VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ
FAKULTA CHEMICKÁ

Habilitační práce v oboru Potravinářská chemie

Sledování vybraných nežádoucích látek ve sladu, mladině a pivu

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Brno 2021

Abstrakt

Tato práce shrnuje hlavní publikované výsledky dlouhodobého výzkumu autorky zaměřeného na problematiku sledování látek, které mohou znehodnotit kvalitu piva i ohrozit zdraví spotřebitele.

V jednotlivých kapitolách jsou charakterizovány vybrané procesní kontaminanty, mykotoxiny, nežádoucí senzoricky aktivní látky a sladové proteiny. Zmíněny jsou také použité moderní analytické instrumentální metody při stanovení těchto látek. Akrylamid jako procesní kontaminant vzniká hlavně během tepelného zpracování potravin a 3-chlorpropan-1,2-diol především při hydrolýze potravin. Obě tyto látky jsou vysoce toxické a jsou zařazeny do skupiny 2A, jako pravděpodobné lidské karcinogeny. Mezi karcinogeny jsou zařazeny také některé sledované mykotoxiny T-2 a HT-2 toxin, deoxynivalenol, ochratoxin A a aflatoxiny. Kromě těchto kontaminantů mohou kvalitu vyrobeného sladu a piva negativně ovlivnit i senzoricky aktivní těkavé látky a jejich prekurzory. Degradací sirných prekurzorů vznikají těkavé látky, jako jsou dimethylsulfid, methional, sirouhlík apod, degradací lipidů vzniká těkavý aldehyd trans-2-nonenal. Pro některé spotřebitele mohou představovat určité riziko sladové proteiny. Gluten (lepek) je složený protein, který se nachází v potravinách zpracovaných z pšenice a příbuzných druhů, včetně ječmene a žita. Lepek je složen z gliadinů (dříve nazývaných prolaminů) a glutelinů. Gliadiny jsou alergenní pro jedince trpící nesnášenlivostí lepku – tzv. celiakií.

Klíčová slova

Akrylamid, 3-MCPD, mykotoxiny, DMS, trans-2-nonenal, gliadin, GC-MS, SPME, ELISA

Abstract

This study summarizes the major published results of the author's long-term research that focused on monitoring substances that may degrade the quality of beer and endanger the consumers´ health.

The individual chapters characterize selected process contaminants, mycotoxins, undesirable sensory active substances and malt proteins. The modern analytical instrumental methods used for the determination of these substances are also described. Acrylamide as a process contaminant is formed mainly during the heat treatment of food and 3-chloropropane-1,2-diol during the hydrolysis of food. Both these substances are highly toxic and are classified in group 2A as probable human carcinogens. Some of the monitored mycotoxins T-2 and HT-2 toxin, deoxynivalenol, ochratoxin A and aflatoxins also belong to the carcinogens. In addition to these contaminants, the quality of the produced malt and beer can also be negatively affected by sensory active volatile substances and their precursors. Degradation of sulfur precursors produces volatiles such as dimethyl sulfide, methional, carbon disulfide, etc., and degradation of lipids forms volatile aldehyde trans-2-nonenal. Malt proteins may pose a risk to some consumers. Gluten is a complex protein found in foods processed from wheat and related species, including barley and rye. Gluten is composed of gliadins (formerly called prolamins) and glutelins. Gliadins are allergenic to individuals suffering from gluten intolerance - so-called celiac disease.

Keywords

Acrylamide, 3-MCPD, mycotoxins, DMS, trans-2-nonenal, gliadin, GC-MS, SPME, ELISA

Poděkování

Na tomto místě bych ráda poděkovala všem svým blízkým za podporu a pomoc při mé práci, tedy rodině, přátelům a kolegům.

Obsah

1 Úvod

Předložená habilitační práce uvádí přehled o látkách, které mohou znehodnotit kvalitu piva a souhrně informuje o publikovaných výsledcích sledování těchto látek ve sladu a pivu.

Pivo patří již po staletí k tradičním českým nápojům. Vždy bylo považováno nejen za osvěžující, ale také za velmi zdravý, výživný a chutný nápoj, který má příznivé dietetické vlastnosti.

Na průkazné analytické i senzorické charakterizaci piva se spolupodílí kvalita pivovarských surovin, technologie výroby sladiny a mladiny i technologie kvašení a zrání piva. V ČR jsou kladeny značné nároky zejména na použití vysoce kvalitních českých pivovarských surovin. Česká republika je zřejmě jedinou zemí ve světě, ve které pivovarští odborníci rozlišují u nových odrůd ječmene, zda je to odrůda sladovnického ječmene vhodná pro exportní slad, nebo pro zachování senzorického charakteru národního nápoje. Rovněž další surovina – český chmel představuje špičku mezi jemnými aromatickými chmely, podobné odrůdy se jinde nedaří vypěstovat. Ke specifickým znakům českého piva pak patří například plnost chuti a zlatožlutá barva. Dalším důležitým atributem je správná pěnivost piva; smetanová hustá pěna chrání pivo před oxidací.

V poslední době je celosvětově, a i v České republice věnována zvýšená pozornost látkám, které ohrožují kvalitu finálního výrobku nebo zdraví spotřebitele. Pivo patří již desítky let v České republice k potravinám, které jsou pečlivě kontrolovány z hlediska zdravotní nezávadnosti. S tím souvisí i kontrola zdravotní nezávadnosti surovin potřebných na výrobu piva ke kterým patří vedle vody a chmele také slad. Kromě zdraví škodlivých látek se kontrolují i látky, které jsou v určitých koncentracích nežádoucí z pohledu narušení senzorických vlastností sladu a následně i piva.

