS can be metabolized to 15-MAS during the penetration of DAS through excised human skin. Metabolism can occur in both the liver and the intestinal tract of animals.

To date, the metabolism of trichothecenes in animals and humans remains to be studied further. In swine, some unknown metabolites of T-2 toxin, such as PM-1–PM-3, are not elucidated yet. In poultry, the unknown T-2 tetraol monoacetate isomer and other uncharacterized metabolites in chickens are not identified. Moreover, data of T-2 metabolism in chicken liver are limited. Similarly, as for DON, although some unknown metabolites were detected in ruminants, their structures are not elucidated as yet. There are little data for *in vivo* metabolism of DON in chickens, to date. Whether NIV is able to be metabolized in animal liver is not illustrated clearly, and data for metabolism of NIV in ruminants are still not complete. In comparison with other trichothecenes, the study on the metabolism of DAS in animals is not extensive, especially in ruminants and poultry. Study on the metabolism of all trichothecenes in human is quite limited, even in the *in vitro* metabolism study.

Although the structures of some metabolites from trichothecenes were elucidated, their toxicity to animals and humans are not clear. Only a few researchers have investigated the possible presence of toxic residues in animal products. Consequently, toxicology and residue studies should be concerned in future investigations. Moreover, most researchers investigated metabolic fates of only one trichothecene in animals; however, animals in nature are often exposed to several mycotoxins simultaneously. Thus, the metabolism of multitrichothecenes in animals should be investigated, because metabolic pathways may be modified when compared with monotrichothecene in animals. In addition, some metabolites were found to be less toxic than the parent trichothecenes, which provides researchers with something to think about on the detoxification of trichothecenes in animal feed or human food. In the future, with more advanced, sensitive instruments coming out, the trend of study on the metabolism of trichothecenes will be more comprehensive, systematic, and thorough.

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Publikace XI

P14 TESTING OF DIFFERENT *SACHAROMYCES* SPECIES FOR THE ABILITY TO SORB DEOXYNIVALENOL

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Introduction

Mycotoxin contamination of food and feed is a high risk to human and animal health[2]. The majority of the causal organisms are producers of mycotoxins such as highly toxic trichothecenes[4]. Four types of trichothecenes are described, type A and type B, which differ in the presence or absence of a keto group at C-8 of the trichothecene skeleton, type C with additional epoxydic group and macrocyclic type D. The most common trichothecene in cereals is type B trichothecene deoxynivalenol (DON).

Deoxynivalenol, also called vomitoxin, is produced by *Fusarium fungi*, such as *F. graminearum* or *F. culmorum*. It has negative effect on animal growth and health. DON inhibits the synthesis of DNA, RNA and proteins at the ribosomal level. High doses causes the vomiting in pigs, lower concentrations in the diet reduces feed intake and animal growth[5]. Different physical and chemical methods have been recommended for detoxification of mycotoxin-contaminated food and feed. Nevertheless, only a few of them have been accepted for practical use. Thermal degradation of trichothecenes is not so effective, because they are relatively stable and they decomposes at 210 °C within 30–40 min[10]. From physical methods are the most frequently used feed additives on sorbent basis, such as bentonite or active charcoil. The disadvantages of adsorbents are their relatively high dosage and sorption of biologically active compounds, e.g. vitamins or trace metals. In addition, the sorbents can bind to only a limited group of mycotoxins and in some cases does not provide required effect[7]. Biological decontamination of mycotoxins using microorganisms is one of the well-known strategies for the management of mycotoxins in food and feed. Biological decontamination of mycotoxins by different microorganisms was reviewed several times.[1,8,11,15] There are two ways of action – sorption on cell walls or enzymatic degradation, for example using epoxydase. De-epoxylated form is less toxic than original DON. Among the different potential decontaminating microorganisms, the genus Saccharomyces represent the unique group, which is widely used in food fermentation and preservation. The aim of this study was screening of ability of different Saccharomyces species to remove deoxynivalenol from liquid medium.

Experimental

Microbial Cultures and Culture Conditions

All cultures were obtained from Culture Collection of Yeast (Bratislava, Slovakia). The cultures were *Saccharomyces cerevisiae* 20_1; isolated from loaf of hornbeam, *Saccharomyces bayanus* 21-31-12; isolated from mushrooms, *Saccharomyces paradoxus* 2 isolated from needles of spruce, *Saccharomyces paradoxus* 21-53-2 isolated from soil and *Saccharomyces paradoxus* isolated from the loaf of locust. All cultures were cultivated in Plate Count Broth (PCB; Merck, Germany) at 30 °C for 48 hours and then subcultured by transfering 4 ml of the culture to the cultivation test tube. Three replicates per sample of inoculum were used at each measuring. On the base of O. D. (600 nm) values that have been taken during the cultivation of yeasts with deoxinyvalenol the analysis of their counts has been done.

Preparation of Yeast Cultures for Testing of DON Sorbtion

All used chemicals were analytical or gradient grade. Standard of deoxynivalenol (DON) was obtained from Sigma-Aldrich, s.r.o. (Czech Republic). The stock solution of DON was prepared by dissolution of 1 mg of DON in 5 ml of acetonitrile to give solution with concentration 0.2 mg ml^{-1}. The solution was stored at –18 °C. Working solutions for calibration curve measurement were prepared by dilution of stock solution.

The amount of 0.5 μg of DON was transferred to test tube and evaporated to dryness. In the next step, 4 ml of cultivation media (PCB) with yeasts were added to the test tube and the sample was cultivated in thermostated box at 30 °C for 4 hours. The concentration of free DON was measured at the beginning of cultivation and after 4 hours of cultivation.

Prior the DON determination it was necessary to remove yeasts from culture medium using ultrafiltration through polytetrafluoroethylene membrane filter (SMI-LabHut Ltd., UK) with pore size 0.20 μm. After this step, the filtrate was diluted with acetonitrile (in ratio 16 : 84). Next, the clean-up with MycoSep® 225 Trich was applied. Briefly, 5 ml of solution was transferred to the glass tube and pushed through the MycoSep® 225 Trich column. 2 ml of this eluate were evaporated to dryness and redissolved in 400 μl of HPLC mobile phase. Mobile phase consisted of 1mM formic acid/acetonitrile (90 : 10, v:v) with flow rate 1 ml min^{-1}.

Chromatographic Determination of DON

The HPLC system HP 1100 (Agilent Technologies, Palo Alto, USA) consisted of vacuum degasser unit (model G1322A), quaternary pump (G1311A), autosampler (G1313A) and quadrupole mass spectrometer (G1946VL) with electrospray ionization was used. The ChemStation software (Rev. A 10.02) controllig chromatographic system and was used for chromatogram evaluation.

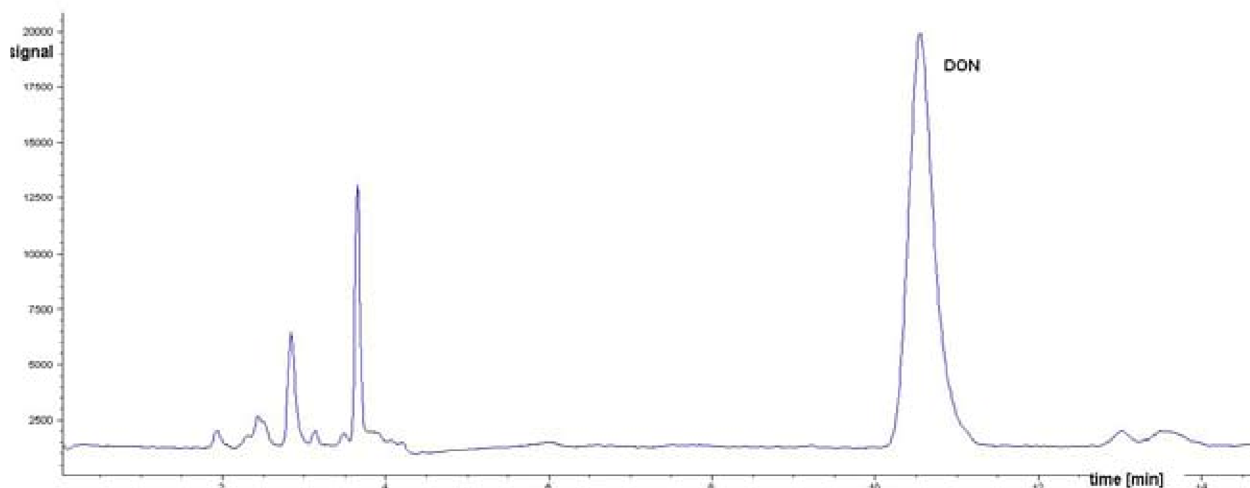

Fig. 1. **Elution of DON in mobile phase**

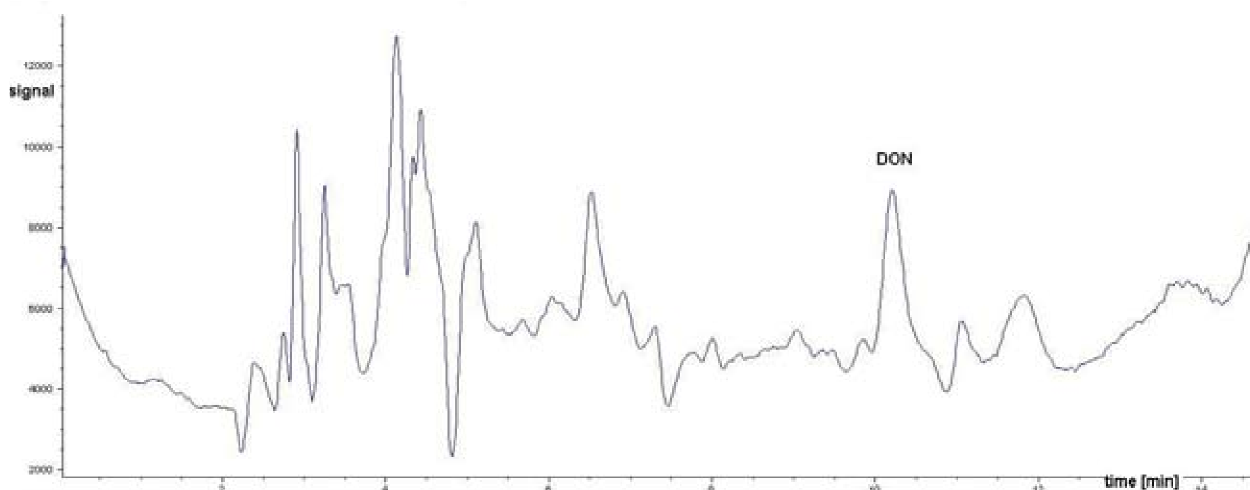

Fig. 2. **Elution of DON in culture medium (Plate Count Broth)**

Analytical determination of DON was performed on reverse-phase chromatographic column LUNA (250 × 4.6 mm, particle size 5 μm; Phenomenex, USA). DON was detected on mass spectrometric detector (MS) as positively charged ions: $[DON+H]^+$, m/z = 297 and $[DON+Na]^+$, m/z = 319). The time of analysis was 15 min, DON was eluted at 10.9 minutes. The separation was performed at laboratory temperature.

The calibration curve was measured using standard addition method. The appropriate amount of DON was added into pure cultivation media solution and processed in the same way as the real samples.

Results

Knowledge on interaction of yeasts with mycotoxins goes back more than three decades[6,12].

The yeast of the genus *Saccharomyces* were described as capable to bind different types of mycotoxins, like aflatoxins[14], ochratoxin A[13], T2 toxin and zearalonon[9].

In this study, isolates of yeasts belonging to different species of the genus *Saccharomyces* were tested for deoxinyvalenol binding.

First of all, the detection of DON in culture medium was tested. It was observed that the detector signal is much lower when analyzed DON in culture medium (PCB) than when analyzed it in pure mobile phase (see Fig. 1. and 2.).

The reason of this phenomenon could be the lowering of deoxynivalenol inonization caused by interfering compounds in culture medium.

The detector signal of initial concentration of deoxynivalenol in culture medium was taken as 100 % for futher calculations.

In all culture batches there was observed decrease in the concentration of DON, which was measured using HPLC.

Of the 5 *Saccharomyces* isolates, all of them were able to sorbe DON.

The percentages of lowering of DON concetrations after cultivation with all *Saccharomyces* isolates are shown in Table I.

Table I
Lowering of DON concentration [%] in culture media with different *Saccharomyses* strains

Yeast strains	Incubation time [hours] 0	4	8
	Concentration [%]		
S. cerevisiae	67.71	71.12	78.15
S. bayanus	67.75	68.07	72.05
S. paradoxus	66.04	67.92	75.65
S. paradoxus	5.83	12.67	18.95
S. paradoxus	57.77	62.61	76.25

The maximum sorbtion of deoxinyvalenol was observed in all yeasts strains immediately after DON addition to culture medium with cultured yeasts.

The next cultivation did not significantly influenced further lowering of DON concentration.

Most of the yeast strains bound more than 70 % (w/w) of deoxinyvalenol. The only isolate from soil (*Saccharomyces paradoxus* 21-53-2) bound less than 20 % (w/w) of the added toxin in PCB.

It follows from Table II that the most significant decrease of live yeast cells was detected at isolate of *S. bayanus* (isolated from muschrooms).

This strain was the most sensitive to deoxynivalenol. On the other hand, the most resistant to deoxynivalol was *S. paradoxus* (isolated from the loaf of locust).

From results obtained in this study it is clear that the ability to sorb deoxynivalenol by several strains of the genus *Saccharomyces* was demonstrated. Commertially, the yeast cell walls are applied as feed additives (Mycosorb). Their detoxifying activity is based on complex formation between glucomannan and mycotoxins.

Tabulka II
The yeasts concentration in cultivation media after addition of DON

Yeast strains	Incubation time (hours) 0	4	8
	Yeast concentration [CFU ml^{-1}]		
S. cerevisiae	1.4×10^7	1.3×10^6	1.1×10^5
S. bayanus	1.9×10^7	1.6×10^5	1.5×10^4
S. paradoxus	1.7×10^6	1.6×10^5	1.3×10^5
S. paradoxus	9.9×10^4	4.0×10^4	5.5×10^3
S. paradoxus	4.5×10^5	1.5×10^5	1.1×10^5

Conclusions

It seems that, according to results of experiments realized till present time, microorganisms are the main living organisms applicable of mycotoxin biodegradation. Further screening of microorganisms for their ability to sorbe deoxynivalenol may lead to detection of more efficient and better applicable yeasts and bacteria.

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Publikace XII

MAIZE EAR ROT, *FUSARIUM* MYCOTOXINS AND ERGOSTEROL CONTENT IN MAIZE HYBRIDS

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Abstract: A field experiment with 26 maize hybrids including two genetically modified (Bt-transformation) ones and their non-transgenic counterparts was aimed to observe infection by maize ear rot caused by *Fusarium* species and infestation by European corn borer. After harvest, the content of mycotoxins (deoxynivalenol, zearalenone, fumonisins) and ergosterol was determined. Climatic conditions of the harvest year 2007 were not favourable for the development of maize ear rot and the mycotoxin content was low. The ergosterol content was significantly correlated with disease extent and severity but there was no significant relationship between maize ear rot and mycotoxins content. A significant relationship was found between zearalenone and European corn borer infestation. The content of mycotoxins in Bt-hybrids was always lower than the mean of all hybrids. None of the tested Bt-hybrids was infested by European corn borer.

Keywords: maize, maize ear rot, *Fusarium*, mycotoxins, European corn borer

Introduction

Fungi of the genus *Fusarium* can cause root, stem and ear rot on maize which can result in severe reduction of the yield and grain contamination by mycotoxins. *Fusarium* species causing maize ear diseases can be basically divided into two groups. The first one includes species responsible for the damage of ears called “maize red ear rot“ - predominantly *F. graminearum*, *F. culmorum*, *F. cerealis* and *F. avenaceum*, the second group includes *F. verticillioides* (syn. *F. moniliforme*), *F. subglutinans* and *F. proliferatum* responsible for “maize pink ear rot“ (Logrieco et al. 2002). The former are common in years and locations with more frequent rainfall and lower temperatures in the summer, while the latter are favoured by drier and warmer weather conditions (Ellend et al. 1997). *F. verticillioides* is the main producer of fumonisins, whereas *F. graminearum* is responsible for production of deoxynivalenol (DON) and zearalenone (ZEA). Climatic factors throughout the maturation season determine the presence of fungal species in maize kernel and, consequently, the presence of mycotoxin in grains (Blandino et al. 2008). Besides the weather, the severity of maize ear rot and subsequent mycotoxin contamination is closely correlated to injury by the European corn borer (ECB) (*Ostrinia nubilalis*) (Munkvold et al. 1999) because injured plant parts are preferably infected by *Fusarium* pathogens. Bt-transformed maize, eg. maize obtained by genetic engineering capable of endogenous production of the δ-endotoxin, is highly resistant against the infestation by ECB. The growing of Bt-transformed maize has a great potential to reduce mycotoxin content in maize. Over the last years, harmfulness of ECB has been increasing in the Czech Republic (CR). In the past, it was present in warmer regions and on maize for grain only. Nowadays, however, it also causes considerable losses to maize for silage.

The objectives of the present study were: (1) to evaluate the infection of hybrids including two Bt-hybrids and their non-transgenic counterparts by maize ear rot, (2) to determine the association between the extent and severity of maize ear rot and

mycotoxin (DON, ZEA, fumonisins) contamination and (3) to verify the reliability of using ergosterol content as a mycotoxin predictor.