Mezi toxické látky, které vznikají během technologického zpracování potravin a jsou sledovány v průběhu sladování a ve sladu patří procesní kontaminanty akrylamid a 3-MCPD (3-chlorpropan-1,2-diol). Ve sladu a v pivu jsou sledovány také toxické sekundární metabolity mikroskopických vláknitých hub – mykotoxiny. Kromě těchto kontaminantů mohou kvalitu vyrobeného sladu a piva negativně ovlivnit i senzoricky aktivní těkavé látky a jejich prekurzory. Patří mezi ně zejména sirné sloučeniny a lipidy. Stopová množství těchto sloučenin, které lze běžně nalézt v potravinách, se spolupodílejí na vytváření aróma a tento vliv lze obecně hodnotit jako příznivý. U sladu, resp. u piva však toto platí jen ve velmi omezené míře a přítomnost těchto látek se hodnotí spíše nepříznivě. Pro některé spotřebitele mohou představovat určité riziko sladové proteiny, které mohou být nebezpečné pro jedince trpící alergií na lepek – tzv. celiakií.

Habilitační práce je sepsaná formou souhrnného komentáře k souboru uveřejněných vědeckých publikací autorky. Výsledky, které jsou v rámci textu prezentovány, zahrnují publikace v zahraničních a domácích impaktovaných a recenzovaných časopisech. Kopie vybraných článků jsou součástí práce jako přílohy.

Obsah a členění práce odpovídá výzkumnému zaměřeni autorky, které zahrnuje oblast potravinářské chemie, speciálně chemie a technologie výroby sladu a piva.

Výše uvedené oblasti výzkumu jsou také v souladu s výzkumnou a pedagogickou činnosti autorky i s tématy obhájených bakalářských a diplomových prací. Do oblasti pivovarství a sladařství v širším kontextu byly také zaměřeny výzkumné projekty, na nichž se autorka podílela jako řešitel či spoluřešitel.

2 Procesní kontaminanaty

Procesní kontaminanty jsou relativně novou a veřejnosti méně známou skupinou toxických látek. Vznikají obecně v průběhu zpracování potravin a mohou způsobit závažná onemocnění.

Mezi toxické procesní kontaminanty, které vznikají během technologického zpracování potravin, patří mimo jiné i akrylamid a 3-MCPD (3-chlorpropan-1,2-diol) [1].

2.1 Akrylamid

V dubnu 2002 zveřejnil Švédský národní úřad (NFA – National Food Administration) spolu se Státní univerzitou ve Stockholmu nález akrylamidu v potravinách zpracovávaných při teplotách nad 120 °C [2]. Nález této neurotoxické a potenciálně karcinogenní látky [3] veřejnost i odborné kruhy nepříjemně překvapil.

Akrylamid je triviální název pro 2-propenamid. V literatuře je možné se setkat s různými synonymy této látky např. 2-propeneamid, vinyl amid, akryl amid, akrylamid monomer, propenamid, ethylen karboxamid, kyselina propenová. Akrylamid je bílá krystalická látka, bez barvy a zápachu, dobře rozpustná ve vodě, metanolu, etylacetátu a nerozpustná v heptanu a benzenu. Pevný akrylamid je stabilní při laboratorní teplotě, ale může polymerizovat při zahřívání nebo při oxidativním působení [4; 5; 6]. Polyakrylamid již není na rozdíl od monomeru toxický.

Polyakrylamid je rozsáhle používán pro úpravu pitných a odpadních vod a k odstranění pevných částic z průmyslových vod. Polymer vytváří vazbu s pevnými částicemi a tyto těžké formy agregátů rychle sedimentují a mohou být následně odstraněny. Polyakrylamid se běžně používá v papírenském průmyslu jako nosič nebo k zadržení pigmentů na papírových vláknech. Uplatňuje se také v kosmetickém průmyslu, přidává se do mýdel a do vlasových přípravků. Dále se používá při syntéze různých barev, při výrobě kontaktních čoček a také se uplatňuje v textilním průmyslu. Minoritně je akrylamid používán pro chromatografii a elektroforézu nebo jako stabilizátor pro náplně do tiskáren [7; 8].