Materials and methods

Twenty-six maize hybrids including two Bt-transformed ones and their corresponding conventional counterparts were grown in five replications at the location in the maize production region of the CR (Cejc) in 2007. The following parameters were measured: disease incidence of maize ear rot as percentage of diseased ears (DI total), disease severity (DS) (a 1-9 scale, where 9-no infection, 1-infected more than 50 kernels per ear), percentage of ears infected only by maize ear rot (DI-1), percentage of ears infected by maize ear rot and ECB (DI-2), and percentage of ears infested by ECB in total (ECB). After harvest, the content of DON and ZEA (ELISA method, kits of R-Biopharm Ridascreen®Fast DON and Ridascreen®ZEA) and fumonisins (ELISA method, a kit of Neogen Corporation Veratox®HS) was determined. The ergosterol content was assessed by HPLC according to Varga et al. (2006). Detection limits are given in Table 1. Statistical analyses were performed using Statistica software (StatSoft, Inc.). Since a non-parametric Spearman statistical procedure was used, no assumptions as to the distributional form of the data, such as normality, were made. To calculate means and medians, the values under LOD were replaced by values that consider LOD for a certain analyte and percentage of samples above LOD.

Results and discussion

There were weather conditions in 2007 that do not favour the infection of maize by maize ear rot and, consequently, mycotoxin content was low (Table 1). Limits for *Fusarium* mycotoxins in food maize (European Commission 2006) were not exceeded in any case. There was no significant relationship between infection by maize ear rot and mycotoxin content (Table 2). The ergosterol content was significantly correlated with both disease severity and extent, and positively, however, insignificantly with DON content. Ergosterol as predominant sterol component of fungal cell membranes, seems to be a good predictor of the toxicological quality of maize. The severity and extent of ear infection by maize ear rot were closely correlated. A significant correlation was confirmed between ZEA content and plant infestation by ECB. The mycotoxin content in Bt-hybrids was always lower than the mean for all 26 hybrids. None of the tested Bt-hybrids was infested by ECB.

Table 1. Maize ear rot, mycotoxins content, corn borer infection and ergosterol content in 26 maize hybrids (maximum, minimum, means and medians), Cejc, 2007

	DON (μg/kg)	ZEA (μg/kg)	Fumonisin (μg/kg)	DI total (%)	DS	DI-1 (%)	DI-2 (%)	ECB (%)	Ergosterol (μg/kg)
Min	< LOD	< LOD	< LOD	14	4.56	4	0	0	< LOD
Max	861	45.05	695	88	8.66	82	24	26	9900
Mean	251	6.32	181	36	7.91	27	8	10	1933
Median	208	0.81	103	34	8.15	24	9	11	1360
LOD	51	1.75	50						35

Table 2. Correlation coefficients between mycotoxins and ergosterol content and infection by maize ear rot and European corn borer in selected maize hybrids (n = 26), Cejc, 2007

Variable	Spearman correlation coefficients with								
	DON	ZEA	Fumonisin	DI total	DS	DI-1	DI-2	ECB	Ergosterol
DON	1.000								
ZEA	0.002	1.000							
Fumonisin	-0.156	-0.048	1.000						
DI total	0.244	-0.147	-0.048	1.000					
DS	-0.245	0.082	0.016	*-*0.981*	1.000				
DI-1	0.226	-0.298	0.017	**0.934*	*-*0.904*	1.000			
DI-2	0.151	0.356	-0.216	0.307	-0.332	-0.022	1.000		
ECB	0.088	*0.404*	-0.213	0.268	-0.291	-0.061	**0.975*	1.000	
Ergosterol	0.378	0.082	-0.102	**0.516*	*-*0.532*	*0.470*	0.208	0.193	1.000

Values significant at P <0.05 are in *italics*, values significant at P <0.01 are in *italics* and marked with asterisk.

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Publikace XIII

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Determination of Fumonisins in Milled Corn Grains Using HPLC–MS

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Abstract

A new method for determination of fumonisins in corn samples was developed and validated. The mycotoxins were extracted by a mixture of methanol–acetonitrile–water (1:1:2, v/v/v) and determined on a liquid chromatograph with mass spectrometric detection. The separation was performed on Zorbax XDB-C_{18} column (150 × 4.6 mm; 5 µm) with a Metaguard ODS-2 precolumn (30 × 4.6 mm; 5 µm) using gradient elution with mobile phase consisting of acetonitrile and 5 mmol/L ammonium acetate (adjusted by acetic acid to pH 3.0). For detection of ($M+H^+$) ions, a quadrupole mass spectrometer in single ion monitoring mode was applied. Developed method showed very good linearity in a tested range of concentration. Detection limit is 62.0 µg FB_1/kg and 58.5 µg FB_2/kg of maize grains. Because the detection limits lie under the maximum permitted EU levels, the method is suitable for determination of fumonisins in milled corn grains.

Introduction

The fumonisins, a family of food-borne carcinogenic mycotoxins, were first isolated in 1988 from cultures of *Fusarium verticillioides* (previously known as *Fusarium moniliforme*) (1), one of the most common field fungi associated with corn and corn-based foods and feeds worldwide (2). A 19–20 carbon amino-polyhydroxyalkyl chain characterizes this group of mycotoxins, which is diesterified with propane-1,2,3-tricarballylic acid (3). Of the 28 analogues FBs identified to date, the fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3) are the most important (Figure 1). These three compounds have similar toxicity, but FB_1 is the most associated with food contamination (usually constituting about 70% of the total FBs content) (2,4,5).

Fumonisins induce equine leukoencephalomalacia (ELEM) in horses, porcine pulmonary edema (PPE) hydrothorax and hepatic syndrome in pigs, and hepatotoxic and carcinogenic effects and apoptosis in the liver of rats (6–8). Fumonisins frequently co-occur with aflatoxins in corn and have been shown to promote aflatoxin carcinogenicity in trout (9). Consumption of fumonisin-contaminated corn correlated with the high incidences of human esophageal cancer in South Africa and China (10–12). The carcinogenic risk of fumonisins to humans was evaluated by the International Agency for Research on Cancer (IARC) in 1993, and these toxins have been classified as potential carcinogens for humans (class 2B carcinogens). Maximum levels for fumonisins ($FB_1 + FB_2$) in maize and maize products are setting in Commission Regulation (EC) No. 1126/2007 (for milling fractions of maize with particle size > 500 µm–1,400 µg/kg and for milling fractions of maize with particle size ≤ 500 µm–2,000 µg/kg) (13).

High-performance liquid chromatography (HPLC) with fluorimetric (14–18) or mass spectrometric (19–22) detection (MS) is the most commonly used method for the analysis of fumonisin representatives. The application of HPLC with evaporative light scattering detector was also reported (23). Furthermore, gas and thin-layer chromatography (24) and capillary electrophoresis (25) can be used as well. Competitive enzyme-linked immunosorbent assays (ELISA) (26), which are easy to perform and do not require extensive equipment, may only be used for the quantitative screening of total fumonisin contents due to its non-selectivity of high cross-reactivity of fumonisin B_1, B_2, and B_3. Next, the drawback of ELISA is comparatively poor precision, linearity, etc.

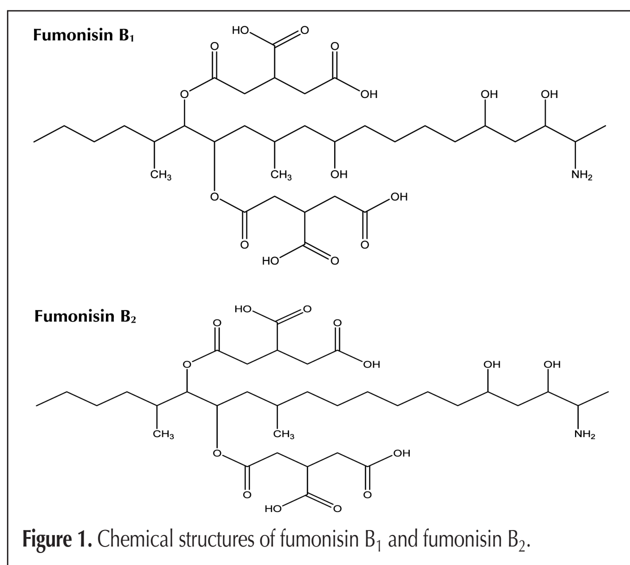

Figure 1. Chemical structures of fumonisin B_1 and fumonisin B_2.

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The extraction of fumonisin compounds from edible matrices has been performed using a mixture of methanol–water, acetonitrile–water, or acetonitrile–methanol–water (27–29). The cleanup, to remove matrix impurities and concentrate the fumonisins, can be done by solid-phase extraction using either reversed-phase (C_{18}) (30) or strong anion-exchange (SAX) cartridges (31,32), or by immunoaffinity columns (18,33). Analytical methods based on HPLC are described previously (21,22,34).

In this work, an innovative procedure for determination of fumonisin B_1 and B_2 in milled corn samples was done. It combines high efficient extraction with methanol–acetonitrile–water using UltraTurrax blending and HPLC–MS.

Experimental

Chemicals and reagents

Analytical-grade purity ammonium acetate, fumonisin B_1 and B_2 standard solution (concentration of FB_1 = 50.1 ± 0.7 µg/mL, FB_2 = 50.2 ± 0.7 µg/mL, in acetonitrile/water = 50/50), and gradient-grade purity solvents acetonitrile and methanol were purchased from Sigma Aldrich (Prague, Czech Republic). All solutions were prepared in deionized water (Demiwa ros, Vatek, Czech Republic) and stored in darkness at 4°C, the standard of pure solid fumonisins at –20°C.

Chromatographic instrumentation

The HPLC system HP 1100 (Agilent Technologies, Palo Alto) consisted of vacuum degasser unit (model G1322A), quaternary pump (G1311A), autosampler (G1313A), and quadrupole mass spectrometer (G1946VL) with electrospray ionization was used. The ChemStation software (Rev. A 10.02) controlled the chromatographic system, and it was used for chromatogram evaluation. The separation was performed on a Zorbax Eclipse XDB-C_{18} column (150 × 4.6 mm; particle size 5 µm) equipped with a Metaguard ODS-2 precolumn (30 × 4.6 mm; 5 µm). Mobile phase consisted of water respective ammonium acetate (1, 2, 5, or 20 mM; pH adjusted by concentrated acetic acid to 3.0) and acetonitrile. Linear gradient elution of fumonisins was applied. The composition of mobile phase at the beginning was 33% acetonitrile, at 8 min was 60%, and at 9 min was again 33%. The column was conditioned prior to each analysis for 6 min. Total time of analysis, including prewash, was 20 min. The flow rate of mobile phase was 0.8 mL/min. The volume of injected sample was 5 µL. All measurements were performed in laboratory thermostated to 20°C.

Extraction

Extraction process is based on modified European Standard EN 114352:2004 "Foodstuffs—Determination of fumonisin B_1 and B_2 in maize based food—HPLC method with immunoaffinity column clean up" (34). Twenty-five milliliters of extraction solution (methanol–acetonitrile–water, 1:1:2, v/v/v) was added to 10 g of milled corn grains and mixed on Heidolph Diax 900 homogenizator for 2 min at speed 3 (Sigma Aldrich). Next, the flask was centrifuged (Universal 32R, Hettich, Germany) at 4000 rpm for 5 min. The supernatant was transferred to a 50-mL volumetric flask. Extraction of solid rest was then repeated with 20 mL and 15 mL of extraction solution. Volume of collected extracts was adjusted in 50 mL. Extracts were filtered through a polytetrafluoroethylene membrane filter (SMI-LabHut Ltd., Gloucestershire, UK) with pore size 0.20 µm prior to HPLC–MS determination.

Preparation of standard solutions and spiked samples

Calibration solutions were prepared fresh from stock solution of fumonisin B_1 and B_2 mixture by dilution with an appropriate amount of mobile phase and stored at 4°C in a dark place until analysis. Spiked samples were prepared by addition of pure mycotoxin standard to maize flour sample.

Results

HPLC–MS method optimization

To obtain the highest sensitivity of the quadrupole mass spectrometric detector, it was necessary to set up all parameters. At the start, the composition of mobile phase was optimized. In screening experiments, there was positive influence of acidic solution on ammonium acetate. Thus, the effect of acidic acetate concentration was tested and compared with ionization in neutral water–acetonitrile mobile phase. Presence of ammonium acetate in mobile phase led to higher ionization. We assumed that the presence of ammonium ions supports the formation of protonated species. The mechanism of this formation process of this species is speculative. For example, the protonated species can be formed via unstable intermediate $(M+NH_4)^+$. The highest ionization was obtained at 5 mmol/L concentration. With a higher concentration of ammonium acetate, there appeared problem with electrospray functionality due to high conductivity of aerosol and discharges in the spray chamber.

The quadrupole mass detector allows setting of the fragmentor (collision) voltage and fragmentation regulation of charged compounds. The trend of abundance of molecular ions as a function of fragmentor voltage has typical shape. At low voltage, adducts of molecular ion with sodium respective to mobile phase constituents are formed on an account of single charged protonated molecular ion. This leads to the molecular signal decreasing. When voltage is increasing, the abundance of molecular ion is increasing, and amount of adducts is lowering. At voltage 300 V, the molecular peak show maximum intensity. Higher voltage leads to higher molecular fragmentation and significant decreasing of signal. The dependence of signal intensity on electrospray capillary voltage is hyperbolic. With increasing voltage, the abundance of molecular ions increases. The highest signal was observed with voltage 6000 V.

Nebulizer pressure did not have a significant influence on ionization. Low nitrogen pressure caused problems with insufficient evaporation of mobile phase under relatively high flow rate (0.8 mL/min). The highest possible pressure (60 psig) was chosen as the optimal value for maximal evaporation.

The ionization obtained at nebulizer gas flow 12 L/min was approximately 20% higher than those under low flow rates. High flow rate supports better mobile phase evaporation and ionization.

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Because fumonisins are relatively stable in heat, high temperature of drying gas can be applied. The influence of temperatures at intervals 150–350°C was investigated. Low temperatures (below 200°C) made ionization difficult because the aerosol was wet and caused discharges in electrospray chamber. The best ionization was obtained at 350°C. The problem was caused due to the high flow rate of mobile phase and relatively low content of organic solvent in it. On the other side, the compromise between time analysis and ionization was reached.

The developed method is faster in comparison with methods using fluorescence detection because it was not necessary to perform the derivatization step. The detection limit (LOD) for both detected fumonisins allows method application in food control without the necessity of preconcentration.

The developed method was then applied on determination of fumonisins in various samples of grounded maize corn. The mycotoxin content in this material ranged between > LOD–10.878 mg FB_1/kg and > LOD–2.114 mg FB_2/kg. Higher concentrations of FB_1 than FB_2 were found in all positive samples. These results correspond to those of analysis of *Fusarium verticillioides* naturally contaminated food samples, where the FB_1 forms 70–80% of total fumonisins and FB_2 15–25% (35).

Table I. Quantification of FB1 and FB2

	LOD		LOQ		RSD %		Calibration
	(µg/mL)	(µg/kg)	(µg/mL)	(µg/kg)	(n = 6)	r^2	equation
FB1	0.0124	62.0	0.041	202.7	1.95	0.9997	$y = 43937x - 390.08$
FB2	0.0117	58.5	0.040	199.1	2.04	0.9997	$y = 44927x - 399.95$

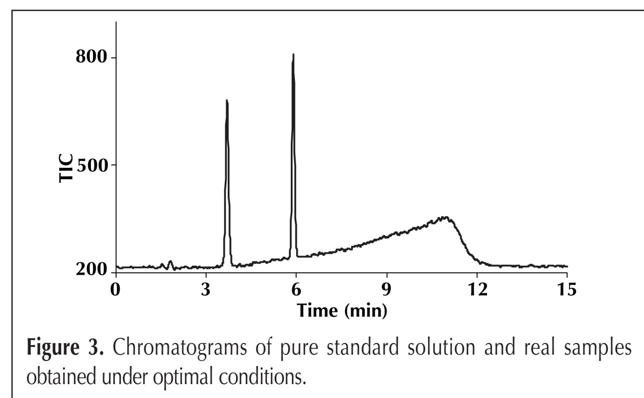

Figure 3. Chromatograms of pure standard solution and real samples obtained under optimal conditions.

Chromatographic method development

Primarily, it was necessary to find the best conditions separation and ionization of fumonisins. In the case of chromatographic separation, it was optimized composition of the mobile phase. Operational range of MS parameters were: nebulizer gas flow (5–13 L/min; step 1 L/min), nebulizer pressure (20–60 psig; step 5 psig), gas temperature (150–350°C; step 50°C), capillary voltage (1000–6000 V; step 500 V), and fragmentor voltage (0–400 V; step 25 V). These parameters were optimized in positive ion mode of electrospray ionization-MS detection. In accordance to Commision Decision 2002/657/EC (36), selected fumonisins were detected as ($FB_1 + H^+$) (722.4 *m/z*) or ($FB_2 + H^+$) (706.4 *m/z*) and confirmed using fragments *m/z* 334 and 352 for FB_1 and *m/z* 336 and 318 for FB_2. The optimization was carried out by flow injection analysis (FIA) or several standard solutions varying in concentration (Figure 2). The optimal settings of electrospray ionization-MS detector were found as follow: nebulizer pressure 60 psig, nebulizer gas flow 12 L/min, gas temperature 350°C, capillary voltage 6000 V, and fragmentor voltage 300 V.

Effect of ammonium acetate

We assumed that the content of acidic ammonium acetate in mobile phase could significantly improve ionization of fumonisins. Thus, we have tested five solutions: deionized water and four solutions of different ammonium acetate concentrations (1, 2, 5, and 20 mmol/L) adjusted to pH 3.0 by concentrated acetic acid. The concentration of buffer 5 mmol/L yields the best results. Chromatogram obtained under the best conditions of pure standard solution is presented on Figure 3.

Calibration curve for pure standards—linearity and limit of detection/quantification

The linearity of developed HPLC determination was investigated in the range of concentration between 5–0.005 µg/mL, which corresponds to 25,000–25 µg/kg. Each selected concentration was measured in triple repetition. The ratio between molecular ions and confirmation fragments was also monitored. Developed method showed very good linearity over the whole range of concentrations. LOD in the mean of 3× the noise level was 0.0124 µg/mL (62.0 µg/kg) for FB_1 and 0.0117 µg/mL (58.5 µg/kg) for FB_2. Then, the limit of quantitation (LOQ, 10× the noise level) reached 0.041 µg/mL (202.7 µg/kg) for FB_1 and 0.040 µg/mL (199.1 µg/kg) for FB_2 (Table I).