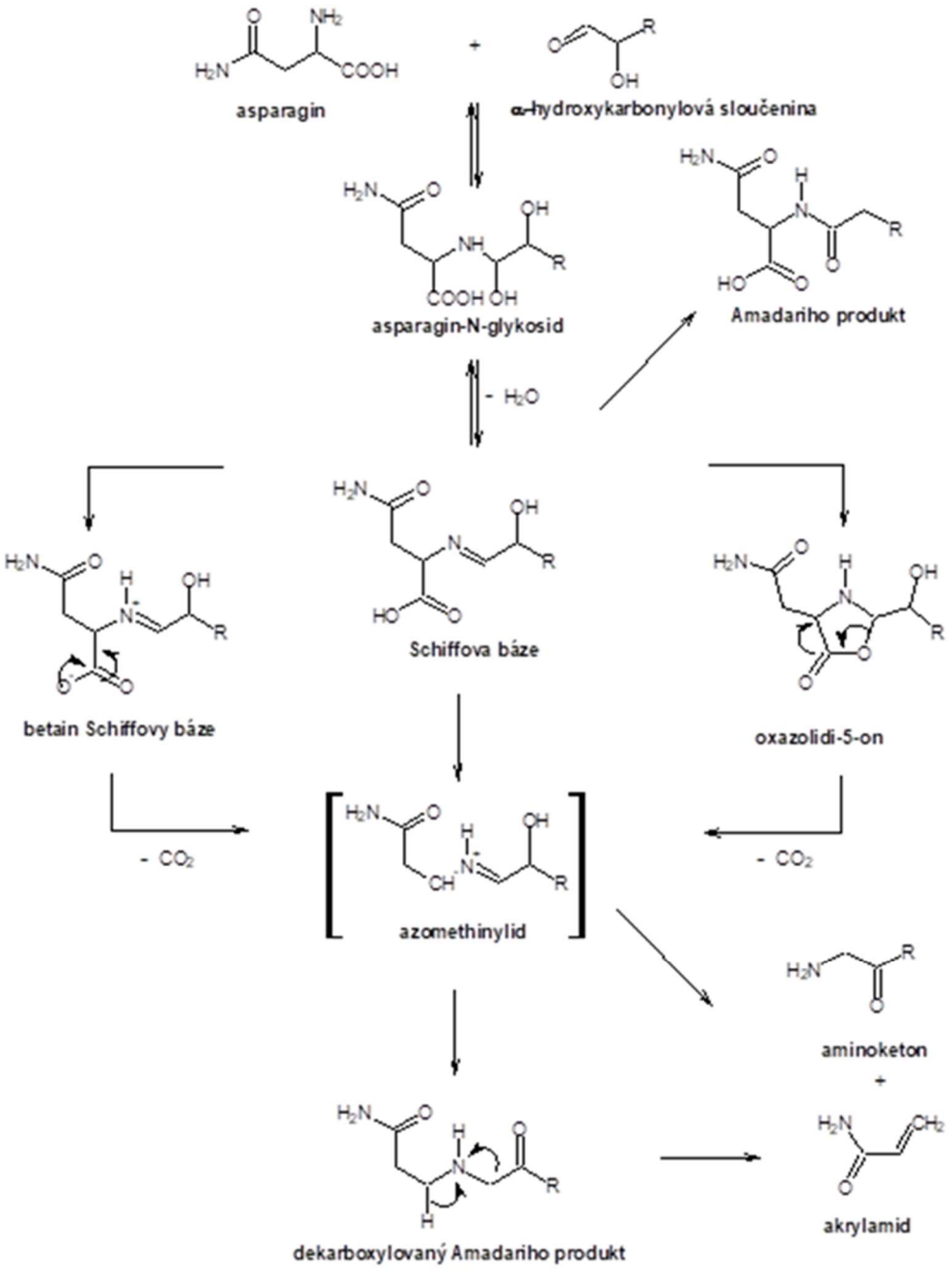
Akrylamid má vysoký potenciál vyvolat široké spektrum toxických účinků, včetně neurotoxických [2; 9; 10]. Na základě provedených studií byl akrylamid zařazen do skupiny 2A, jako pravděpodobný lidský karcinogen [2]. In vivo může být akrylamid metabolizován na epoxid glycidamid, který vykazuje stokrát až tisíckrát vyšší reaktivitu s DNA, než samotný akrylamid [11].

Akrylamid může být absorbován kůží, sliznicí, plícemi nebo zažívacím ústrojím [12]. Je považován za vysoce nebezpečnou látku z hlediska možné perorální expozice pro člověka. Lidská populace je perorální expozicí akrylamidem vystavena zejména při konzumací pitné vody a kouření [13; 14]. Expozice akrylamidem pro člověka je však pravděpodobně mnohem vyšší konzumací potravin obsahujících škrob, které byly tepelně zpracované [15; 16]. Expozice pitnou vodu je poměrně nízká [17]. EU stanovila maximální přípustný obsah akrylamidu v pitných vodách na 0,1 $\mu g.l^{-1}$ [18].

Evropský úřad pro bezpečnost potravin (EFSA) vydal v roce 2015 k otázce přítomnosti akrylamidu v potravinách vědecké stanovisko, které potvrdilo, že akrylamid představuje kvůli neurotoxickým a potenciálně karcinogenním účinkům zdravotní riziko [19].

2.1.1 Mechanismus vzniku akrylamidu

Akrylamid v potravinách vzniká v průběhu Maillardovy reakce a jeho prekurzory jsou redukující cukry a aminokyselina asparagin (Obr. 1) [20].



Obr. 1: Mechanismus tvorby akrylamidu z asparaginu v přítomnosti α-hydroxy karbonylových sloučenin [20].

Reakční mechanismus vzniku akrylamidu v potravinách závisí na složení potravin a na podmínkách jejich zpracování [21; 22; 23; 24]. Za hlavní mechanismus vzniku akrylamidu jsou považovány reakce mezi volnou kódovanou neesenciální aminokyselinou asparaginem a redukujícími cukry při teplotách nad 120 °C, kdy vzniká celá řada velmi reaktivních karbonylových sloučenin [21; 7; 25; 26; 27].

Rozeznávají se tři fáze reakcí vedoucích k tvorbě akrylamidu [28; 25]:

- Počáteční fáze zahrnuje tvorbu glykosylaminu, následovanou Amadoriho přesmykem
- Ve střední fázi dochází k dehydrataci a fragmentaci sacharidů doprovázející Streckerovu degradaci aminokyselin.
- V závěrečné fázi prochází Streckerův aldehyd dalšími redukčními a dehydratačními reakcemi za vzniku akrylamidu.

Vedle těchto možností bylo na modelových systémech prokázáno, že za určitých podmínek se na tvorbě akrylamidu podílí i akrolein a kyselina akrylová po reakci s asparaginem v potravinách bohatých na lipidy [25].

Při zkoumání modelového systému směsi glukózy a asparaginu byl zjištěn vliv teploty a také doby zahřívání. Tvorba akrylamidu byla nízká v teplotním rozsahu 120 až 140 °C, pokud se zvýšila na 160 až 180 °C, obsah akrylamidu se v matrici dramaticky zvýšil. Po dosažení teploty 180 °C dochází k následnému poklesu tvorby akrylamidu. Pokles tvorby akrylamidu při vyšších teplotách lze vysvětlit tím, že akrylamid jako meziprodukt Maillardovy reakce dále reaguje za vzniku jiných sloučenin [26].

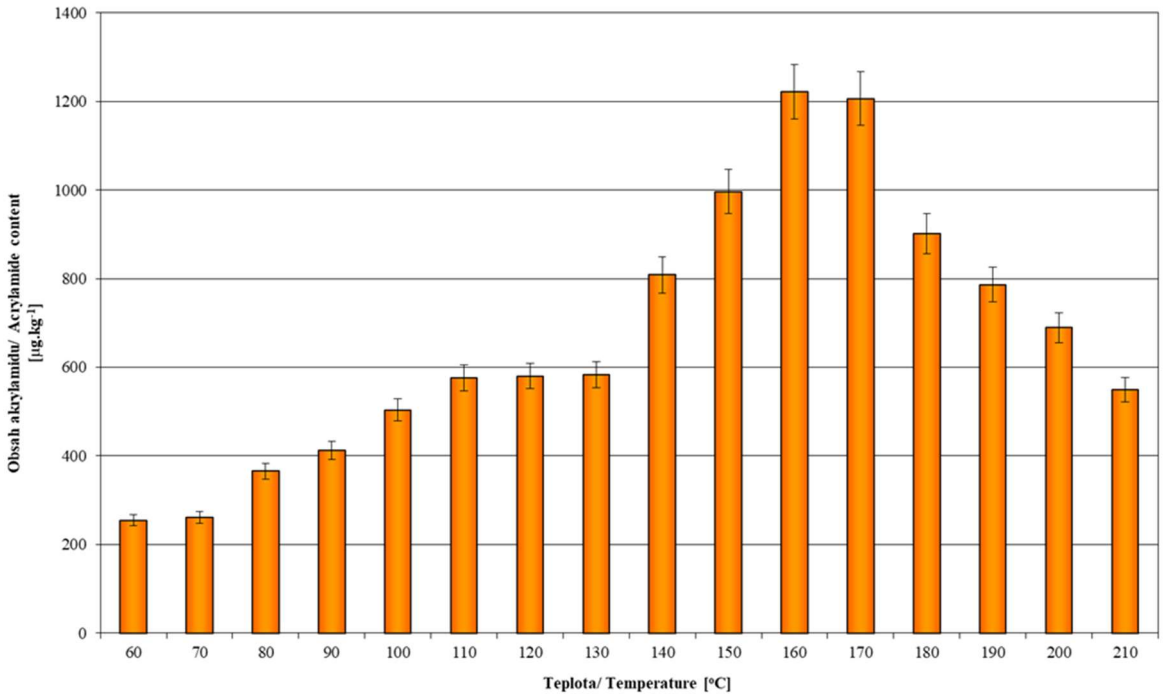
Surovinou pro výrobu sladu jsou obilky ječmene, které obsahují především škrob a dusíkaté sloučeniny. V průběhu sladování se v ječmeni působením enzymů zvyšuje obsah redukujících cukrů, během hvozdění dochází vlivem teploty k biochemickým změnám a vznikají melanoidinové látky [29]. To vytváří předpoklady pro tvorbu akrylamidu. Vzhledem k těmto skutečnostem jsme sledovali závislost tvorby akrylamidu na teplotě hvozdění a obsahy akrylamidu ve speciálních sladech, které se praží při vyšších teplotách. Naše poznatky byly popsány v publikaci[1,2] uvedené v **Příloze č.1**. Závislost tvorby akrylamidu na teplotě ukazuje Obr. 2. Výsledky prokázaly, že k tvorbě akrylamidu dochází již při teplotách od 60 °C. O vzniku akrylamidu při nízkých teplotách rozhoduje doba jejich působení. Slad se předsouší až 10 hodin při teplotách od 55 až 65 °C, poté následuje vyhřátí na dotahovací teplotu, která je pro daný druh sladu specifická (80 až 225 °C) trvající od 1,5 hodiny do 2,5 hodiny. Maximální tvorba akrylamidu byla zjištěna v teplotním intervalu 150 až 170 °C. Při teplotách nad 170 °C dochází k poklesu tvorby akrylamidu. Snížení tvorby akrylamidu při vyšších teplotách lze zdůvodnit tím, že akrylamid jako meziprodukt Maillardovy reakce dále reaguje za vzniku dalších sloučenin reakcí neenzymatického hnědnutí [28; 25].

[1] MIKULÍKOVÁ, Renata, Klara SOBOTOVA. Determination of acrylamide in malt with GC/MS. *ACTA CHIMICA SLOVENICA*. 2007, **54**(1), 98-101. ISSN 1318-0207.

[2] KELLNER, Vladimír, Renata MIKULÍKOVÁ a Pavel ČEJKA. 15 Nežádoucí a zdraví škodlivé látky sladu. BASAŘOVÁ, Gabriela, ed. *Sladařství: teorie a praxe výroby sladu*. Praha: Havlíček Brain Team, 2015, s. 395-432. ISBN 978-80-87109-47-2.

Vyšší obsahy akrylamidu byly zjištěny ve speciálních a barevných sladech (450–1700 μg/kg), což souvisí s vyšší teplotou hvozdění při jejich výrobě (105–220 °C).

Obsah akrylamidu v analyzovaných vzorcích piva byl pod mezí detekce použité GC/MS metody (< 25 μg/l)[3].



Obr. 2: Závislost tvorby akrylamidu na teplotě hvozdění (pražení) sladu[4,5].

2.1.2 Výskyt akrylamidu v potravinách a surovinách a možnosti jeho stanovení

Ke vzniku akrylamidu v potravinách včetně piva a pivovarských surovin dochází během jejich tepelného zpracování, a to především při smažení, grilování, pečení nebo fritování surovin bohatých na škroby. Při této tepelné úpravě dochází i ke vzniku důležitých žádoucích senzoricky aktivních sloučenin, které dodávají produktům charakteristické zbarvení, chuť a vůni [30]. Akrylamid neobsahují syrové nebo vařené potraviny [31].