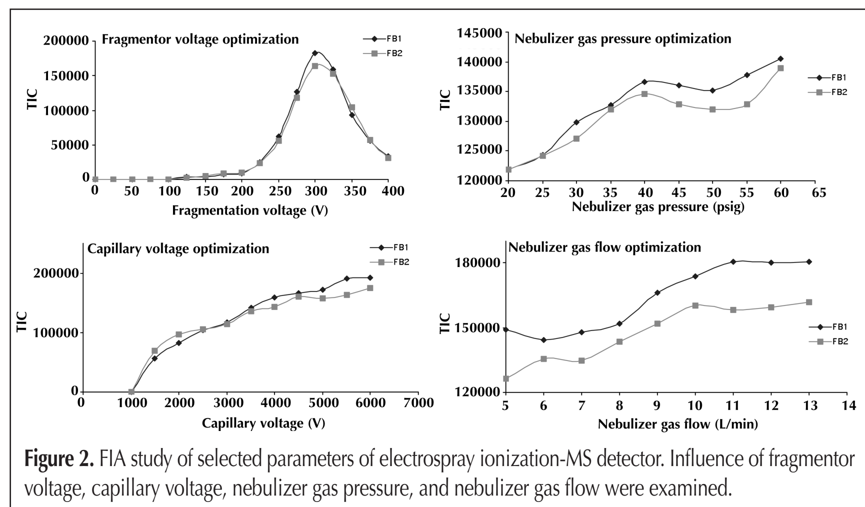

Figure 2. FIA study of selected parameters of electrospray ionization-MS detector. Influence of fragmentor voltage, capillary voltage, nebulizer gas pressure, and nebulizer gas flow were examined.

Extraction process

In this work, we searched for an extraction method with the highest yield of mycotoxins. The method used in European Standard uses a two-step extraction on an orbital shaker for 20 min each time. The recovery reported for this approach is 75.6 and 72.0% for fumonisin B_1 and B_2, respectively. Our developed method is faster and yields higher recovery. It uses

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Table II. Compound Recovery of Fumonisin During Extraction

Number of extraction	100 µg/kg		2000 µg/kg (limit EU)		5000 µg/kg	
	FB1 (%)	FB2 (%)	FB1 (%)	FB2 (%)	FB1 (%)	FB2 (%)
1	61.1 ± 2.0	53.5 ± 1.9	62.7 ± 1.5	56.9 ± 0.7	63.2 ± 1.6	56.2 ± 1.7
2	88.1 ± 2.1	83.3 ± 1.8	91.2 ± 1.8	85.0 ± 1.7	91.7 ± 1.7	88.3 ± 1.8
3	94.7 ± 2.2	93.9 ± 2.1	100.0 ± 1.9	95.3 ± 1.8	100.6 ± 1.8	99.6 ± 2.3
4	98.2 ± 2.2	96.4 ± 2.4	102.2 ± 2.2	99.0 ± 2.3	102.9 ± 2.1	101.0 ± 2.3

Table III. Precision and Recovery of Determination of FB1 and FB2 in Maize Samples (*n* = 5)

Compound	Analyte content (µg/kg)		Measured (µg/kg)	Recovery (%)
	Maize	Spike		
Intra-day (n = 5)				
FB1	485.2 ± 17.5	101.2 ± 2.3	594.0 ± 18.9	101.3
		2013.2 ± 62.3	2558.4 ± 59.6	102.4
		5092.3 ± 142.5	5728.1 ± 144.5	102.7
Inter-day (n = 25)				
	492.5 ± 21.4	107.3 ± 3.9	600.5 ± 23.2	100.2
		1995.6 ± 71.3	2518.0 ± 69.2	101.2
		5021.2 ± 159.3	5563.3 ± 159.2	100.9
Intra-day (n = 5)				
FB2	263.6 ± 8.4	102.1 ± 3.2	358.9 ± 15.9	98.3
		2024.0 ± 58.9	2239.6 ± 67.5	97.9
		5008.3 ± 104.2	5177.0 ± 162.3	98.2
Inter-day (n = 25)				
	259.9 ± 11.3	99.7 ± 3.9	352.8 ± 29.2	98.1
		2079.3 ± 67.9	2339.2 ± 79.5	99.3
		5052.4 ± 122.3	5264.5 ± 173.1	99.1

more invasive extraction with an UltraTurrax homogenizer. Each sample was extracted with 25, 20, and 15 mL of extraction solvent for 5 min. The recovery observed in spiked material was 94.7–100.6% for FB_1 and 93.9–99.6% for FB_2. Addition of one more extraction step had no significant influence on recovery. Three extractions were selected as an optimum condition.

Recovery studies were performed on spiked samples at three concentration levels—lower, equal to, and higher than the limit required in EU for maize flour. The recovery was similar in all concentration levels.

In comparison with some other methods validated for fumonisin determination, the recovery is high. Often, yield is in the range from 50 to 105%.

Extraction development

Samples of milled maize spiked with fumonisins standard of three levels, below EU limit, limit, and up to the limit (100, 2000, and 5000 µg/kg), were used for monitoring of fumonisins recovery during single extraction steps. Each determination was done in five repetitions. The recovery of fumonisins during four extraction steps is shown in Table II.

Three extraction steps were selected as sufficient for fumonisins determination in maize samples. In comparison with procedure by European Standard (ES), the precision of developed method is approximately four-times higher. These results can be caused by difference of used instrumentation for extraction. Although ES recommends extraction in an orbital shaker for 20 min, we had applied an UltraTurrax blender for 5 min. This phenomenon was observed earlier (37). During European interlaboratory comparison study for the determination of fumonisins, higher recoveries were obtained using shaking than with blending.

Accuracy, precision, and recovery

Accuracy, precision, and recovery of the fumonisins determination were evaluated with real samples spiked with appropriate amount of standards to give concentrations between 100–5000 mg/kg. Coefficients of variation of inter-day (five days) and intra-day were determined from an analysis of six spiked samples. Each day, calibration curves were measured, and the concentrations of analytes were calculated from these curves. Accuracy was evaluated by comparing measured concentrations with the known concentration of fumonisins (Table III).

Conclusion

In this work, two extraction processes of fumonisin B_1 and B_2 from maize samples were compared. Extraction method using triple extraction with acetonitrile–methanol–water solution in a homogenizer had significantly higher recovery than the one on orbital shaker. The recovery of developed extraction method was 96.4–102.9% in tested range of concentration, which was chosen around the EU limits for maize flour. New HPLC–MS method for fumonisin determination was developed, optimized, and applied for analysis of real samples. Developed methodology allows detection of 62.0 µg FB_1/kg and 58.5 µg FB_2/kg. Method was validated and successfully applied for analysis of reference material and real samples.

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Publikace XIV

VÝVOJ METODIKY EXTRAKCE NA TUHÉ FÁZI A HPLC-MS PRO STANOVENÍ DEOXYNIVALENOLU V JEČMENI A SLADU

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Klíčová slova: mykotoxin, deoxynivalenol, HPLC-MS, ječmen, slad

Úvod

Mykotoxiny jsou toxické sekundární metabolity mikroskopických vláknitých hub, vyvolávající různé toxické syndromy, souhrnně nazývané mykotoxikózy. Mykotoxiny se vyskytují na všech úrovních potravního řetězce[1]. Současné výzkumy dokazují, že riziko mykotoxinů existuje na celém světě, včetně průmyslově vyspělých států[2]. Z chemického hlediska se mykotoxiny řadí mezi vysoce stabilní nízkomolekulární organické sloučeniny nebílkovinné povahy[3].

Deoxynivalenol (DON) je mykotoxin produkovaný plísněmi rodu *Fusarium* (*F. graminearum, F. culmorum*), které jsou běžnými patogeny obilnin[4]. DON patří k celosvětově nejrozšířenějším trichothecenům[5]. Z toxikologického hlediska jsou akutní negativní zdravotní účinky deoxynivalenolu charakterizovány zejména střevními potížemi a zvracením[2]. Během výroby piva deoxynivalenol přechází z kontaminovaného ječmene do sladu a následně pak do sladiny. V průběhu dalších kroků zpracování již nedochází k jeho odstranění či degradaci. Částečně se jeho koncentrace v pivu může snížit sorpcí na komponenty buněčné stěny (zejména β-D-glukomanany) pivních kvasinek. Není zcela jisté, jak dalece může konzumace piva obsahujícího mykotoxiny ohrozit lidské zdraví[6], ale jeho přítomnost, kromě jiných faktorů, může vést ke vzniku nežádoucího jevu zvaného „gushing", nebo-li přepěňování piva[7,8]. Tento úkaz je velmi komplexní a může souviset i s produkcí specifických metabolitů plísní, mezi které patří např. relativně malé proteiny zvané hydrofobiny[9,10].

Přímé stanovení DON ve vzorcích rostlinného původu bez jejich předchozí úpravy je vzhledem ke složitosti matrice velmi obtížné. Proto jsou pro přípravu vzorku k analýze užívány různé postupy, které mají za úkol vliv této matrice minimalizovat. Nejčastěji používanou metodou je extrakce na tuhou fázi (solid phase extraction, SPE)[11]. Zde bývá s různou výtěžností aplikována celá řada sorbentů, jako jsou např. aktivní uhlí-alumina, iontově výměnné pryskyřice, silica, Florisil[12], grafitizované saze (GCB), MycoSep kolonky a imunoafinitní kolonky[13].

K vlastnímu stanovení DON v potravinách se v poslední době používají chromatografické metody, jako je např. plynová chromatografie s detektorem elektronového záchytu (ECD)[12] nebo s hmotnostním detektorem, vysokoúčinná kapalinová chromatografie s hmotnostní detekcí či superkritická fluidní chromatografie[14]. Kromě těchto metod jsou často aplikovány imunologické postupy, např. radioimunologická analýza (RIA) či enzymová imunoanalýza (ELISA)[15].

Cílem práce bylo vyvinutí a zavedení metodiky pro extrakci mykotoxinů z ječmene a sladu a následně i jejich stanovení pomocí vysokoúčinné kapalinové chromatografie s hmotnostní detekcí. Metodika byla poté využita k hodnocení kontaminace zrna ječmene a z něj vyrobeného sladu a k posouzení vlivu sladování na obsah DON.

Experimentální část

Přístroje

Stanovení obsahu DON ve vzorcích bylo prováděno na kapalinovém chromatografu Agilent HP1100 (Agilent, Palo Alto, USA), sestávajícího z vakuové odplyňovací jednotky (model G1322A), kvartérního čerpadla mobilní fáze (G1311A), automatického dávkovače vzorku (G1313A), UV-VIS detektoru s nastavitelnou vlnovou délkou (G1314A), fluorescenčního detektoru (G1321A) a kvadrupólového hmotnostního detektoru (G1946VL) s ionizací elektrosprejem.

Standard DON byl zakoupen u firmy Sigma-Aldrich, s. r. o. (Praha, ČR). Z něj byl poté rozpuštěním v acetonitrilu připraven zásobní roztok o koncentraci 0,2 mg ml^{-1}. Roztok byl uchováván při teplotě –18 °C. Metoda HPLC-MS stanovení DON byla vyvíjena s roztoky čistého standardu. Ty byly připravovány vždy čerstvé ze zásobního roztoku DON v acetonitrilu o koncentraci 0,2 mg ml^{-1} ředěním. Veškeré použité chemikálie odpovídaly chromatografické čistotě a byly dodány firmou Sigma-Aldrich, s. r. o.

Vzorky ječmene a sladu

Vzorky ječmene a sladu byly získány od firmy Agrotest fyto, s.r.o (Kroměříž, Česká republika). Jednalo se o vzorky několika odrůd ječmene pěstovaných v různých

oblastech České republiky a po různých předplodinách. Sladování bylo prováděno v laboratoři VÚPS a.s., Sladařského ústavu v Brně. Pro kontrolu byl u všech vzorků obsah DON stanoven nejprve komerčními kity ELISA (RIDASCREEN ® FAST DON; výrobce R-Biopharm, Darmstadt, Německo) s limitem detekce (LOD) pro ječmen 12 μg kg^{-1}, pro slad 62 μg kg^{-1} a limitem kvantifikace (LOQ) pro ječmen 42 μg kg^{-1} a pro slad 190 μg kg^{-1}. Výtěžnost metody pro obě matrice odpovídá 110 %.

Příprava vzorku

Navážka 1 g ječmene byla jemně rozemleta a extrahována 10 ml směsi acetonitril (ACN)/voda (84/16, v/v) po dobu 2 min na míchačce (MS2 Minishaker IKA, USA) a vzniklá suspenze byla centrifugována při 10 000 ot min^{-1} po dobu 10 min (centrifuga Universal 32 R, Hettich Zentrifugen, Německo). Pevný zbytek byl opět extrahován 2 × 7 ml směsi ACN/voda (84/16, v/v), centrifugován, extrakty spojeny a odpařeny do sucha na vakuové odparce. Odparek byl rozpuštěn v 1 ml směsi ACN/voda (84/16), smíchán s 5 ml vody a přefiltrován přes membránový polytetrafluorethylenový filtr (SMI-LabHut Ltd., UK) o velikosti pórů 0,20 μm. Takto připravený roztok byl přečištěn extrakcí na tuhé fázi (SPE) na vakuovém manifoldu (Supelco, USA). K extrakci byly na základě dat z literatury[13] vybrány kolonky Envicarb (Supelco, USA). Ty byly předem kondiciovány 10 ml vody a 0,3 ml methanolu. Matrice byla vymyta 3 ml směsi ACN/voda (84/16) a následně byl DON vymyt 2,5 ml methanolu, 5 ml methanolu okyseleného 0,095 ml kyseliny mravenčí a nakonec opět 2,5 ml methanolu. Získaný extrakt byl po odpaření pod mírným proudem dusíku do sucha rozpuštěn v 0,75 ml mobilní fáze (1 mmol l^{-1} kyselina mravenčí/ ACN, 90/10, v/v). Takto připravený vzorek byl použit k vlastnímu stanovení na HPLC/MS.

Analýza pomocí HPLC/MS

K separaci byla použita dle cit.[16] chromatografická kolona LUNA C18(2) o rozměrech 250 × 4,6 mm, plněná částicemi o velikosti 5 μm (Phenomenex, USA). Mobilní fáze pro eluci DON měla složení 1 mmol l^{-1} kyselina mravenčí/ACN (90/10, v/v) a průtok 1 ml min^{-1}. Detekce probíhala na MS detektoru v pozitivním módu (DON jako $[DON + H]^+$, m/z = 297 a $[DON + Na]^+$, m/z = 319). Nalezené optimální hodnoty jednotlivých parametrů MS detektoru jsou uvedeny v tabulce I. Čas potřebný k analýze činí 15 min a eluční čas deoxynivalenolu je roven 10,9 min. Proces separace byl prováděn při laboratorní teplotě.

Kalibrace

Kalibrační křivka byla sestrojena metodou standardního přídavku. Postup zpracování vzorku pro kalibraci odpovídal postupu použitého pro úpravu reálného vzorku. Před vlastním zpracováním vzorku bylo k navážce obiloviny přidáno požadované množství standardu DON. Více k problematice kalibrace pomocí metody standardního přídavku např. v publikaci[17].

Výsledky a diskuse

Extrakce a čištění vzorku

Při zpracování rostlinné matrice bylo vycházeno z publikovaného postupu[13]. Podle potřeby byly jednotlivé kroky modifikovány na námi použitou instrumentaci. Extrakce DON byla nejprve vyvíjena s čistým standardem a teprve poté aplikována na reálné vzorky. K extrakci byly vybrány SPE kolonky Envicarb od firmy Supelco, LiChrolut EN od firmy Merck s. r. o. (Říčany, ČR) a komerční jednorázová kolonka MycoSep 225 (Romer Labs, Tulln, Rakousko). U všech typů kolonek byla sledována výtěžnost DON a také reprodukovatelnost extrakčního procesu. Dále byl testován i vliv objemu rozpouštědla, ve kterém byl vzorek rozpuštěn před průchodem kolonou, na množství zachyceného DON. Výtěžnost byla sledována pro tři koncentrační hladiny, a to podlimitní (500 μg kg^{-1}), limitní (1250 μg kg^{-1}) a nadlimitní (5000 μg kg^{-1}). Nejvyšší výtěžnost DON v oblasti koncentrací kolem 1250 μg kg^{-1} obilniny (maximální povolený limit z Nařízení Komise Evropského společenství č. 1881/2006 (cit.[18]) vykazovaly kolonky Envicarb (84,6 %), zatímco kolonky LiChrolut a MycoSep měly hodnoty výtěžností velmi podobné (65,9 a 64,1 %). Jako nejvhodnější pro další práci byla proto zvolena kombinace kolonky Envicarb a vzorku rozpuštěného v 5 ml vody. K vymytí DON sloužil methanol a methanol okyselený kyselinou mravenčí (0,19 ml/10 ml

Tabulka I
Parametry hmotnostního detektoru

Parametr	Optimální hodnota	Rozmezí sledovaných hodnot
Průtok sušícího plynu, l min^{-1}	13	5 až 13
Tlak rozprašovače, kPa (Psig)	345 (50)	138 až 414 (20 až 60)
Napětí na kapiláře elektrospreje, V	4500	1500 až 6000
Teplota, °C	350	150 až 350
Fragmentor, V	125	0 až 400

methanolu). Byl studován vliv objemu elučního činidla na výtěžnost DON. Nejlepších výsledků bylo dosaženo při použití 2,5 ml methanolu, 5 ml okyseleného methanolu a nakonec opět 2,5 ml methanolu.

Chromatografické stanovení deoxynivalenolu

Důležitým krokem při vývoji chromatografické separace bylo stanovení nejvhodnějšího složení mobilní fáze. Byla použita izokratická eluce při poměru vodné fáze a ACN (90:10). U vodné fáze byl sledován vliv přídavku kyseliny mravenčí o různé koncentraci (0; 0,01; 0,1 a 1 mmol l^{-1}). Nejlepších výsledků bylo dosaženo při použití mobilní fáze s 1 mmol l^{-1} kyselinou mravenčí. Ta za použitých podmínek metody nejvíce podporovala ionizaci molekuly DON, potřebnou pro její stanovení na hmotnostním detektoru. Citlivost metody byla dále zvýšena opětovnou optimalizací jednotlivých parametrů hmotnostního detektoru (viz výše).