Z potravin mají největší obsahy akrylamidu hlavně brambory zpracovávané při vysoké teplotě pečením, smažením nebo pražením. Mezi takto tepelně upravené výrobky patří především bramborové hranolky, bramborové lupínky (chipsy), pečené brambory apod. Také

[3] MIKULÍKOVÁ, Renata, Zdeněk SVOBODA, Sylva BĚLÁKOVÁ a Simona MACUCHOVÁ. Monitoring of acrylamide in the course of malting and in beer. *Kvasny Prumysl*. 2008, **54**(6), 181-185. ISSN 00235830. Dostupné z: doi:10.18832/kp2008011

[4]MIKULÍKOVÁ, Renata, Klara SOBOTOVA. Determination of acrylamide in malt with GC/MS. *ACTA CHIMICA SLOVENICA*. 2007, **54**(1), 98-101. ISSN 1318-0207.

[5] KELLNER, Vladimír, Renata MIKULÍKOVÁ a Pavel ČEJKA. 15 Nežádoucí a zdraví škodlivé látky sladu. BASAŘOVÁ, Gabriela, ed. *Sladařství: teorie a praxe výroby sladu*. Praha: Havlíček Brain Team, 2015, s. 395-432. ISBN 978-80-87109-47-2.

sladké cereální výrobky (pražené) a obilné výrobky denní spotřeby jako je chleba a pečivo obsahují vyšší hodnoty akrylamidu. Cereální pražené výrobky představují významný zdroj akrylamidu vzhledem k jejich vyšší spotřebě, a to především u dětí a mládeže. Nezanedbatelné jsou obsahy akrylamidu v kávě a kávovinových náhražkách, pernících, krekrech, oplatcích, sušenkách a pražených skořápkových plodech [32; 33; 34; 35; 36; 37]. Překvapivé jsou vysoké nálezy obsahu akrylamidu v sušených švestkách (od 730 do 1680 $\mu g \cdot kg^{-1}$), které detekovali Amrein a kol [38].

Vzhledem ke složitým potravinovým matricím je nutno při stanovení akrylamidu používat spolehlivé a citlivé analytické metody, které jsou schopné stanovit stopová množství akrylamidu. [39; 40; 41]

Nejčastěji používané metody pro stanovení akrylamidu jsou plynová a kapalinová chromatografie ve spojení s hmotnostním detektorem [42; 43; 44; 45; 46].

Obsah akrylamidu může být v některých analyzovaných vzorcích nízký a složité potravinové matrice mohou obsahovat rušící látky, proto je vhodné při jeho stanovení provést pro dosažení vyšší selektivity a nižších detekčních limitů derivatizaci analytu. Známa je derivatizace použitím silylačního činidla N,O-bis(trimethylsilyl)trifluor acetamidu (BSTFA) za vzniku těkavé formy akrylamidu – N,O-bis(trimethylsilyl)akrylamidu (BTMSA) [47]. Další derivatizace, která se používá je reakce akrylamidu s xanhydrolem za vzniku N-xanthyl akrylamidu [48]. Nejznámější a nečastější způsob derivatizace akrylamidu je bromace, která se používá při GC-MS analýze [49]. Výhodou bromace akrylamidu je, že produktem je více těkavá a méně polární sloučenina s lepšími hmotnostními charakteristikami. Výsledný derivát je snáze extrahován z vodných roztoků a může být lépe detekován GC – MS metodou [50]. Konverze akrylamidu na 2,3-dibrompropionamid byla provedena přidáním bezvodého bromidu draselného, kyseliny bromovodíkové a nasyceným roztokem bromové vody. Přidáním trietylaminu byl nestabilní 2,3-dibrompropionamid převeden na více stabilní derivát 2-brompropenamid [51; 52].

Jak již bylo zmíněno, akrylamid byl analyzován ve sladu a pivu a zavedené optimalizované metody byly aplikovány i na analýzu v dalších potravinách a surovinách. Výsledky sledování obsahu akrylamidu ve vzorcích potravin rozdělených do jednotlivých skupin podle složení matrice byly publikovány[6]. Mezi analyzovanými potravinami byly bramborové lupínky, káva, chléb, sušenky, krekry, bramborové hranolky předsmažené a smažené bramborové hranolky. Pro analýzu byla použita metoda GC/MSD, před níž byl akrylamid derivatizován bromací. Přítomnost akrylamidu byla potvrzena ve většině analyzovaných vzorků. Akrylamid nebyl detekován pouze v instantní kávě a v slunečnicovém chlebu.

Obsah akrylamidu v brambůrkách se pohyboval v intervalu 160–1530 $\mu g \cdot kg^{-1}$. Podobné výsledky uvádí i souhrnná zpráva EFSA z let 2007 až 2009 [53] . Doporučení Evropské komise č. 2013/647/ES uvádí směrné hodnoty obsahu akrylamidu v brambůrkách 1000 $\mu g \cdot kg^{-1}$ [54].

[6] SVOBODA, Zdeněk, Renata MIKULÍKOVÁ, Olga CWIKOVÁ, Sylvie BĚLÁKOVÁ a Karolína BENEŠOVÁ. Monitoring of Acrylamide Content in Selected Foods. *Kvasny Prumysl*. 2015, **61**(7-8), 206-211. ISSN 00235830. Dostupné z: doi:10.18832/kp2015021

V mleté kávě se hodnoty akrylamidu pohybovaly v rozmezí od 240 μg·kg^{-1} do 358 μg·kg^{-1}, což odpovídá výsledkům kontrolního měření provedeného SZPI během let 2005–2011, kdy pražené mleté kávy obsahovaly akrylamid v množství od 87 do 460 μg·kg^{-1}. Tyto hodnoty potvrdil i Andrzejewski et al. [55] ve své studii, kdy v mleté kávě zjistil koncentrace akrylamidu v rozmezí od 40 do 400 μg·kg^{-1}. Nejnižší obsah akrylamidu (240 μg·kg^{-1}) obsahovala 100% Arabica, což může být způsobeno nižším obsahem volného asparaginu než v kávě typu Robusta [20]. V instantní kávě nebyl akrylamid detekován. Doporučení Evropské komise č. 2013/647/ES uvádí směrné hodnoty obsahu akrylamidu v pražené kávě 450 μg·kg^{-1} [54].