LOD vyvinuté metody pro stanovení obsahu DON ve vzorcích ječmene (sladu) je roven 25 μg kg^{-1} = 0,033 μg ml^{-1} (30 μg kg^{-1} = 0,040 μg ml^{-1}) a limit kvantifikace 83 μg kg^{-1} = 0,110 μg ml^{-1} (100 μg kg^{-1} = 0,133 μg ml^{-1}). V porovnání s výchozí prací[16], kde sloužil k analýze systém HPLC-UV, se nám LOD s roztokem čistého standardu podařilo snížit téměř desetkrát (LOD HPLC/UV = 0,095 μg DON/ml, LOD HPLC/MS = 0,01 μg DON/ml). Limit detekce publikovaných metod určených k analýze obilovin je následující: ve vzorcích pšenice LOD = 50 μg kg^{-1} (cit.[19]), LOD = 40 μg kg^{-1} (cit.[20]), kukuřice LOD = 60 μg kg^{-1} (cit.[11]).

Byla provedena validace metody v rámci jednoho dne (10 měření), kdy relativní směrodatná odchylka (RSD) nepřesáhla 6,08 % a v rámci pěti dnů (25 měření) s RSD 6,54 %.

Tabulka II
Srovnání metod ELISA a HPLC-MS při stanovení obsahu deoxynivalenolu ve vzorcích ječmene a sladu (množství DON v μg kg^{-1})

Odrůda ječmene	Předplodina	ELISA[a,b]		HPLC/MS[a,b]	
		ječmen	slad	ječmen	slad
Bojos	kukuřice	138	pod LOQ	pod LOQ	pod LOQ
Diplom	cukrovka	pod LOQ	pod LOD	pod LOD	pod LOD
Jersey	pšenice	51	pod LOD	pod LOD	pod LOD
Jersey	kukuřice	pod LOQ	pod LOD	pod LOD	pod LOQ
Jersey	mák	pod LOD	pod LOD	pod LOD	pod LOD
Malz	cukrovka	92	pod LOD	104	pod LOQ
Malz	cukrovka	50	pod LOD	pod LOD	pod LOD
Malz	pšenice ozimá	58	pod LOD	pod LOD	pod LOD
Malz	kukuřice	53	349	pod LOD	112
Prestige	cukrovka	56	pod LOD	103	109
Prestige	cukrovka	pod LOQ	pod LOD	pod LOD	pod LOD
Prestige	pšenice ozimá	55	pod LOD	pod LOD	pod LOD
Prestige	pšenice	pod LOQ	pod LOD	pod LOD	pod LOD
Prestige	ječmen jarní	835	295	641	499
Prestige	slunečnice	42	pod LOD	pod LOQ	127
Prudentia	kukuřice	pod LOQ	pod LOQ	177	231
Sebastian	–	53	193	pod LOD	pod LOQ
Sebastian	cukrovka	63	pod LOQ	pod LOQ	pod LOQ
Sebastian	kukuřice	59	299	pod LOQ	pod LOQ
Tolar	soja	103	pod LOD	pod LOQ	pod LOQ
LOQ[b]		42	190	83	100
LOD[a]		12	62	25	30

[a] LOD – limit detekce; [b] LOQ – limit kvantifikace

Výsledky měření

20 vzorků ječmene a z nich vyrobených 20 vzorků sladu bylo analyzováno na obsah DON. Při použití SPE extrakce a HPLC-MS analýzy převyšovalo množství DON LOQ v devíti případech (viz tab. II). Analýzami provedenými pomocí kitů ELISA RIDASCREEN® FAST DON, se podařilo kvantifikovat DON celkem v 18 vzorcích. Při porovnávání výsledků je nutné přihlédnout k faktu, že LOQ byl nižší u vzorků ječmene než u sladu (ELISA 5,2× nižší, HPLC/MS 1,2× nižší), tudíž nebylo možné vyhodnotit velmi nízké koncentrace DON v materiálu po sladování. V případech, kdy se množství DON pohybovalo pod LOQ ve výchozím materiálu, nebylo jeho množství s jedinou výjimkou stanovitelné ani po procesu sladování. Z naměřených hodnot u pozitivních variant je zřejmé, že obsah DON ve sladu někde převýšil jeho obsah v korespondujících vzorcích ječmene, v některých případech byl naopak nižší. Schwarz a spol.[21] sledovali změny obsahu ergosterolu a DON v průběhu sladování. Hodnoty obsahu DON v zeleném sladu činily 18–114 % hodnoty obsahu DON v původních vzorcích ječmene, u uhvozděného sladu bylo rozpětí těchto hodnot 16–100 %. U kontaminovaného ječmene se může obsah DON zvýšit v průběhu máčení, klíčení a na počátku hvozdění ječmene, kdy jsou nastoleny ideální podmínky pro růst plísní a další produkci mykotoxinů[4,5,22]. Na straně druhé může dojít k poklesu množství DON a to při máčení zrna, díky odstranění prachu a splavků, a také vyloužením deoxynivalenolu do máčecí vody[6,10]. Vztahy mezi obsahem DON v ječmeni a ve sladu jsou obecně velmi různorodé a závisí jak na odrůdě ječmene, tak také na technologických podmínkách sladování[23].

Závěr

Cílem práce bylo vyvinutí a zavedení metodiky pro extrakci a stanovení DON v obilovinách dostupnou instrumentací. K SPE extrakci DON z rostlinného materiálu byla vybrána velmi účinná kolonka Envicarb (Supelco). K vlastnímu stanovení byl použit kapalinový chromatograf s hmotnostní detekcí. LOD pro vzorky ječmene (sladu) je roven 25 $\mu g\,kg^{-1}$ (30 $\mu g\,kg^{-1}$) a LOQ 83 $\mu g\,kg^{-1}$ (100 $\mu g\,kg^{-1}$). Doba analýzy odpovídá 15 min, což ji předurčuje jako vhodné řešení pro větší série analýz. Vyvinutá metoda je relativně levná, rychlá a má dostatečnou citlivost, zaručující stanovení nízkých koncentrací DON v obilovinách. Aplikací tohoto postupu jsme získali hodnoty obsahu DON ve vzorcích ječmene a z něj vyprodukovaného sladu. Naměřené výsledky jednoznačně neprokázaly pokles nebo nárůst obsahu DON v procesu sladování. Obsah DON ve všech analyzovaných vzorcích byl pod maximálním povoleným limitem daným legislativou.

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A. Ježková[a], J. Karasová[b], V. Dohnal[a,b,c], and I. Polišenská[d] *([a] Department of Food Technology, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Brno, [b] Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Hradec Králové, [c] Department of Chemistry, Faculty of Science, J.E. Purkyně University, Ústí nad Labem, [d] Agrotest Fyto Ltd, Kroměříž)*: **Development of Solid-Phase Extraction and HPLC/MS Methods for Deoxynivalenol Determination in Barley and Malt**

A method for the determination of deoxynivalenol (DON) in cereals has been developed and tested. The method is based on solid phase extraction in a column and subsequent separation by HPLC-MS. DON was detected with a MS detector in positive mode. The method is inexpensive, fast and highly sensitive. The method was used for the determination of deoxynivalenol in barley and malt, with the detection limit 25−30 μg kg^{-1}. Changes in the DON content after malting were followed using the method.

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- Marketingové průzkumy, monitoring a vyhodnocování konkurenčních aktivit
- Organizace a vedení marketingových akcí a kampaní
- Udržování a posilování dobrého jména společnosti

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- Angličtina – schopnost plynule hovořit i na odborné úrovni
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- Výborné obchodní a prezentační dovednosti, dobrý mluvený i psaný projev pro komunikaci s představiteli výzkumných ústavů, lékaři, obchodními manažery
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ORIGINAL PAPER

Fluctuation in the ergosterol and deoxynivalenol content in barley and malt during malting process

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Abstract This paper describes determination of the deoxynivalenol and ergosterol in samples from different varieties of barley and, consequently, malt produced from this barley. In total, 20 samples of barley and 20 samples of barley malt were analyzed. The alkaline hydrolysis with consequent extraction into hexane was applied to obtain the ergosterol from cereals. Extraction to acetonitrile/water and subsequent solid-phase extraction (SPE) were used for deoxynivalenol. The determination of the samples was performed on high-performance liquid chromatography using UV detection (ergosterol) and mass spectrometric detection (deoxynivalenol). The influence of the malting process on the production of two compounds of interest was assessed from obtained results. Ergosterol concentration ranged 0.88–15.87 mg/kg in barley and 2.63–34.96 mg/kg in malt, where its content increased to 95% compared to samples before malting. The malting process was observed as having a significant effect on ergosterol concentration (P=0.07). The maximum concentration of deoxynivalenol was found to be 641 µg/kg in barley and 499 µg/kg in malt. Its concentration was lower than the legislative limit for unprocessed cereals (1,250 µg/kg). The statistic effect of the malting process on deoxynivalenol production was not found. Linear correlation between ergosterol and deoxynivalenol content was found to be very low (barley R=0.02, malt R=0.01). The results revealed that it is not possible to consider the ergosterol content as the indicator of deoxynivalenol contamination of naturally molded samples.

Keywords Ergosterol · Deoxynivalenol · Barley · Malt · Mold

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Introduction

Contamination of cereals by toxic fungal metabolites due to the fungal infection is one of the most occurring risks in cereal production. Fungi may have negative effect on cereal properties, namely to their hygienic and nutritional quality. The contamination of cereals by ascomycetes (e.g., *Fusarium* sp.) happens predominantly on the field during harvest. The risk of contamination is higher, when high temperature and humidity occurs [1]. Plant variety selection, crop rotation, post-harvest management and soil treatment plays a very important role in the prevention of fungal infection on the field [2]. In addition, fungal growth can be observed also during storage [3–5].

Negative effect of fungi on corn is not considered only as a decrease of quantity and quality of contaminated cereals, but it is also accompanied with production of toxic

metabolites—mycotoxins. The most significant and the most occurring mycotoxin, that contaminates food and feed produced from cereals, is deoxynivalenol (DON) [6] (Fig. 1). This mycotoxin may have negative effect on animals and human [7]. It is produced by *Fusarium* fungi, e.g., *Fusarium graminearum* and *Fusarium culmorum* [8]. In addition, the presence of *Fusarium* or other micromycetes in barley and malt showed connection to their technological quality resulting in phenomenon called "gushing".

One of the most important steps in malt production is crop soaking to attain approximately 45% humidity. These conditions were found optimal not only for barley germination, but also for fungi growth and potential mycotoxin production [9].

One of the most used markers of fungal infection in plant material is ergosterol. This steroid compound forms an important part of the fungal cell wall, while it is not presented in higher plants [10, 11]. In contrast to microbiological methods, the determination of ergosterol is suitable both for quantification of live fungal cells and for inactive fungal cultures. Many studies dealt with ergosterol and mycotoxin content in contaminated corn without homogenous results [7, 11, 12].

In this paper, the contamination with DON of barley and, consequently, malt produced from this barley was observed. Additionally, the ergosterol content was monitored in the same sample to detect possible implication for barley and malt contaminated by DON. The aim of the work was to evaluate the possibility of use of ergosterol quantification as a marker of fungal contamination and also consequent deoxynivalenol formation by these fungi during the malting process.

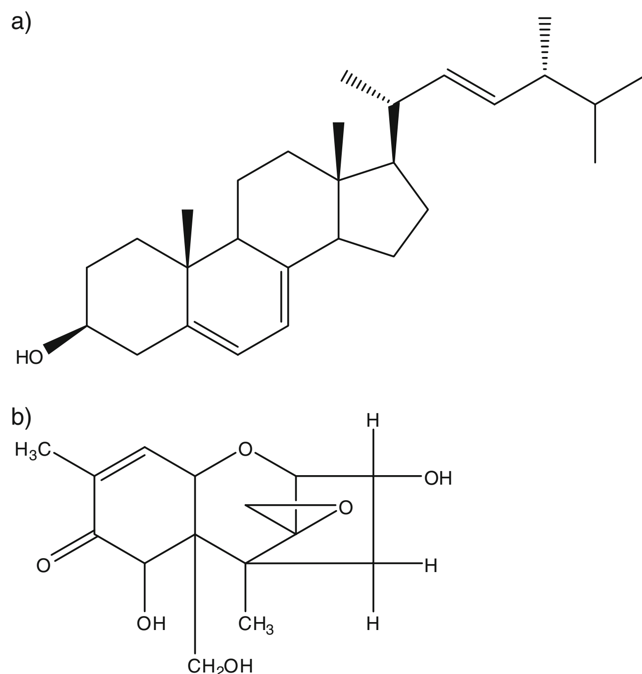

Fig. 1 Chemical structures of **a** ergosterol and **b** deoxynivalenol

Experimental

Chemicals

Formic acid (99%), potassium hydroxide, *n*-hexane, toluene of analytical grade purity, and acetonitrile and methanol of gradient grade purity were obtained from Sigma-Aldrich. Standards of deoxynivalenol and ergosterol were supplied by Sigma-Aldrich. Deionized water was prepared using Demiwa 10 ros (Watek, s.r.o., Ledec nad Sazavou, Czech Republic). The stock solutions of DON and ergosterol were prepared in methanol. These solutions were kept at −12 °C and protected against light. The experimental solutions were prepared freshly before experiment.

Instrumentation

The magnetic stirrer MM 4 (Lavat, Chotutice, Czech Republic), centrifuge Universal 32 R (Hettich Zentrifugen, Germany), rotary vacuum evaporator BH4 basic (IKA Labortechnik, Germany), vacuum manifold (Supelco, USA) and minishaker MS2 (IKA, USA) were used for the extraction of ergosterol and DON. A pH meter pH526 (WTW, Germany) was used for buffer pH measurement. Calibration buffers were supplied by Radiometer analytical, S.A. (France). Chromatographic analysis was performed on HPLC Agilent 1100 series (Agilent Technologies, Palo Alto, USA) that consisted of a vacuum degasser (model G1322A), quaternary pump (G1311A), autosampler unit (G1313A), UV–Vis variable-wavelength detector (G1314A), fluorescence detector (G1321A), and mass spectrometer (G1946VL). Nitrogen generator (model NM18LA) was delivered by Peak Scientific Ltd. (Scotland).

Barley and malt samples

Barley samples from various strains were collected during autumn 2007 in different parts of Czech Republic. Micromalting process was performed in the Research Institute of Brewing and Malting in Brno (Czech Republic) in the micromalting plant of KVM (Czech Republic) using modified MEBAK method for laboratory malting [13].

HPLC conditions

Determination of ergosterol was performed on Agilent HPLC on reversed-phase column Zorbax SB-C18 (4.6× 30 mm; particle size 1.8 μm; Agilent Technologies, USA)

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as was described by Varga et al. [10]. The mobile phase consisted of methanol/water (97.5:2.5, *v*/*v*) and isocratic elution with flow rate 0.6 mL/min was used. Injected sample volume was 2 µL. The UV detection at a wavelength of 282 nm was applied.

DON was determined using modified method of Jedličková [14] on chromatographic column LUNA C18(2) (250× 4.6 mm, particle size 5 µm; Phenomenex, USA). Mobile phase consisted of 1 M acetic acid/acetonitrile (90/10, *v*/*v*) and flow rate was 1 mL/min. Quadrupole mass spectrometer was used for detection in positive ion mode. DON was detected as molecular ion $[DON+H]^+$, $m/z=297$. Total time of analysis was 15 min and elution time of DON was 10.9 min. Separation was performed at the laboratory temperature.

MS conditions

Mass spectrometer conditions for DON determination were as follows: drying gas flow rate 13 L/min, nebulizer pressure 50 psig (345 kPa), capillary voltage 4,500 V, drying gas temperature 350 °C and fragmentation voltage 125 V.

Extraction of ergosterol

Slightly modified method of Varga et al. [10] was used for ergosterol extraction from barley and malt samples [15]. Briefly, 250 mg of homogenized sample was mixed with 2 mL of 10% methanolic solution of potassium hydroxide and incubated in vial at 80 °C for 90 min. After cooling to laboratory temperature, 0.5 mL of water and 1 mL of *n*-hexane was added and the vial was shaken for 1 min. The vial was centrifuged, the upper layer was removed. The lower layer was again extracted with 1 mL of *n*-hexane and the extraction process was repeated twice. Collected *n*-hexane layers were combined and evaporated under nitrogen pressure. Prior to analysis, there evaporated samples were dissolved in 400 µL of methanol/toluene solution (75:26, *v*/*v*) and analyzed by HPLC.

Extraction of deoxynivalenol

The extraction of DON was according to Ježková et al. [16]. Briefly, 1 g of homogenized barley or malt was extracted with 10 mL of acetonitrile/water solution (84:16, *v*/*v*) for 2 min. Resulting homogenate was centrifuged at 10,000 rpm for 10 min. The solid phase was twice extracted using 7 mL of same solution. Combined extracts were evaporated to dryness on vacuum evaporator and dissolved in 1 mL of acetonitrile/water solution (84:16, *v*/*v*) and 5 mL of water. This mixture was filtrated through polytetrafluoroethylene syringe membrane (SMI-LabHut Ltd., UK) with pore size 0.20 µm and purified using solid-phase extraction (SPE) on Envicarb columns (Supelco, USA). Prior to extraction, the SPE columns were pre-conditioned with 10 mL of water and 0.3 mL of methanol. The sample was passed through column and the column matrix was eluted by 3 mL of acetonitrile/water solution (84:16, *v*/*v*). Finally, the DON was eluted from the column using 2.5 mL of methanol, 5 mL methanol with 95 µL of formic acid and 2.5 mL of neutral methanol. The extract was evaporated to the dryness under slight flow of nitrogen. It was dissolved in 0.75 mL of mobile phase (1 mM formic acid/acetonitrile, 90/10, *v*/*v*) and used for HPLC/MS analysis.