Koncentrace akrylamidu ve vzorcích chleba byla stanovena v rozmezí 78–125 μg·kg^{-1}. Chléb obsahuje akrylamid především ve své kůrce a jeho obsah závisí zejména na druhu a stupni vymletí mouky [56], fermentaci, době a teplotě kvašení, použití kyseliny citronové nebo mléčné, době a teplotě pečení [57]. Množství akrylamidu v chlebu se obecně pohybuje od 15 až do 161 μg.kg^{-1} (v silně propečeném pšeničném chlebu). Žitný chléb upečený za stejných podmínek obsahoval vyšší koncentraci akrylamidu, a to 68–205 μg.kg^{-1} [56; 57]. Doporučení Evropské komise č. 2013/647/ES uvádí směrné hodnoty obsahu akrylamidu v pšeničném chlebu 80 μg·kg^{-1} a v jiném než pšeničném chlebu 150 μg·kg^{-1} [54].

Vzorky sušenek vykazovaly nízký až střední obsah akrylamidu. Koncentrace akrylamidu v sušenkách se pohybovala v rozmezí 100–259 μg·kg^{-1}. Konings et al. [57] ve své práci naměřili nižší koncentraci akrylamidu v sušenkách 44–121 μg·kg^{-1}, naproti tomu Taeymans et al. [8] naměřili výrazně vyšší obsah akrylamidu v sušenkách (170–560 μg·kg^{-1}) v porovnání s našimi výsledky. Doporučení Evropské komise č. 2013/647/ES uvádí směrné hodnoty obsahu akrylamidu pro sušenky 500 μg·kg^{-1} [54].

Obsah akrylamidu ve vzorcích krekrů se pohyboval v rozmezí 118–470 μg·kg^{-1}. Obdobné výsledky jsou uvedeny v závěrečné zprávě SZPI z monitorování akrylamidu v krekrech z let 2008–2010, kdy byl zjištěn obsah akrylamidu v intervalu 94–715 μg·kg^{-1}. Doporučení Evropské komise č. 2013/647/ES uvádí směrné hodnoty obsahu akrylamidu pro krekry jiné než bramborové 500 μg·kg^{-1} [54].

Obsah akrylamidu v předsmažených bramborových hranolcích se pohyboval v intervalu 66–137 μg·kg^{-1}. Nízké koncentrace akrylamidu v předsmažených produktech potvrzují i závěrečné kontrolní zprávy SZPI z let 2007 a 2010, které uvádějí koncentraci akrylamidu v rozmezí 56–220 μg·kg^{-1}. Pro porovnání a zjištění, jaké je množství akrylamidu v hranolcích předsmažených a po usmažení, byly dva vzorky podrobeny tepelné úpravě, kdy byly po dobu 10 minut při teplotě 180 ºC smaženy ve slunečnicovém oleji. Po tepelné úpravě se koncentrace akrylamidu v hranolcích zvýšila více než desetkrát. Vzorek, který před smažením vykazoval množství akrylamidu pouze 137 μg·kg^{-1}, obsahoval po fritování 1588 μg·kg^{-1} akrylamidu. Stejný efekt byl zaznamenán i u druhého vzorku, kde se původní koncentrace 101 μg·kg^{-1} akrylamidu zvýšila po smažení na hodnotu 1583 μg·kg^{-1}. Výsledky experimentu se smažením hranolků podporují snahu o aplikaci technologických postupů v potravinářském průmyslu minimalizující tvorbu akrylamidu, které ve své práci uvádí např. Sanny et al. [58]. Doporučení Evropské komise č. 2013/647/ES uvádí směrné hodnoty obsahu akrylamidu pro hranolky k přímé spotřebě 600 μg·kg^{-1} [54]. Přítomnost akrylamidu byla potvrzena ve většině analyzovaných vzorků.

2.1.3 Možnosti eliminace akrylamidu

Vzhledem k možným nežádoucím účinkům akrylamidu na lidské zdraví je usilováno o snížení jeho množství v potravinách, a to především výběrem vhodných surovin a úpravou technologických postupů výroby potravin [58; 59; 60]. Mezi základní doporučení pro snížení obsahu akrylamidu patří agronomické zásahy, kterými lze omezit obsah redukujících cukrů a aminokyseliny asparaginu ve výchozí surovině [61]. Obsah asparaginu v mouce a obsah škrobu v bramborách a obilovinách (včetně ječmene) závisí nejen na odrůdě, ale i na klimaticko-půdních podmínkách pěstování. Někteří autoři ve svých studiích uvádějí vliv odrůdy, hnojení a zavlažování na snižování obsahu akrylamidu [62; 63]. V současnosti je výzkum zaměřený i na šlechtění obilnin a brambor se sníženým obsahem prekurzorů vzniku akrylamidu [64]. Vzhledem k významu odrůdy ječmene ve vztahu k obsahu prekursorů vzniku akrylamidu byla hledána optimální rychlá metoda pro stanovení odrůdové čistoty sladovnického ječmene. Výsledky porovnání metod jsou uvedeny v publikaci[7] v **Příloze č. 2.**

Nejdůležitějším faktorem z hlediska obsahu akrylamidu v potravinách je finální úprava potravin, kdy se doporučuje při tepelném zpracování potravin používání nižších teplot po delší dobu [26; 65]. Rychlost tvorby akrylamidu po dobu pečení cereálních výrobků závisí na obsahu vody v těstě a na zvoleném teploně-časovém profilu zpracování [66]. Vhodné je nastavit podmínky zpracování tak, aby při požadované konečné vlhkosti byl výrobek méně propečený [67; 68].

Dalším a neméně důležitým krokem je vhodná úprava technologického postupu výroby potravin a úprava receptury. Zde má velký význam především změna pH roztoků používaných při blanšírování [69; 70], anebo přídavek jiných látek (např. glycin, asparaginasa) [38; 61; 57; 56; 71; 72].