Results and discussion

The content of mycotoxin deoxynivalenol and ergosterol was determined in 20 samples of barley or malt produced from this barley. All measurements were performed with three replications and the standard deviations were calculated (Table 1). The content of ergosterol varied from 0.88 to 15.87 mg/kg of barley and from 2.63 to 34.96 mg/kg of malt (Fig. 2.). Ergosterol does not belong to the toxic compounds; hence its limits in food were not determined. Based on data of Abramson et al. [7], Cahagnier et al. [17] and Tothill et al. [18], the content of ergosterol in cereals without contamination by fungi should range between 2 and 6 mg/kg. In our experiment, this upper level (6 mg/kg) was exceeded in 70% of samples. Regarding the obtained results, the trend of increasing ergosterol content during the malting process was observed in 95% of the samples. Statistical analysis confirmed the influence of malting process on increasing of ergosterol concentration on level of significance $\alpha=0.07$ (Fig. 3). These results are similar with results of other authors [11]. Schwarz et al. [19] observed increase of ergosterol and deoxynivalenol production during malting process in 5 days. Increased ergosterol concentration was related to the fungal growth, which is one of risk factors during beer “gushing” phenomenon [20, 21].

In the case of DON, the lowest concentrations in barley and malt were under the limit of detection (25 µg/kg for barley; 30 µg/kg for malt). The upper concentrations were found 641 µg/kg for barley and 499 µg/kg for malt. The legislative limit for unprocessed cereals (1,250 µg of DON/kg) was not exceeded. Regarding DON concentration, its decrease during malting process was observed in 10% of samples and its increase in 20% of samples. Statistic evaluation showed that there was no significant difference in DON content between barley and malt (Fig. 4).

DON content in barley and malt generally depends on complex conditions [22]. The risk of fungi occurrence and DON production grows together with increasing grain humidity during the malting process. Additionally, there

Table 1 Content of ergosterol and DON in barley and malt samples

Barley variety	Preceding crop	Ergosterol [mg/kg]		Deoxynivalenol [mg/kg]	
		barley	malt	barley	malt
Malz	Sugar beet	2.45±0.22	5.95±0.58	0.104±0.006	0.025±0.003
Prestige	Sugar beet	8.56±0.48	13.26±0.79	0.103±0.007	0.109±0.013
Prestige	Wheat	11.76±1.00	5.50±0.44	0.017±0.004	0.017±0.005
Prestige	Barley	6.90±0.42	9.22±0.84	0.641±0.063	0.499±0.040
Prestige	Wheat	3.79±0.21	6.45±0.55	0.048±0.005	0.050±0.006
Malz	Wheat	12.32±1.19	14.17±1.33	0.024±0.005	0.024±0.007
Prudentia	Corn	4.19±0.23	7.16±0.43	0.177±0.023	0.231±0.027
Prestige	Sugar beet	6.89±0.62	8.07±0.78	0.042±0.006	0.038±0.006
Jersey	Corn	6.46±0.33	9.09±0.71	0.036±0.004	0.041±0.007
Tolar	Soya	13.38±1.12	13.74±0.83	0.030±0.004	0.024±0.006
Diplom	Sugar beet	15.30±0.84	15.79±0.82	0.019±0.005	0.023±0.007
Malz	Sugar beet	4.32±0.27	7.07±0.49	0.029±0.008	0.038±0.009
Sebastian	Sugar beet	15.87±1.46	17.60±0.91	0.037±0.006	0.035±0.007
Sebastian	–	15.33±1.04	34.96±2.03	0.026±0.005	0.022±0.005
Jersey	Poppy	0.88±0.08	2.63±0.23	0.019±0.004	0.021±0.006
Prestige	Sunflower	4.48±0.41	12.12±0.82	0.042±0.006	0.127±0.010
Sebastian	Corn	5.57±0.32	19.02±1.25	0.019±0.004	0.039±0.006
Malz	Corn	12.00±1.19	15.40±0.80	0.024±0.005	0.112±0.014
Bojos	Corn	8.12±0.68	11.76±0.72	0.038±0.006	0.032±0.006
Jersey	Wheat	4.27±0.39	4.66±0.27	0.029±0.004	0.031±0.006

might be risk of enzymatic reactions, where DON is released from its analogues [23]. On the other side, major part of DON may be excluded from malt to water [24], which decreased the DON concentration. In the experiment of Schwarz et al. [19], the DON content in malt was lower or equal to its content in barley. On the contrary, Havlova et al. [25] observed significantly higher DON level in malt compared to barley.

The correlation between ergosterol and DON contents in barley and malt was very low (R=0.02 or 0.01) which is contrast with other authors [7, 25, 26]. This fact might be explained by barley contamination by other species than *Fusarium* such as *Aspergillus* and *Penicillium* [27]. Other limiting factors in DON production were experimental conditions, where the production of DON in identical experimental conditions can significantly varied between different fungal strains [28]. Not only the variability of fungal species but also host plant variety resistance against fungal infection may influence the mycotoxin production. There is evidence for this in the work of [29]. Their experiment with ^{14}C-labeled DON added to embryo callus cultures showed that a resistant cultivar is able to degrade the toxin more efficiently than a susceptible one.

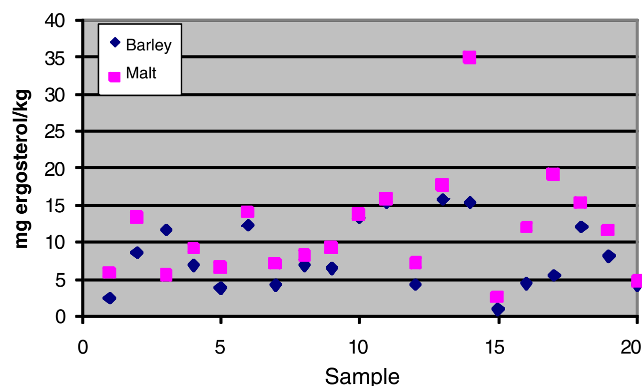

Fig. 2 Level of ergosterol in samples of barley and malt

Abramson et al. [7] observed a linear correlation between ergosterol and DON production in wheat samples—for wheat durum the correlation coefficient was 0.76 and for winter wheat 0.83. These samples were contaminated by *F. graminearum* by more than 90%. In the study of artificially infected rice, Perkowski [26] found correlation coefficient between DON and ergosterol content equal to 0.57. Certain authors [25] found also relationship between these two compounds. In the case of other mycotoxins, the ergosterol content in the pure cultures of selected micro-organisms has a relation to ochratoxin A or aflatoxin B_1 [11, 30]. Such correlation was not found for aflatoxin G_1.

In conclusion, the problem of ergosterol and DON formation in barley and malt is very complex and still unclear and needs to be investigated more deeply. There play role factors such as plant variety and associated resistance, fungal species, growth conditions etc. Also, ergosterol formation is

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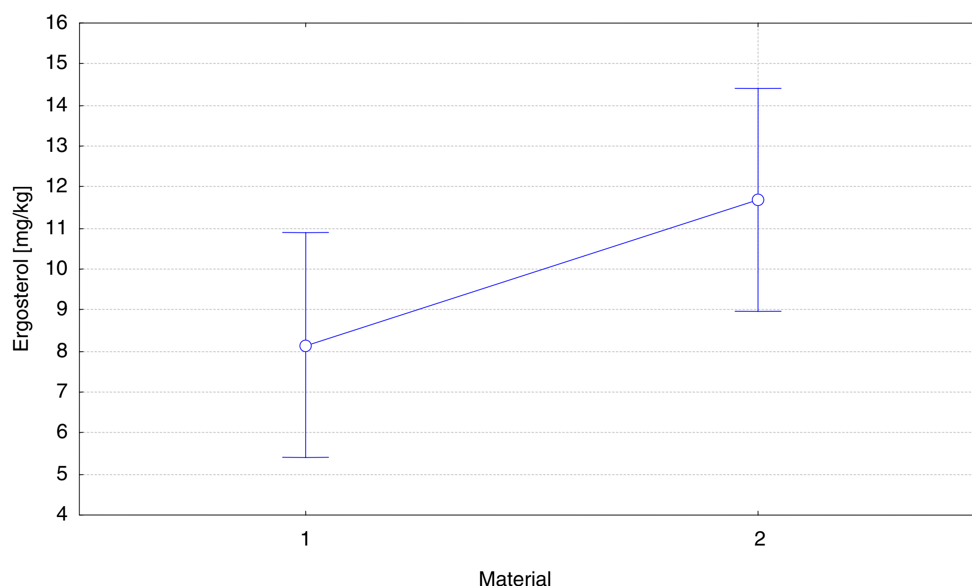

Fig. 3 Changes in ergosterol content in barley (*1*) and malt (*2*)

not constant during the life-time of the fungi. The amount of ergosterol is constant when fungi reach certain development stadia. In addition, the content of ergosterol in dry matter varies strain by strain. The ergosterol content in barley and malt cannot be used as a contamination marker of DON, when the barley is naturally infected.

Conclusions

The present study was focused on the monitoring of the deoxynivalenol and ergosterol content in barley and malt that was produced from this barley. Two aims were established. Firstly, the influence of malting process to the amount of both compounds was searched. Secondly, the possible use of ergosterol content as a marker of deoxynivalenol contamination in cereals was determined. Different varieties of barley from different parts of the Czech Republic were used.

Twenty samples of barley and 20 samples of corresponding malt were analyzed. Ergosterol concentration ranged between 0.88 and 15.87 mg/kg in barley and between 2.63 and 34.96 mg/kg in malt. Ergosterol content increased in 95% of the samples after malting. The results were statistically analyzed and the malting process was observed to have a significant effect on ergosterol concentration (P=0.07).

The amount of deoxynivalenol reached values <25 to 641 µg/kg in barley (25 µg/kg—limit of detection for barley; LOD), and <30 to 499 µg/kg in malt (30 µg/kg—LOD for malt). The legislative limit for deoxynivalenol in unprocessed cereals (1,250 µg/kg) [31] was not exceeded in analyzed samples. The statistic effect of malting to deoxynivalenol content was not found.

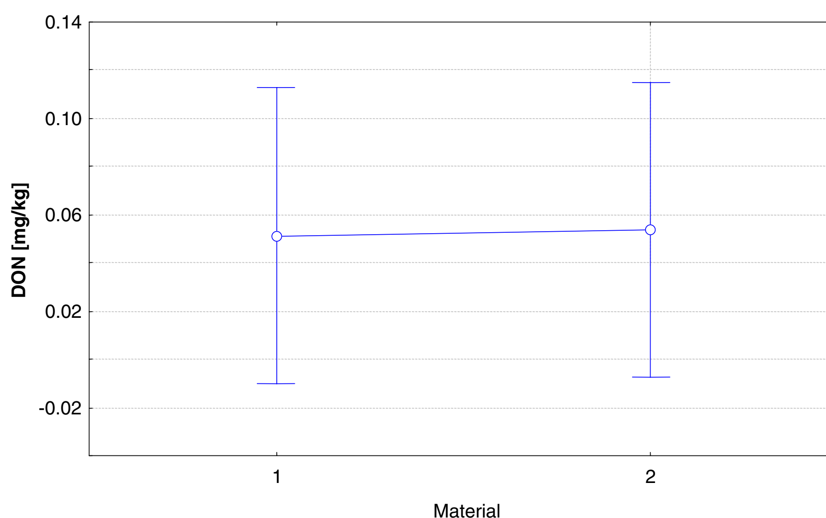

Fig. 4 Changes in DON content in barley (*1*) and malt (*2*)

The concentration changes of deoxynivalenol content did not overreach the legislative limit for unprocessed cereals, which is 1,250 μg/kg (Anonym, 2006). The ergosterol content is not limited until now, but its changes are contributed to the fungal growth and thus possibility of formation of toxic compounds.

The relationship between ergosterol and deoxynivalenol content was not established. The linear correlation provided very low coefficients (barley 0.02, malt 0.01). The ergosterol content as the indicator of deoxynivalenol contamination cannot be considered for naturally molded samples.

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Liquid Chromatography

RAPID AND SENSITIVE METHOD FOR CITRININ DETERMINATION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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Citrinin is a toxic product of secondary metabolism of fungi, such as certain **Aspergillus, Penicillium,** ***and*** **Monascus** ***species that are usually contaminating cereals. A new sensitive liquid chromatographic method with fluorescence detection was developed, validated, and applied for citrinin determination. The method is based on reversed-phase separation at pH 2.5, where citrinin exhibits the highest fluorescence quantum yield. In this setup, no derivatization step is needed. The method shows linearity in the range between 0.2 μg/mL and 0.1 mg/mL. The detection limit reached is 90 ng/mL (3.6 × 10^{-7} M). Validated method was successfully applied on analysis of spiked and real cereal samples.***

Keywords: Cereals; Citrinin; Fluorescence detection; Liquid chromatography

INTRODUCTION

Citrinin (CIT) is a toxic metabolite produced by various fungi species, such as *Penicillium verrucosum, P. citrinum*, eventually *Monascus sp.* (Zheng, Xin, and Guo 2008), which is used for food red coloring (Hajjaj et al. 2000). Together with another mycotoxin, ochratoxin A (OTA), citrinin can contaminate food such as rice (Tanaka et al. 2007). The typical concentrations of CIT found in cereals and cereal based food are between 0.1 and 1130 mg/kg (Zheng et al. 2008; Tanaka et al. 2007).

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Citrinin, together with OTA, has several toxic effects predominantly to kidney and liver. They are responsible for human and animal kidney diseases and tumors, and they are potential causative agents in human endemic Balkan nephropathy. The exposition of renal cells to citrinin results in inhibition of cell proliferation and leads to genotoxicity endpoints (DNA fragmentation and chromosome aberrations). These toxic effects of citrinin are amplified by OTA (Bouslimi et al. 2008).

At the present time, there is no legislative limit worldwide for citrinin content in foods, crops, or feeds. In order to protect public health, there is a need for new rapid and sensitive analytical method developed for citrinin determination.

Various chromatographic methods are applied in citrinin determination. Thin layer chromatography with fluorescence detection was used, for example, for CIT assay in cheeses (Hassanin 1993) and bacteria cultivation media samples (Viñas, Dadon, and Sanchis 1993; Abramson et al. 1999). The derivatization using p-anisaldehyde producing yellow-green spots can be also used (Viñas et al.). The detection limits for TLC determination after reaction with pyridine, acetanhydride, or aluminum chloride are 10–50 ng/g (Bao-Jun et al. 2006).

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Recently, the high performance liquid chromatography (HPLC) was employed for citrinin determination in complex matrices. Citrinin was determined in various samples, such as cheeses (Franco et al. 1996), cereal products (Meister 2004), silages (Schneweis et al. 2001), or biological fluids (Orti et al. 1986).

The most used detection method is fluorescence (LOD 0.5 ng/ml) (Vail and Homann 1990; Franco et al. 1996; Kpodo, Sørensen, and Jakobsen 1996; Zheng et al. 2008), but also another optical methods such as UV (LOD 1–5 mg/mL) (Hajjaj et al. 2000; Reinhard and Zimmerli 1999) or luminescence (Vazquez et al. 1996) are applied. Time resolved luminescence detection was examined after addition of a butanolic solution of Tb^{3+}, yielding a limit of detection (LOD) equal to 2×10^{-6} M, which was significantly lower than direct fluorescence (1×10^{-4} M) and comparable with UV detection (2×10^{-6} M).

Several HPLC methods are using acidic mobile phase for citrinin chromatographic separation to reach the lowest detection limits. The highest fluorescence intensity of citrinin can be observed in solutions with pH 2.5. Unfortunately, the limitation of stationary phase stability of common chromatographic columns at this pH value should be considered. Another solution to this problem is to use post-column mobile phase acidification. Ion-pair chromatography with post-column acidification by 1 M hydrochloric acid (LOD 0.9×10^{-7} M) (Franco et al. 1996) has been reported.

In this work, the liquid chromatography column with extended range of operational pH was examined for citrinin separation using mobile phase adjusted to pH 2.5 by acetic acid and fluorescence detection. The developed method was then applied in cereal based samples analysis.

EXPERIMENTAL

Chemicals

Chromatographic grade purity formic acid (99%), acetic acid, acetonitrile, sulfuric acid (98%), hydrochloric acid (35%), and citrinin standard were obtained

from Sigma-Aldrich, s.r.o. (Prague, Czech Republic). Demineralized water was prepared using Demiwa 10 ros (Watek, s.r.o., Ledec nad Sazavou, Czech Republic). Stock solution of citrinin was prepared by dissolving of 1 mg of citrinin in 5 ml of acetonitrile. The solution was kept at −12°C and protected against light. Working solutions were prepared fresh daily during the experiment.

Calibration Solutions

Calibration solutions were prepared fresh from stock solution of citrinin by dilution with the appropriate amount of mobile phase. To avoid photolysis of citrinin, all solutions were stored at 4°C in a dark place until analysis. Spiked samples were prepared by addition of pure mycotoxin standard into wheat flour samples and stored for one day. After this period spiked samples were analyzed.

Equipment

A pH-meter pH526 (WTW, Germany) was used for buffer pH measurement. Calibration buffers were supplied by Radiometer analytical, S.A. (France). Fluorescence spectra were recorded on a HPLC chromatograph Agilent 1100 series (Agilent Technologies, Palo Alto, USA) consisting of a vacuum degasser (model G1322A), quaternary pump (G1311A), autosampler unit (G1313A), variable-wavelength detector (G1314A), fluorescence detector (G1321A), and mass spectrometer (G1946VL). A nitrogen generator model NM18LA was delivered by Peak Scientific Ltd. (Scotland). A chromatographic column with an extended pH operational range, Zorbax Extend-C18 (3.0 × 100 mm, 3.5 μm) (Agilent, Palo Alto, USA), was used for the chromatographic separations.

Chromatographic Conditions

The isocratic elution was applied for chromatographic determination of citrinin. Mobile phase consisted of 60% of acetic acid solution with pH adjusted to 2.5 and 40% of acetonitrile. The flow rate was set to 0.4 mL/min. The fluorimetric detection used excitation at wavelength 331 nm and the emission was measured at 500 nm. The volume of the injected sample was 1 μl. The elution time under experimental conditions was 4.36 min.

Samples of Wheat Flour

Twenty samples of milled wheat were collected during harvest in 2007 in various areas of the Czech Republic.

Citrinin Extraction

The extraction of citrinin and extract purification procedure described in Molinié et al. (2005) was applied for citrinin extraction. Briefly, 20 g of sample was extracted for 20 min on an orbital shaker (363 rpm) by a mixture of 144 mL of acetonitrile, 16 mL of 4% aqueous solution of potassium chloride acidified by

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0.32 mL of concentrated sulfuric acid. Next, the suspension was filtrated through a paper filter Whatman no. 4 and extracted on an orbital shaker for 1 min by 100 mL of hexane. This step was repeated two times. Then, 20 mL of chloroform was added to the lower (aqueous) phase and shaken for 20 min. The chloroform phase was removed and the upper phase re-extracted twice.

The pooled chloroform extracts were alkalinized and extracted three-times with 50 mL of 5% sodium bicarbonate and shaken for 10 min. Subsequent aqueous extracts were combined and acidified to pH 1.5 by concentrated hydrochloric acid and allowed to stand 20 min. This solution was extracted three times by chloroform (100, 50, and 50 mL). Chloroform extracts were pooled and evaporated near to dryness under vacuum on a rotary evaporator at 40°C. Two ml of methanol were added, the solution transferred to a vial, and evaporated to dryness under nitrogen flow. Prior to the analysis, the sample was dissolved in 500 μL of methanol.

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RESULTS AND DISCUSSION

Method Development

Citrinin is a compound with a structure containing coumarinic moiety (Fig. 1). Its molecule possesses a carboxylic group in which dissociation has a strong effect on citrinin fluorescence intensity. Franco et al. (1996) observed that the highest fluorescence quantum yield is obtained at a pH equal approximately to 2.5 and decreases in more acidic respective alkaline solutions. The reason for this spectroscopic behavior can be explained by dissociation of the carboxylic group in an alkaline environment, respectively, by protonation of oxygen atoms in strongly acidic solutions. Thus, the highest fluorescence is produced by a neutral citrinin molecule. At this state, there are hydrogen bonds formed between hydrogen and oxygen atoms resulting in the formation of two new conjugated six-membered cycles and stabilization of planar structure of molecule. This highly conjugated rigid planar system has very favorable fluorescent properties.

The fluorescence spectra of 0.01 mg/mL aqueous solution of citrinin with pH 2.5 were measured. There were selected optimal wavelengths for detection: excitation equal to 331 nm and emission at 500 nm.

In addition, the separation of non-dissociated citrinin on reversed-phase columns can lead to higher separation efficacy due to elimination of charge–charge

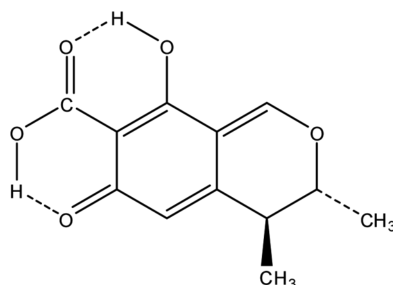

Figure 1. Chemical structure of citrinin at pH 2.5.

interactions and interactions between analyte and free silanol groups, which usually leads to peak deformations, such as peak tailing. The separation was performed on Zorbax XDB C18 Extend column, which has an extended operational range of pH. For all these reasons, it was decided to adjust the pH of mobile phase to 2.5.

The acetic acid was selected as the acidifying agent due to its solubility in acetonitrile and water. The sensitivity and separation efficacy of method using formic acid was higher in comparison with other acidifiers—hydrochloric acid and formic acid (Fig. 2).

Acetonitrile and methanol, as organic modifiers of the mobile phase, were tested. There were no observed notable differences in separation efficacy. The acetonitrile was selected for further optimization based on its better elution properties.

Calibration Curve for Pure Standards—Linearity and Limit of Detection/Quantification

The linearity of developed HPLC determination was investigated in a range of concentration between 100 µg/mL to 0.2 µg/mL, which corresponded to 2.5 mg/kg, respectively 5.0 µg/kg. Each of the six concentrations was measured in triple repetition. The developed method showed very good linearity in the whole proposed range of concentrations. The limit of detection in the mean of the 3× noise level was 90 ng/mL (2.25 µg/kg) for citrinin. The limit of quantification (10× noise level) was 0.3 µg/ml (7.5 µg/kg) (Table 1).

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Method Validation

Accuracy, precision, and recovery of the citrinin determination were evaluated with real samples spiked with an appropriate amount of pure standard solution to give concentrations between 2.5 mg/kg and 25 µg/kg. The spiked wheat samples

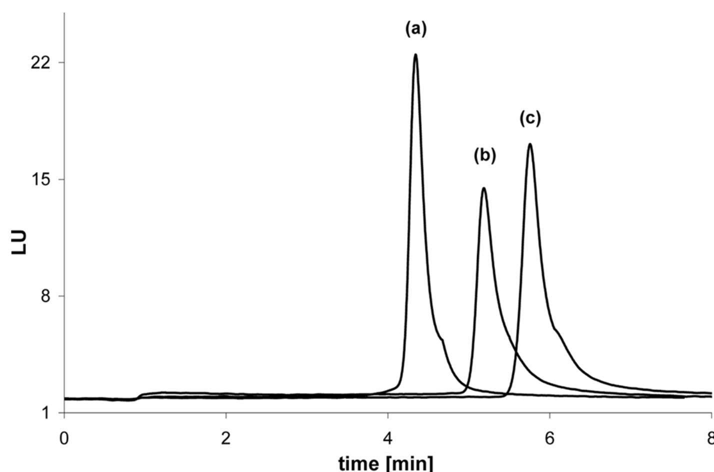

Figure 2. Influence of acetic (a), formic (b), and hydrochloric (c) acid on peak shape and retention properties of citrinin. Mobile phase 60% aqueous solution of acid, pH 2.5, 40% acetonitrile, flowrate 0.4 mL/min. Fluorescence detection at 331/500 nm.

Table 1. Quantification of citrinin with limits of detection, limits of quantification, RSD and calibration curve parameters

	LOD		LOQ		RSD		
	ng/ml	μg/kg	μg/ml	μg/kg	(%; n = 6)	r^2	Calibration equation
Citrinin	90	2.25	0.3	7.5	2.37	0.997	$Y = 15210x + 5.247$

Table 2. Precision and recovery of determination of citrinin in wheat flour samples (n = 5)

	Analyte content [μg/kg]			
	Wheat flour (matrix)	Spike	Measured [μg/kg]	Recovery [%]
Intra-day (n = 5)	<LOD	25.1 ± 1.3	19.2 ± 1.2	76.5
		504.3 ± 21.3	400.2 ± 17.1	79.3
		2532.2 ± 105.5	2068.6 ± 79.5	81.7
Inter-day (n = 25)	<LOD	25.2 ± 1.4	19.4 ± 1.2	76.9
		501.6 ± 23.5	388.8 ± 21.2	77.5
		2551 ± 111.1	2107.3 ± 83.2	82.6

were analyzed after one day. Coefficients in variation of inter-day (5 days) and intra-day were determined from analysis of the six spiked samples. Each day the calibration curve and the concentrations of citrinin were measured and calculated from these curves. Accuracy was evaluated by comparing measured concentrations with the known concentration of citrinin (see Table 2).

Analysis of Spiked and Cereal Samples

The developed method was applied on 20 wheat samples collected during the summer of 2008. There were no detectable amounts of CIT found in any analyzed sample. This could be caused by very dry weather during the year of the sample collection and low occurrence of fungal infections on crop production.

CONCLUSIONS

A new, very sensitive chromatographic method for citrinin determination in cereal samples was developed. The method takes advantage of the high fluorescence of analyte in acidic solutions. The detection limit reached by this method is 90 ug of citrinin. The developed method was validated using spiked wheat flour samples and applied on analysis of real wheat samples. The method can be used in the monitoring of citrinin in order to minimize mycotoxin occurrence and help to protect public health.

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Occurrence of ochratoxin A and citrinin in Czech cereals and comparison of two HPLC methods for ochratoxin A detection

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The aims of the study were to obtain information about the occurrence of ochratoxin A (OTA) and citrinin (CIT) in cereals harvested in the Czech Republic and to compare two analytical procedures for detecting OTA. A total of 34 cereal samples, including two matrix reference materials (R-Biopharm, Germany), were analysed. The results were compared with the limit for raw cereal grains used as a foodstuff according to Commission Regulation No. 1881/2006, which allows a maximum OTA level of $5\,\mu g\,kg^{-1}$. Compared were two methods based on the high-performance liquid chromatography principle, one using the immunoaffinity columns OchraTest™ (VICAM) and the second based on solvent partition (PART), both followed by fluorescence detection. The highest OTA contents were found in two barley samples. According to the method employed, the results for the first sample (malting barley) were VICAM = $31.43\,\mu g\,kg^{-1}$ and PART = $44.74\,\mu g\,kg^{-1}$. For the second sample (feeding barley) they were VICAM = $48.63\,\mu g\,kg^{-1}$ and PART = $34.40\,\mu g\,kg^{-1}$. Two samples of bread wheat had an OTA content approaching the legal limit (VICAM = $4.71\,\mu g\,kg^{-1}$ and PART = $6.03\,\mu g\,kg^{-1}$; VICAM = $4.12\,\mu g\,kg^{-1}$ and PART = $3.95\,\mu g\,kg^{-1}$). CIT was analysed using the PART method only, and its highest content ($93.64\,\mu g\,kg^{-1}$) was found for the malting barley sample with high OTA content ($44.74\,\mu g\,kg^{-1}$ as analysed using PART).

Keywords: high-performance liquid chromatography (HPLC); mycotoxins; ochratoxin A; cereals

Introduction

On a world scale, five mycotoxins or groups of mycotoxins are considered to be important in human health. These are aflatoxins, ochratoxin A (OTA), fumonisins, trichothecenes, and zearalenone (Miller 1996). OTA is the only toxin among them produced by *Penicillium* species. It can also be produced by several species of the genus *Aspergillus*: *A. ochraceus* (now named *Aspergillus alutaceus*), *A. carbonarius* and *A. niger* (Pitt 1987; Madhyastha et al. 1990). *P. verrucosum* has been identified as the only producer of OTA in cereals in temperate climates (Ciegler et al. 1973; Lund and Frisvad 2003; Scudamore 2005). Another mycotoxin, citrinin (CIT), can occur in cereals together with OTA, as *P. verrucosum* is able to produce both of these mycotoxins, but CIT production is also possible by other *Penicillium* (Scott et al. 1972; Pitt 1987; Abramson et al. 1990) and *Aspergillus* species (Scott 1977; Bettina 1984). Simultaneous contamination with OTA and CIT of both raw cereals (Abramson et al. 1999; Vrabcheva et al. 2000) and cereal-based products (Molinié et al. 2005) has been identified.

The International Agency for Research on Cancer (IARC) (1993) classifies OTA as possibly carcinogenic to humans (Group 2B) based on sufficient evidence of carcinogenicity in experimental animals. OTA has nephrotoxic effects, and it has been shown to cause mycotoxic porcine nephropathy (Krogh 1976). It has other adverse effects that include mutagenicity (DeGroene et al. 1996; Palma et al. 2007) and genotoxicity (Creppy et al. 1985; Pfohl-Leszkowicz et al. 1991; Lebrun and Föllmann 2002; Pfohl-Leszkowicz and Manderville 2007). CIT is also nephrotoxic (Krogh et al. 1973; Frank 1992) and genotoxic (Segvic-Klaric et al. 2007; Iwahashi et al. 2007; Pfohl-Leszkowicz et al. 2008). It enhances OTA renal toxicity in pigs (Krogh et al. 1973), the incidence of renal cell tumours in male mice (Kanizawa 1984), and kidney adenomas in male rats (Arrai and Hibino 1983), although CIT alone has not been

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proven carcinogenic. Both OTA and CIT seem to be implicated in Balkan endemic nephropathy in some Balkan areas (Petkova-Bocharova and Castegnaro 1991; Vrabcheva et al. 2000; Pfohl-Leszkowicz et al. 2002, 2007). The incidence of and mortality from urothelial urinary tract tumours have been correlated with the geographical distribution of Balkan endemic nephropathy in Bulgaria and Yugoslavia. A relatively high frequency of contamination of cereals and bread with ochratoxin A has been reported in an area of the former Yugoslavia where Balkan endemic nephropathy is present (IARC 1976, 1983, 1987, 1993). OTA–DNA adducts were found in kidney, liver, and spleen of mice and rat orally exposed to OTA (Pfohl-Leszkowicz et al. 1991, 1993) and also in pig fed OTA contaminated food (Miljkovic et al. 2002).

Due to documented negative effects on human health, OTA content is limited in unprocessed cereals intended for food production (the limit is $5\,\mu g\,kg^{-1}$) and in products derived from raw cereals, including processed cereal products and cereals intended for direct human consumption (the limit is $3\,\mu g\,kg^{-1}$) and also in baby food (the limit of $0.5\,\mu g\,kg^{-1}$) (European Commission 2006a). In cereals and cereal products intended for animal feeding, only guidance values are given: for cereals and cereal products it is $0.25\,mg\,kg^{-1}$, but there are different values for complementary and complete feedstuffs for pigs ($0.05\,mg\,kg^{-1}$) and for poultry ($0.10\,mg\,kg^{-1}$) (European Commission 2006b). No limits have been established for CIT.

OTA is fairly stable to heat. In cereal products, up to 35% of the toxin survives autoclaving for up to 3 h (IARC 1976). It currently can be found in cereal-based products (Wolff 2000; Lombaert et al. 2003) also together with CIT (Molinié et al. 2005). Beer, through malting barley or other kinds of cereals used for malt production, could also be contaminated with OTA (Tangni et al. 2002; Anselme et al. 2006). As Harcz et al. (2007) conclude, there is a risk of exposure to a significant amount of OTA with respect to the tolerable daily intake (TDI) especially in such countries with high beer consumption habits as Belgium, the Czech Republic, the UK, Germany, Ireland, Austria, and Denmark.

Based on risk assessment performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), cereals and cereal products contribute more than 50% of human OTA exposure (JECFA 2001). Lund and Frisvad (2003) report that cereals normally account for 50–80% of the average consumer intake of OTA. A basic and reasonable way to reduce the consumer intake of OTA is to monitor it thoroughly in raw cereals and to prevent its entry into the food chain.

Frisvad and Viuf (1986) investigated 70 samples of barley from Denmark and found OTA content varying from zero to $7380\,\mu g\,kg^{-1}$. According to reports of Scott et al. (1972) and Krogh et al. (1973), detected OTA contamination levels in cereals range from 0.03 to 27.5 ppm. As Scott (1994) reported, concentrations of CIT are often several times higher than are those for accompanying OTA. A detailed survey of OTA incidence in cereals in Europe by Rizzo et al. (2002) found that the highest reported levels were in wheat (239 samples analysed) and rye (228 samples analysed) from Poland, and a maximum level for both cereals was $2400\,\mu g\,kg^{-1}$. Birzele et al. (2000) observed the occurrence of OTA using the enzyme-linked immunoabsorbant assay (ELISA) method within the limit of detection (LOD) of $0.4\,\mu g\,kg^{-1}$ for 43 freshly harvested wheat samples in Germany. They found 21% positive samples and a maximum estimated value of $0.7\,\mu g\,kg^{-1}$. Conkova et al. (2006) examined 105 cereal samples which were taken immediately after harvest from selected localities of Poland (45 samples) and eastern Slovakia (60 samples). OTA was analysed using the high-performance liquid chromatography (HPLC) method with the LOD of $0.015\,\mu g\,kg^{-1}$ and no positive sample was found. In Belgium, cereal grain samples were reported to be contaminated up to $25.4\,\mu g\,kg^{-1}$ (Chandelier et al. 2004). Malir et al. (2006) studied the occurrence of OTA in 114 cereal samples taken at the time of harvest in the Czech Republic, among which ten were positive (the LOD of the method used was $0.4\,\mu g\,kg^{-1}$) and the maximum level was $37.0\,\mu g\,kg^{-1}$. Analysis of 125 wheat samples from France revealed the presence of OTA in 60% of samples ranging from trace to $69\,\mu g\,kg^{-1}$. The highest amount was found in a sample collected in a farm located in northern part of France and badly stored (Bédouret et al. 2001; Pfohl-Leszkowicz et al. 2007). Half of the samples contaminated by OTA were also contaminated by CIT.

The reliability and comparability of analytical results for monitoring studies are crucially important. The percentage of reported positive samples depends on the LOD of the method employed. Several methods for OTA analyses in raw cereals have been developed and validated (Visconti and DeGirolamo 2005). The methods are mainly based on thin-layer chromatography (Nesheim et al. 1973; Levi 1975) or, now more frequently, on liquid chromatography (LC) with fluorescence detection without (Nesheim et al. 1992) or with immunoaffinity clean-up (IMA) (Entwisle et al. 2000). For simultaneous analyses of OTA and CIT, a method suitable for wheat and barley was described by Lepom (1986), and a versatile method suitable for raw cereals and cereal products using partition was developed by Molinié et al. (2005). In the method of Molinié et al., the mycotoxins are extracted by acetonitrile instead of chloroform and at a lower pH (1.5 versus 4.0). These modifications are particularly important for CIT for which the recovery reached 80% in the method of Molinié et al. and is only 60% with chloroform. Purification on immunoaffinity could lead

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to underestimation, notably when OTA has been extracted in alkaline conditions, because it is converted onto open-ring OTA which could not cross-react with antibodies anymore (Pfohl-Leszkowicz et al. 2006; Castegnaro et al. 2006). The current status of methods for OTA detection and of methods development is reviewed in detail by Shephard et al. (2009).

Methods based on antigen–antibody reaction, including ELISA, have also been developed for both OTA and CIT analysis. Numerous procedures have been published that use monoclonal or polyclonal antibodies in raw cereals, e.g. for OTA in barley (Morgan et al. 1983) or in wheat (Lee and Chu 1984). Currently, an entire range of ELISA microtitre plate kits is sold which is intended for both OTA and CIT detection in cereals, feeds, beer and other matrices.