Snížení obsahu uhličitanu amonného, který se používá jako běžná kypřící látka při přípravě perníků, protože zabezpečí charakteristický vzhled, texturu a aróma výrobků, vede ke snížení obsahu akrylamidu. Hydrogenuhličitan amonný je výrazným akcelerátorem vzniku akrylamidu, pro to je vhodné ho částečně nahradit sodnými nebo draselnými alternativami. Mechanismus účinku hydrogenuhličitanu amonného je založený na zvýšené tvorbě sacharidových fragmentů (glyoxalu a methylglyoxalu), které rychleji reagují s asparaginem. Ve vzorcích průmyslově vyrobených perníků byl stanoven výrazný pokles akrylamidu, ale výsledný produkt měl netypicky světlou barvu a výraznou slanou chuť [73].

Na základě těchto informací jsme zjišťovali závislost obsahu aditiv na organoleptických vlastnostech vyrobených perníků. Kombinací aditiv byl snížen obsah akrylamidu ve

[7] ŠEDO, Ondrej, Michal KOŘÁN, Michaela JAKEŠOVÁ, Renata MIKULÍKOVÁ, Michal BOHÁČ a Zbyněk ZDRÁHAL. Rapid assignment of malting barley varieties by matrix-assisted laser desorption–ionisation – Time-of-flight mass spectrometry. *Food Chemistry*. 2016, **206**, 124-130. ISSN 03088146. Dostupné z: doi:10.1016/j.foodchem.2016.03.056

vyrobených perníčcích pod mez detekce, aniž by došlo k výraznému snížení senzorických parametrů. Naše poznatky byly shrnuty a uvedeny v publikaci[8] v **Příloze č. 3**.

Výsledné množství akrylamidu v potravinách je možné ovlivňovat v jednotlivých stádiích jejich technologického zpracování. Eliminace akrylamidu je ovlivněna mnoha faktory. Mezi základní faktory patří výběr vhodné suroviny a uplatnění správných agronomických postupů, kterými lze omezit obsah redukujících cukrů a aminokyseliny asparaginu ve vstupní surovině.

Na základě Nařízení komise (EU) 2017/2158 ze dne 20. listopadu 2017, kterým se stanoví zmírňující opatření a porovnávací hodnoty pro snížení akrylamidu v potravinách musí provozovatelé potravinářských podniků, kteří vyrábějí a uvádějí na trh bramborové hranolky, bramborové lupínky, chléb, cereálie, jemné pečivo, kávu, náhražky kávy, potraviny pro malé děti a obilné příkrmy pro kojence uplatnit zmírňující opatření za účelem snížení množství akrylamidu na co nejnižší rozumně dosažitelnou míru [74]. Opatření ke snížení obsahu akrylamidu jsou uplatňována i v průběhu technologie výroby sladu a piva. Jedná se hlavně o regulaci teploty a doby hvozdění sladu.

2.2 3-chlorpropan-1,2 diol (3-MCPD)

3-chlorpropan-1,2-diol (3-MCPD) je bezbarvá kapalina s příjemnou vůní snadno rozpustná ve vodě a etanolu s bodem varu 213 °C. Její relativní molekulová hmotnost je 110,54 $g \cdot mol^{-1}$. Patří do skupiny chlorhydrinů glycerolu nebo také chlorpropanolů. Mezi nejčastěji se vyskytující chlorpropanoly patří kromě 3-MCPD také 1,3-dichlor-2-propanol (1,3-DCP). Nalezeny byly ale i 2-chlor-1,3-propandiol (2-MCPD) a 2,3-dichlor-1-propanol (2,3-DCP). Dále byly v potravinách objeveny monoestery a diestery chlorpropanolů, kde jsou jedna nebo dvě hydroxylové skupiny acylovány zbytkem mastné kyseliny [75; 76].

3-MCPD je považován za potencionální karcinogen s mutagenními, chemosterilačními a nefrologickými účinky. 3-MCPD je na základě studií zařazen do 2A skupiny, jako potenciální lidský karcinogen. EFSA v roce 2001 stanovila maximální denní příjem (ADI) na 2 $\mu g \cdot kg^{-1}$ tělesné hmotnosti [77; 78].

V současné době existuje jediný legislativní limit, který stanovuje maximální hodnoty pro množství 3-MCPD v kyselých hydrolyzátech bílkovin a v sójových omáčkách 20 $\mu g \cdot kg^{-1}$ výrobku s 40 % sušiny (Nařízení komise (ES) č. 1881/2006). Evropskou komisí však bylo vydáno doporučení komise EU č. 2014/661 o monitorování hladin 2- a 3-MCPD a jejich esterů v potravinách, což dokládá závažnost dané problematiky nejen v ČR, ale i v rámci EU.

2.2.1 Výskyt 3-MCPD v potravinách

Chlorpropanoly vznikají v potravinách, které současně obsahují vyšší obsah tuků a soli, mají nízkou aktivitu vody a jsou vystavené působení vyšších teplot [79; 80]. Prekurzorem pro vznik

[8] KOMPRDA, Tomáš, Antonin PRIDAL, Renata MIKULÍKOVÁ, Zdeněk SVOBODA, Olga CWIKOVÁ, Šárka NEDOMOVÁ a Vladimír SÝKORA. A combination of additives can synergically decrease acrylamide content in gingerbread without compromising sensory quality. *Journal of the Science of Food and Agriculture*. 2017, **97**(3), 889-895. ISSN 00225142. Dostupné z: doi:10.1002/jsfa.7811

chlorpropanolů v potravinách jsou tuky. Především to jsou tři lipidické složky: triacylglyceroly, fosfolipidy a glycerol. Nezbytná je přítomnost chloridů, které jsou v podobě soli přítomné téměř v každé potravině [81]. Primárními produkty reakcí mezi těmito složkami jsou diestery MCPD, které se hydrolyzují na monoestery MCPD. Reakcí esterů MCPD s chloridy vznikají estery DCP a volné chlorpropanoly následně vznikají hydrolýzou esterů [82; 83].