The aims of the current study were to detect the levels of contamination with OTA and CIT in unprocessed cereals harvested in the Czech Republic and to compare analytical procedures based on LC for OTA analysis in raw cereal grain with and without immunoaffinity clean-up.

Materials and methods

Sample collection

Set I

The first set of samples (numbers 1–10) includes ten samples of freshly harvested winter wheat (2006 harvest) selected from samples provided by farmers to a laboratory of Agrotest fyto, s.r.o. for analysis in relation to monitoring the quality of wheat intended for bread-making harvested in the Czech Republic. Samples delivered at the end of monitoring (i.e. later than 25 August 2006) were selected. The samples were collected by farmers who had been instructed on the principles of good sampling according to Commission Regulation (EC) No. 401/2006 specifying the methods of sampling and analysis for the official control of mycotoxins levels in foodstuffs (European Commission 2006c). After delivering to the laboratory, wheat moisture content was determined according to Commission Regulation (EC) No. 824/2000 of 19 April 2000 establishing procedures for the taking-over of cereals by intervention agencies and laying down methods of analysis for determining the quality of cereals (European Commission 2000). The samples were then left in their original packing and stored in a dry environment at 15–18°C until OTA analyses in November–December 2006. Nine of the ten samples analysed were samples of bread wheat and one sample was wheat for feeding. The characteristics of the samples (harvest date, delivery date, and moisture content) together with results are given in Table 1.

Set II

The second set (numbers 11–34) contains 22 raw cereal grain (twelve wheat and ten barley) samples, all but two (numbers 11 and 12) of which were taken from storage facilities, and two matrix reference materials. Sample 11 was naked barley intended for producing wholemeal foods originating from organic farming. It was taken from a tractor trailer on a farm where it had been temporarily stored. Barley sample 12 was taken from a lot offered to a malt house for malt production. Having checked its malting quality in the malt house laboratory, it had been refused due to a higher content of admixtures and impurities, as well as a mouldy smell. The other 20 samples were collected from randomly selected storage facilities across the Kromeriz district and sampled by warehousers according to Commission Regulation (EC) No. 401/2006. Sampling and analyses were carried out during November–December 2008. The samples are characterised and the results presented in Table 2. Reference materials (RM) (wheat meal P64/OW803 with the determined OTA value of $1.8 \pm 0.6\,\mu g\,kg^{-1}$ and P64/OW806 with the determined OTA value of $7.7 \pm 2.0\,\mu g\,kg^{-1}$) were purchased from R-Biopharm AG (Darmstadt, Germany).

Methods of analyses, chemicals

HPLC-VICAM method (VICAM)

Analyses were performed by a laboratory of the State Veterinary Institute in Olomouc (Czech Republic), which is accredited for this determination according to EN ISO/IEC 17025:2005 being employed for official control of OTA levels. OchraTest^{TM} immunoaffinity columns were used. Guidelines from the manufacturer's of VICAM – OchraTest^{TM} (Watertown, MA, USA) and the OchraTest^{TM} WB HPLC Instruction Manual (VICAM 2005), paragraph 4.3 HPLC Procedure for Corn, Milo & Feeds, were followed. Also used were a methanol : water (80 : 20) extraction mixture, 4.6×75 mm Symmetry C18 column (Waters, Milford, MA, USA), C18 sorbent, flow rate of $0.8\,ml\,min^{-1}$ at 25°C. An Agilent 1100 fluorescence detector was used (Santa Clara, CA, USA), with excitation and emission wavelengths of 333 and 477 nm, respectively. Chemicals were of HPLC grade and purchased from Sigma Aldrich (Czech Republic). The method validation in the laboratory was carried out at the extent needed to comply with EN ISO/IEC 17025:2005 requirements. Performance characteristics are summarised in Table 3. Recovery was determined using a repeated analysis of three spiked wheat samples at the OTA concentrations of 2.5, 5.0 and $10.0\,\mu g\,kg^{-1}$, which was carried out on one day by the same operator. The average recovery for these three concentrations was $87.0\% \pm 6.1\%$. All results are corrected for this recovery. A limit of

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Table 1. Ochratoxin A and citrinin in cereals harvested in the Czech Republic, Set I, samples from farmers, 2006 harvest, analyses in November–December 2006 (VICAM – method using the immunoaffinity columns OchraTest^{TM}; PART – method based on solvent partition without purification on immunoaffinity columns).

Number	Sample description	Date of:		Moisture content[a] (%)	Ochratoxin A ($\mu g\,kg^{-1}$)		Citrinin ($\mu g\,kg^{-1}$) PART	Percentages of admixtures and impurities[b]			
		Harvest	Delivery to the laboratory		VICAM	PART		Category 1	Category 2	Category 4	Category 5
1	Bread wheat	22 August	25 August	11.31	<LOD	<LOD	<LOD	0.88	1.58	32.74	0.46
2	Bread wheat	3 August	29 August	12.05	4.71 ± 1.18	6.03 ± 1.51	<LOQ	1.45	1.37	0.00	4.82
3	Bread wheat	26 August	30 August	12.21	<LOD	<LOD	<LOD	1.68	4.54	9.73	0.26
4	Wheat for feeding	24 August	30 August	12.03	<LOD	<LOQ	<LOD	4.48	2.17	47.60	2.51
5	Bread wheat	24 August	6 September	11.82	<LOD	<LOD	<LOD	3.68	0.32	25.36	2.02
6	Bread wheat	5 September	18 September	11.74	<LOD	<LOD	<LOD	7.69	0.95	15.60	2.18
7	Bread wheat	1 September	19 September	12.41	<LOD	<LOD	<LOD	0.89	5.20	0.65	2.01
8	Bread wheat	1 September	5 October	11.25	<LOD	<LOD	<LOD	0.43	2.96	1.09	1.75
9	Bread wheat	27 August	6 October	10.90	<LOD	<LOD	<LOD	1.63	2.07	0.78	1.69
10	Bread wheat	6 September	13 October	11.38	<LOD	<LOQ	<LOD	4.78	0.42	10.90	1.67

Notes: [a]Moisture content at the time of a sample's delivery to the laboratory.
[b]Categories of materials that are not basic cereal of unimpaired quality, according to Commission Regulation (EC) No. 824/2000: 1, broken grains; 2, impurities consisting of grains; 4, sprouted grains; and 5, miscellaneous impurities.

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Table 2. Ochratoxin A and citrinin in cereals harvested in the Czech Republic, Set II, samples from storage facilities, analyses in November–December 2008 (VICAM – method using the immunoaffinity columns OchraTest™; PART – method based on solvent partition without purification on immunoaffinity columns).

Number	Sample description	Harvest year	Ochratoxin A (μg kg^{-1})		Citrinin (μg kg^{-1})	Percentage of admixtures and impurities[e]			
			VICAM	PART	PART	Category 1	Category 2	Category 4	Category 5
11	Naked barley, foodstuff[a]	2008	0.49 ± 0.12	0.46 ± 0.12	<LOQ	1.37	1.16	0.00	0.00
12	Malting barley[b]	2007	31.43 ± 7.86	44.74 ± 11.19	93.64 ± 28.09	1.53	1.04	0.00	14.35
13	Barley for feeding	2007	0.67 ± 0.17	0.35 ± 0.09	<LOQ	0.90	0.69	0.00	0.22
14	Barley for feeding	2007	<LOD	<LOD	<LOD	2.58	4.11	0.00	0.53
15	Barley for feeding	2007	<LOD	0.58 ± 0.15	<LOD	27.52	15.38	0.00	6.09
16	Barley for feeding	2007	2.52 ± 0.63	1.03 ± 0.26	5.25 ± 1.58	3.47	6.41	0.00	1.06
17	Barley for feeding	2007	<LOD	<LOD	<LOD	0.85	0.71	0.00	0.11
18	Malting barley	2007	<LOD	<LOD	1.82 ± 0.46	2.34	2.25	0.00	0.20
19	Malting barley	2007	<LOQ	<LOQ	<LOQ	4.09	1.93	0.00	0.15
20	Barley for feeding	2007	48.63 ± 12.16	34.40 ± 8.60	13.17 ± 3.29	18.21	3.35	0.00	3.91
21	Bread wheat	2005	4.12 ± 1.03	3.95 ± 0.99	<LOQ	2.20	4.00	0.00	5.34
22	Wheat for feeding	2007	<LOQ	<LOQ	<LOD	6.37	3.09	0.00	0.85
23	Wheat for feeding	2007	<LOD	0.26 ± 0.07	<LOD	2.64	3.84	0.00	0.61
24	Wheat for feeding	2007	<LOD	<LOD	<LOD	5.42	5.39	0.00	1.17
25	Bread wheat	2007	0.33 ± 0.08	0.30 ± 0.08	<LOD	3.98	1.64	0.00	1.92
26	Wheat for feeding	2007	<LOD	<LOQ	<LOD	5.49	0.39	0.00	1.61
27	Wheat for feeding	2007	<LOD	<LOD	<LOD	1.92	2.09	0.05	0.80
28	Wheat for feeding	2007	<LOD	<LOD	<LOD	2.39	1.54	0.00	0.77
29	Wheat for feeding	2007	<LOD	<LOQ	<LOD	1.07	1.71	0.00	0.26
30	Wheat for feeding	2007	<LOD	<LOQ	<LOD	4.97	4.06	0.06	0.95
31	Wheat for feeding	2007	<LOD	<LOD	<LOD	15.68	5.71	0.00	0.68
32	Wheat for feeding	2007	<LOD	<LOQ	<LOD	17.49	4.09	0.00	0.53
33	Reference material[c]	–	8.75 ± 2.19	12.73 ± 3.18	<LOD	–	–	–	–
34	Reference material[d]	–	2.54 ± 0.64	4.52 ± 1.13	<LOD	–	–	–	–

Notes: [a]Sample obtained from organic farming.
[b]Sample obtained from a malt house laboratory.
[c]P64/OW806 (7.7 ± 2.0)μg kg^{-1}, wheat.
[d]P64/OW803 (1.8 ± 0.6)μg kg^{-1}, wheat.
[e]Categories of materials that are not basic cereal of unimpaired quality, according to Commission Regulation (EC) No. 824/2000: 1, broken grains; 2, impurities consisting of grains; 4, sprouted grains; and 5, miscellaneous impurities.

Table 3. Performance characteristics of two HPLC methods used for analyses of ochratoxin A (OTA) (VICAM – method using the immunoaffinity columns OchraTest™; PART – method based on solvent partition without purification on immunoaffinity columns).

Method	Recovery (%)	Repeatibility (%)	Inter-day variability (%)	Limit of detection (LOD) ($\mu g\,kg^{-1}$)	Limit of quantification (LOQ) ($\mu g\,kg^{-1}$)
VICAM	87.0 ± 6.1	7.2	12.0	0.15	0.25
PART	75.6 ± 6.6	7.5	12.5	0.05	0.20

quantification (LOQ) of $0.25\,\mu g\,kg^{-1}$ and a limit of detection (LOD) of $0.15\,\mu g\,kg^{-1}$ were calculated based on a blank sample signal when a value of maximum variation in a chromatogram basic line for the blank sample in the region given by a 20-multiple of OTA peak half-width and calibration line slope were used for calculation. The average repeatability of 7.2% was determined from five repeated measurements of spiked samples at the concentrations of 2.5, 5.0 and $10.0\,\mu g\,kg^{-1}$ performed by the same operator on one day and with the same HPLC system. Inter-day variability was measured on the same set of samples on five successive days resulting in a reproducibility of 12.0%. All samples were measured in duplicate and the results are presented as mean ± measurement uncertainty, which was estimated at 25%. For the purposes of this contribution, results obtained using this method are considered to be reference results, meaning that if only one final OTA level is shown in the text, and it is not specified in any other way, then it is from HPLC-VICAM analysis.

HPLC method based on solvent partition (PART)

Analyses were performed by a Laboratory of Chemical Engineering UMR CNRS/INPT/UPS n° 550 in Toulouse (France). The method according to Molinié et al. (2005) was employed. All reagents were of HPLC grade and purchased from ICS (France). Deionized water was used to prepare all aqueous solutions and for HPLC. OTA free of benzene and CIT were from Sigma Chemicals (France). The HPLC analysis used a Gilson 811B dynamic chromatography pump coupled to a Spectra Physics 2000 fluorescence spectrophotometer and an ICS autosampler. In order to optimise sensitivity for the analysis of OTA and CIT, which have different excitation and emission fluorescence parameters (OTA of 335 and 465 nm, CIT of 331 and 500 nm, respectively), analyses were performed using HPLC conditions adapted for each toxin. A C18 spherisorb column (3 µm C18, 46 × 250 mm) was used, preceded by a C18 pre-column from ICS. To reduce the risk of false-positives, the system was run isocratically using two different phases for the analyses of OTA. Phase 1 was methanol/acetonitrile/sodium acetate (5 mM)/acetic acid (300/300/400/28); flow rate was $0.7\,ml\,min^{-1}$; and the elution time of OTA was about 9 min. Phase 2 was H_3PO_4 (0.33 M)/acetonitrile/propan-2-ol (600/400/50); flow rate was 0.7 min, and the elution time of OTA was about 18 min. A third phase was used for determining CIT: H_3PO_4 (0.33 M)/acetonitrile/propan2-ol (700/300/50); flow rate was 0.7 min; and the elution time of CIT was about 19 min. Validation of this method was carried out and validation data have been published previously (Molinié et al. 2005). Performance characteristics are summarised in Table 3 in comparison with the VICAM method. OTA recovery was calculated based on analysis of five different cereal samples spiked with $3\,\mu g\,kg^{-1}$ (corresponding to value from European Union legislation) and $10\,\mu g\,kg^{-1}$ on the same day, by the same operator and with the same HPLC system. The average OTA recoveries were 76.1% ± 5.7% and 75.1% ± 11.9%, respectively, for cereal samples spiked with OTA at the concentration levels of 3 and $10\,\mu g\,kg^{-1}$. The results presented are corrected for an average recovery 75.6% ± 6.6%. OTA repeatability was calculated based on the results of these samples analysed five times on the same day and amounts to 7.5%. OTA inter-day variability was measured by the means of samples spiked with $3\,\mu g\,kg^{-1}$ and one naturally contaminated sample analysed on five successive days by the same operator and with the same HPLC system resulting in a value of 12.5%. All samples were measured in duplicate and results are presented as mean ± measurement uncertainty, which was estimated to be 25%. The dose–response curve for OTA analysis was measured by analysis of solutions containing OTA in concentrations from $0.5\,\mu g\,l^{-1}$ to $1\,mg\,l^{-1}$ (corresponding to concentrations from $12.5\,ng\,kg^{-1}$ to $24.9\,\mu g\,kg^{-1}$ in cereals). The coefficient of linearity (R^2) was 0.997. The LOD was $0.05\,\mu g\,kg^{-1}$ and the LOQ was $0.20\,\mu g\,kg^{-1}$ at nine-times background. CIT recovery was calculated based on the analysis of five different cereal samples spiked with 3 and $10\,\mu g\,kg^{-1}$ on the same day by the same operator and with the same HPLC system. The average recovery was 80.3% ± 5.0%. The results are corrected for this recovery. CIT repeatability was calculated based on the analysis of these samples analysed five times on the same day on the level of 8.1%. For CIT inter-day variability calculation, samples spiked with $3\,\mu g\,kg^{-1}$ and one naturally contaminated sample were analysed on five successive days by the same operator and with

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the same HPLC system. The average CIT reproducibility was 13.6%; the measurement uncertainty was estimated to be 30%. The dose–response curve for CIT was measured by analysis of solutions containing CIT in concentrations from $50\,\mu g\,l^{-1}$ to $2.25\,mg\,l^{-1}$ (corresponding to concentrations from 1.12 to $56.25\,\mu g\,kg^{-1}$ in cereals). R^2 was 0.98. The LOD was $0.5\,\mu g\,kg^{-1}$ and the LOQ was $1.5\,\mu g\,kg^{-1}$ at seven-times background. Recovery tests for both toxins simultaneously were carried out by means of analysing five different samples spiked with both toxins at the concentration of $3\,\mu g\,kg^{-1}$ each on the same day by the same operator with the same HPLC system. The average recovery was $81.3\% \pm 4.2\%$ for OTA and $80.1\% \pm 5\%$ for CIT. The confirmation of the presence of OTA in samples detected at $2\,\mu g\,kg^{-1}$ was performed using two techniques: the carboxypeptidase technique for producing α-ochratoxin (α-OT). Briefly, an aliquot taken from the purified extract of a sample where OTA was detected using the two HPLC methods was dried, and residue dissolved in 0.975 ml of a buffer solution of 0.04 M Tris-HCl, 1 M NaCl, pH 7.5. The amount of $25\,\mu l$ of carboxypeptidase in water ($100\,U\,ml^{-1}$) was added and the solution incubated at room temperature overnight. The samples were analysed by the HPLC chromatographic conditions used above for the analysis of OTA. The peak of OTA disappeared, whereas the peak of α-OT appeared. Some samples were analysed by LC-MS/MS by an external laboratory (data not shown).

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Measurement of admixtures and impurities, and determination of moisture content

For all samples the parameter 'Matter other than basic cereals of unimpaired quality' was determined and for sample Set I also 'Moisture content'. Both parameters were analysed according to Commission Regulation (EC) No. 824/2000 of 19 April 2000 establishing procedures for the taking-over of cereals by intervention agencies and laying down methods of analysis for determining the quality of cereals (European Commission 2000). Values of 'Matter other than basic cereals of unimpaired quality' which were found for the samples were compared with maximum permitted values according to Commission Regulation (EC) No. 824/2000. For wheat they are as follows: 1, broken grains 5%; 2, impurities consisting of grains 7%; 4, sprouted grains 4%; and 5, miscellaneous impurities 3%. For barley, they are: 1, broken grains 5%; 2, impurities consisting of grains 12%; 4, sprouted grains 6%; and 5, miscellaneous impurities 3%.

Statistical analysis

Data were analysed by the Statistica program (Statsoft, Inc.), version 8.0. Measured values were plotted in a scatter plot with identity ($x = y$) and regression line; inter-method differences were correlated to measured VICAM values. A pairwise t-test and correlation analysis were used to determine the statistical significance of differences between the two methods. Values below the LOD were eliminated from a comparison. $p < 0.05$ was accepted as being significant (if not otherwise stated). Data are presented as mean $\pm$ measurement uncertainty (95% confidence interval).

Results

Sample analyses – Set I

The results are summarised in Table 1.

OTA analysis – VICAM method

Nine of the wheat samples analysed had an OTA content lower than the LOD of the method used ($0.15\,\mu g\,kg^{-1}$). For sample 2, an OTA content of $4.71 \pm 1.18\,\mu g\,kg^{-1}$ was found.

OTA and CIT analysis – PART method

Three samples were found to have an OTA content above the LOD of the method used ($0.05\,\mu g\,kg^{-1}$). The highest OTA content was determined in sample 2 ($6.03 \pm 1.51\,\mu g\,kg^{-1}$), and two additional positive samples (numbers 4 and 10) had an OTA content between the LOD and the LOQ. CIT content between the LOD and the LOQ was found for sample 2; for all other samples CIT values were under the LOD.

Sample analyses – Set II

The results are summarised in Table 2.

OTA analysis – VICAM method

Using this method, nine samples were found to be OTA positive, of which two samples had OTA values between the LOD and the LOQ. The maximum level was for sample 20 (barley for feeding), at $48.63 \pm 12.16\,\mu g\,kg^{-1}$. The second highest sample was number 12 (malting barley with poor quality parameters), with an OTA level of $31.43 \pm 7.86\,\mu g\,kg^{-1}$. The third highest sample was bread wheat, number 21. Its OTA level was $4.12 \pm 1.03\,\mu g\,kg^{-1}$. In analysing RM P64/OW806 with a determined value of $7.7 \pm 2.0\,\mu g\,kg^{-1}$, the concentration $8.75 \pm 2.19\,\mu g\,kg^{-1}$ was found. In analysing RM P64/OW803 with a determined value of $1.8 \pm 0.6\,\mu g\,kg^{-1}$, the concentration $2.54 \pm 0.64\,\mu g\,kg^{-1}$ was found.

OTA and CIT analysis – PART method

Using this method, 15 samples were found to be OTA positive, of which six samples had OTA values between

the LOD and the LOQ. The maximum level was for sample 12 (malting barley with poor quality parameters), at $44.74 \pm 11.19\,\mu g\,kg^{-1}$. The second highest sample was number 20, with an OTA level of $34.40 \pm 8.60\,\mu g\,kg^{-1}$. The third highest sample was bread wheat, number 21, with an OTA level of $3.95 \pm 0.99\,\mu g\,kg^{-1}$. In analysing RM P64/OW806 with a determined value of $7.7 \pm 2.0\,\mu g\,kg^{-1}$, the concentration $12.73 \pm 3.18\,\mu g\,kg^{-1}$ was found. In analysing RM P64/OW803 with a determined value of $1.8 \pm 0.6\,\mu g\,kg^{-1}$, the concentration $4.52 \pm 1.13\,\mu g\,kg^{-1}$ was found. CIT content above the LOD ($0.5\,\mu g\,kg^{-1}$) was found for eight samples, of which four had CIT values between the LOD and the LOQ. The maximum CIT concentration ($93.6 \pm 28.09\,\mu g\,kg^{-1}$) was found for sample 12, which also had the highest OTA content as analysed using the same method.

Comparison of results obtained by VICAM and PART methods

For the 24 sample measurements (Set II samples and two RMs), *t*-test ($p = 0.20$) and correlation analysis showed no significant bias between the VICAM and the PART methods. Good correlation was observed between measured values ($r = 0.913$) (Figure 1), but not between inter-method differences and measured values ($r = 0.268$).

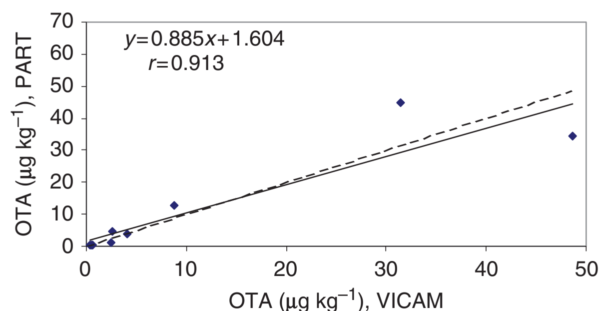

Figure 1. Scatter plot of OTA content measured by VICAM and PART method (VICAM – HPLC using the immunoaffinity columns OchraTest™; PART – based on solvent partition without purification on immunoaffinity columns). Dashed line = identity line.

Discussion

Comparison of analytical procedures based on LC for OTA analysis in raw cereal grain with (VICAM) and without (PART) immunoaffinity clean-up

Two methods based on the HPLC principle, one using the immunoaffinity columns OchraTest™ (VICAM 2005) and the other based on solvent partition (Molinié et al. 2005), both followed by fluorescence detection, were used to analyse 32 samples of raw cereals and two reference materials (RMs). RMs P64/OW803 (OTA = $1.8 \pm 0.6\,\mu g\,kg^{-1}$) and P64/OW806 (OTA = $7.7 \pm 2.0\,\mu g\,kg^{-1}$) from R-Biopharm AG (Germany) were analysed. As the RMs used were not certified, only their properties such as homogeneity and stability could be taken into consideration and the results of both methods were compared only one with the other. The matrix of both RMs was wheat wholemeal. The values obtained using the VICAM method, 2.54 ± 0.64 and $8.75 \pm 2.19\,\mu g\,kg^{-1}$, respectively, were lower that the results of the PART method, $4.52 \pm 1.13\,\mu g\,kg^{-1}$ and $12.73 \pm 3.18\,\mu g\,kg^{-1}$ (Table 2). The reason could be that the recovery of OTA in acidic conditions is of 80%, whereas in neutral and alkaline conditions OTA is converted into open-ring OTA which is no more recognised by antibodies and thus are lost (Pfohl-Leszkowicz et al. 2006; Castegnaro et al. 2006). In the first set of samples, the VICAM method detected only one OTA positive sample, number 2, and the OTA level was determined to be $4.71 \pm 1.18\,\mu g\,kg^{-1}$. The PART method detected a slightly higher level ($6.03 \pm 1.51\,\mu g\,kg^{-1}$) and another two positive samples, with OTA levels above the limit of detection (LOD), but under the limit of quantification (LOQ). In Set II, the highest OTA content using VICAM was assessed in sample 20 ($48.63 \pm 12.16\,\mu g\,kg^{-1}$) and the second highest in sample 12 ($31.43 \pm 7.86\,\mu g\,kg^{-1}$). Based on results obtained using the PART method, the order was the opposite, as the highest content was found in sample 12 ($44.74 \pm 11.19\,\mu g\,kg^{-1}$) and the second highest in sample 20 ($34.40 \pm 8.6\,\mu g\,kg^{-1}$). In the second set of samples, the VICAM method detected nine positive samples. The PART method confirmed this finding in all these samples and identified another six positive ones, with two having OTA in concentrations of $0.26 \pm 0.07\,\mu g\,kg^{-1}$ and $0.58 \pm 0.15\,\mu g\,kg^{-1}$ and four being between the LOD and the LOQ. Statistical analysis for the 24 sample measurements (Set II samples and two RMs), *t*-test ($p = 0.20$) and correlation analysis showed no significant bias between the VICAM and PART methods and good correlation between measured values, suggesting a good agreement between both methods. A comparison of inter-method differences and measured values show no trend in absolute differences.

OTA and CIT occurrence in raw cereals

OTA and CIT are naturally produced by fungi, which can contaminate cereals, beans, nuts, spices, and coffee during harvest, transport, processing, and storage. Studies in the field have found OTA usually – but not always – to be absent in cereals at harvest (Elmholt 2003; Molinié 2004). If it is detected in samples immediately after harvest, this is generally due to insufficient care for the sample between its collection

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and analysis (Scudamore 2005) or to unclean harvesting machines. Results of the current study correspond with these findings. Among ten wheat samples (Set I) that were analysed shortly after harvest (a maximum after 4 months) and not stored, there was only one sample with an increased OTA content ($4.71 \pm 1.18\,\mu g\,kg^{-1}$) and it was also the only one that was CIT positive, but with a low CIT content not exceeding the LOQ of the method ($1.50\,\mu g\,kg^{-1}$). Subsequent analyses of the content of admixtures and impurities revealed that this sample contained a high amount of weed seeds (included in category 5 Miscellaneous impurities, according to Commission Regulation (EC) No. 824/2000; European Commission 2000). Samples of Set I were chosen from the harvest 2006, which was characterised by unusually rainy weather between 3 and 10 August 2006 in the whole country, and samples delivered to the laboratory later than 25 August were taken for analysis. Rainy weather during the harvest corresponds to a high content of sprouted grains in these samples, which reached up to 47.6% for sample 4. The only sample with a positive OTA and CIT contents (number 2) from this series was harvested on 3 August and did not contain any sprouted grains as the only one. There could be two possible reasons for positive OTA and CIT contents. Either the high content of weed seeds could have led to a temporary post-harvest increase in wheat grain moisture and/or the sample was harvested during the beginning of the rain and was not dried. The moisture content of the sample upon arrival at the laboratory was at a standard 12.5%, but nearly 4 weeks had passed between harvest and the sample's delivery to the laboratory. It could be supposed that the sample had not been treated appropriately immediately after harvest.

Of Set II, only sample 11, naked barley from an organic farming system intended for the production of wholemeal foods, had not been stored for a longer time. It was positive for OTA content, but the concentration was low ($0.49 \pm 0.12\,\mu g\,kg^{-1}$). CIT content was between the LOD and the LOQ. This sample was taken from a tractor trailer on a farm where it had been temporarily left after harvest for approximately 2 weeks. The other nine barley samples were from the 2007 harvest, i.e. they had been stored approximately one year before analysis. The highest content was found in a barley sample for animal feeding, number 20, at $48.63 \pm 12.16\,\mu g\,kg^{-1}$. For OTA in cereals intended for animal feeding, only guidance values are given – in this case $0.25\,mg\,kg^{-1}$ (European Commission 2006b). This value was not exceeded. This sample also contained a higher amount of CIT ($13.17 \pm 3.29\,\mu g\,kg^{-1}$). A high content of broken grains (18.21% in comparison with the maximum limit of 5%) and also miscellaneous impurities (3.91% in comparison with a maximum limit of 3%) were found in this sample. The second highest OTA content ($31.43 \pm 7.86\,\mu g\,kg^{-1}$) and the highest CIT content ($93.64 \pm 28.09\,\mu g\,kg^{-1}$) were determined in sample 12, the barley offered to a malt house but not accepted due to a high admixtures and impurities content and smell of mould. As it is a raw material for food production, OTA content should meet the maximum limit according to Commission Regulation No. 1881/2006 for unprocessed cereals, $5\,\mu g\,kg^{-1}$. Thus, the limit was exceeded by more than six times. This sample was found to have an above-limit content of miscellaneous impurities (14.35% in comparison with a maximum limit of 3%), which consisted mainly of matter passing through a sieve with apertures of 1.0 mm (i.e. mostly dust). In total, among ten barley samples analysed, six were OTA positive and five exceeded the LOQ. Of twelve wheat samples in Set II, three were OTA positive and two exceeded the LOQ. Sample 21 (bread wheat, 2005 harvest) contained OTA at $4.12 \pm 1.03\,\mu g\,kg^{-1}$. This sample also had a higher content of miscellaneous impurities (5.34%). The second above-LOQ OTA sample was sample 25 (bread wheat), with OTA $= 0.33 \pm 0.08\,\mu g\,kg^{-1}$.

Within the current study, in total 32 raw cereal samples – ten of barley and 22 of wheat – were analysed, of which six barley (60%) and four wheat (18%) samples were OTA positive. Among eight cereal samples with an OTA value above the LOQ, four exceeded the permitted level of 3% for miscellaneous impurities and one of them (barley number 20) simultaneously for broken grains. Four barley samples had been intended for food production, of which three were OTA positive and one sample exceeded the limit of $5\,\mu g\,kg^{-1}$ by more than six times. Among eleven wheat samples for food use, three were OTA positive, while two approached the limit of $5\,\mu g\,kg^{-1}$.

The detected maximum OTA levels for barley ($48.63 \pm 12.16\,\mu g\,kg^{-1}$) and wheat ($4.71 \pm 1.18\,\mu g\,kg^{-1}$) are in a broad range of maximum OTA values for raw cereals as reported by various authors (Rizzo et al. 2002). In the Czech Republic, Malir et al. (2006) reported a maximum value of $37.0\,\mu g\,kg^{-1}$. Ten samples in their survey were positive (above an LOD of $0.4\,\mu g\,kg^{-1}$), comprising 8.8% of all 114 samples examined. In our survey, we detected ten positive samples (above an LOD of $0.25\,\mu g\,kg^{-1}$) among 32 examined, i.e. 31%, but the choice of the samples was not fully random and the extent of the sample set was small. If we had evaluated the results obtained using a PART method with an LOD of $0.05\,\mu g\,kg^{-1}$, 18 samples would have been OTA positive, i.e. 56%. The reported percentage of OTA-positive cereal samples varies largely. According to a survey by Pittet (1998), for example, it ranges from 4% to 88%. These levels are influenced by the LOD of the analytical method used, as well as by the number of analysed samples and sampling technique. It is also important whether

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only freshly harvested samples are analysed or a survey involves samples that have been stored for some time.

Citrinin is known to be a contaminant in a variety of cereals (Abramson 1997) and could have the same producer (*P. verrucosum*) as OTA, but very few studies have included parallel analyses of both toxins. Vrabcheva et al. (2000) monitored incidences of OTA and CIT in cereal samples from villages where Balkan endemic nephropathy had occurred. Maximum OTA levels in their survey were found for oats (140 and $85\,\mu g\,kg^{-1}$) and for wheat (39 and $31\,\mu g\,kg^{-1}$) using enzyme-linked immunoabsorbant assay (ELISA) and high-performance liquid chromatography (HPLC) methods, respectively. In samples simultaneously contaminated with OTA and CIT, CIT levels were two to 200 times higher than those of OTA. A maximum CIT content ($420\,\mu g\,kg^{-1}$) was found in wheat having the highest OTA content. The fact that when simultaneous contamination of cereals occurs CIT concentrations often exceed by several times the OTA concentrations is also reported by Scott (1994). This is also confirmed for breakfast cereals by findings of Molinié et al. (2005), who found more OTA-positive than CIT-positive samples. Nevertheless, if CIT were present, its content was higher than that of OTA. In their study, for a sample with a maximum CIT content ($42\,\mu g\,kg^{-1}$), OTA content was $4.1\,\mu g\,kg^{-1}$. In the current study, of 32 cereal samples analysed for CIT content, nine were positive and four exceeded the LOQ for CIT ($1.5\,\mu g\,kg^{-1}$). Three of these four samples were simultaneously contaminated with OTA above the LOQ and the CIT:OTA content ratios for these samples were 0.27, 2.08 and 2.98 for OTA as analysed by VICAM, and 0.38, 5.10 and 2.09 for OTA as analysed by PART. The maximum CIT content ($93.64 \pm 28.09\,\mu g\,kg^{-1}$) was found in a barley sample with an OTA content measuring 31.43 ± 7.86 and $44.74 \pm 1.18\,\mu g\,kg^{-1}$ as analysed using VICAM and PART, respectively. All samples with CIT values above the LOQ exceeded the permitted level of 3% for miscellaneous impurities and one of them (barley number 20) simultaneously for broken grains. The highest amount of CIT ever reported was found in French wheat for a farm sample at a level of $520\,\mu g\,kg^{-1}$ (Molinié 2004; Pfohl-Leszkowicz et al. 2007).

The results tend to show that an increased amount of admixtures and impurities in a sample can indicate a higher risk of OTA's occurrence. This fact can be associated, for instance, with the content of weed seeds and parts that are harvested together with a cereal crop. This content can increase grain moisture above a critical level after harvest and thus allow OTA production under favourable temperature conditions. Also, dust seemed to be a risk factor in those samples analysed. As reported by Tangni and Pussemier (2006), dust acts as both an OTA and CIT contaminant and inoculum. They recommend taking precautionary measures not only by controlling and maintaining moisture at a reasonable level during storage of the raw grains, but also by paying close attention to cleaning the storage spaces before placing the new harvests there. They concluded that the presence of dust in grains can be considered a threat to exceeding the legal limits. Cereals can also be contaminated during harvest, as *P. verrucosum* can survive in the field and proliferate on soil organic matter (Elmholt and Hestbjerg 1999). Given appropriate environmental conditions, this may also constitute a risk for grain contamination. OTA content in cereals depends on a variety of factors, including the source of *P. verrucosum* infection, whether or not water activity rises above a critical value (Magan and Aldred 2005), temperature, and storage time. Timely cleaning of cereals and reducing the moisture content below a critical limit can prevent increased OTA from appearing.

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1999 – 2002	Katedra analytické chemie, PřF MU Brno, titul doktor, Ph.D. (*Metod development in capillary electrophoresis and applications*)
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2008 – Stanovení obsahu ochratoxinu A a citrininu v obilninách, IGA AF MZLU v Brně MP 32/AF.

Akademické stáže:

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6/2007	Laboratoire de génie chimique, Ecole Nationale Supérieure Agronomique de Toulouse (INP/ENSAT), Toulouse, Francie (1 týden, program BARRANDE, studijní pobyt, prof. Leszkowicz)
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Vybrané publikace:

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Dohnal, V., Ježková, A., Jun, D., Kuča, K.: Metabolic pathways of T-2 toxin, *Current Drug Metabolism 9(1), 2008, strana 77-82. (IF=4,490)*

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Počet učebních textů a skript:	3
Počet kapitol v monografiích:	1