Přítomnost 3-MCPD ve velké škále zpracovaných potravin byl poprvé popsán v roce 2004 [84]. Největší množství 3-MCPD bylo nalezeno v bílkovinných hydrolyzátech připravených kyselou hydrolýzou, tedy ve výrobcích jako sójová nebo ústřicová omáčka [85; 86]. Dále byl stanoven i v širokém spektru dalších potravin jako jsou sušenky, pekařské výrobky, káva, výrobky z masa a ryb, polévky, uzené výrobky a v některých tradičních přísadách jako jsou speciální tmavé slady, upravené škroby a extrakty masa. V menším množství se chlorpropanoly nacházejí i v tavených a grilovaných sýrech a fermentovaných salámech [87; 88; 89; 90; 91; 92; 93].

2.2.2 Sledování 3-MCPD ve sladu

V souvislosti s výš uvedenými fakty byl sledován obsah 3-MCPD i ve sladech a tyto výsledky byly publikovány[9]. K analýze sladů byla použita metoda GC/MSD, před níž byl 3-MCPD derivatizován kyselinou fenylborovou [94; 95; 96].

Obsah 3-MCPD ve sladech se pohyboval v intervalu <10,0–95,0 $\mu g \cdot kg^{-1}$. Ve světlých plzeňských sladech, sladech mnichovských a ve sladech pšeničných světlých byl obsah 3-MCPD pod mezí stanovení. Tyto hodnoty jsou podstatně nižší, než uvádí literatura [97] a tato skutečnost je pravděpodobně způsobena odlišnou technologií sladování. V karamelových světlých sladech byly hodnoty 3-MCPD v intervalu <10,0– 5,0 $\mu g \cdot kg^{-1}$. Což jsou hodnoty, které nepřekračují stanovený maximální limit pro množství 3-MCPD v kyselých hydrolyzátech bílkovin a v sójových omáčkách, který je 20 $\mu g \cdot kg^{-1}$ výrobku s 40 % sušiny [98]. Nejvyšší hodnoty 3-MCPD byly u barvicích sladů 55,0–95,0 $\mu g \cdot kg^{-1}$ a korespondují s výsledky uváděnými v literatuře [97]. Vznik 3-MCPD při pražení a snížení jeho obsahu v barvicích sladech lze minimalizovat výběrem vhodných podmínek v procesu pražení sladu.

Vzhledem k tomu, že ředění sladu při výrobě finálního produktu piva se pohybuje od 1:10 (tj. 1 kg sladu na 10 kg produktu) do 1:100 [99], nepředstavuje obsah 3-MCPD ve světlých a barvicích sladech potenciální nebezpečí pro spotřebitele.

[9] MIKULÍKOVÁ, Renata, Zdeněk SVOBODA, Karolína BENEŠOVÁ a Sylvie BĚLÁKOVÁ. 3-MCPD Process Contaminant in Malt. *Kvasny Prumysl*. 2018, **64**(1), 6-9. ISSN 00235830. Dostupné z: doi:10.18832/kp201802

3 Mykotoxiny

Mykotoxiny, produkty sekundárního metabolismu mikroskopických vláknitých a některých dalších hub, jsou látky nebílkovinné povahy, toxické pro člověka a další živé organismy [100].

Mykotoxiny jsou strukturně rozmanité komplexní organické sloučeniny o nízké molekulové hmotnosti (až na výjimky nižší než 700 $g.mol^{-1}$). Mykotoxiny jsou sekundární metabolity vláknitých hub a ve srovnání s aminokyselinami, lipidy, nukleovými kyselinami nebo proteiny nejsou nezbytné pro růst a vývoj mikrobiálních producentů (proto název sekundární metabolity). V současné době je známo přes 300 mykotoxinů [101].

Patří mezi významné toxiny přírodního původu, které mohou kontaminovat široké spektrum potravin a krmiv [102], a vyvolat různé toxické syndromy nazývané souhrnně mykotoxikosy [103; 104]. Nejstaršími popsanými mykotoxikosami jsou ergotismus, alimentární toxická aleukie (ATA) a onemocnění z tzv. červené rýže [105]. Toxické vlastnosti mykotoxinů způsobují u lidí i zvířat závažné nefrotoxické, neurotoxické, kancerogenní, imunosupresivní a estrogenní účinky [100].

Vyskytují se v zemědělských plodinách, především obilovinách (pšenice, ječmen, žito, oves), rýži, kukuřici, olejnatých semenech (mák, řepka, hořčice, sojové boby, slunečnice) a v potravinářských výrobcích obsahujících tyto suroviny [106; 107]. Mezi hlavní faktory, které ovlivňují rozsah případné kontaminace zemědělských plodin mykotoxiny v polních podmínkách, patří vlastní rezistence kultivarů vůči napadení mikroskopickými vláknitými houbami, míra fyziologického stresu, kterému jsou vystaveny rostliny (nedostatečná nebo neharmonická výživa, nedostatek či nadbytek vláhy, zasolení půdy, znečištění ovzduší, napadení jinými ostatními chorobami a živočišnými škůdci, atd.), virulence patogenní houby, typ produkovaného mykotoxinu, schopnost rostliny degradovat mykotoxiny, dobrá zemědělská praxe (osevní postup zejména předplodina, typ orby, hnojení) a interval mezi sklizní a obdobím vhodným pro produkci mykotoxinů [108; 109; 110].

Významným producentem mykotoxinů jsou mimo jiné houby rodu *Fusarium*, které jsou současně významnými patogeny zemědělských plodin. U obilnin napadají paty stébel, listy a klasy. V posledním období dochází ke zvýšeným výskytům fusarióz v klasech obilnin [111]. Houbami rodu *Fusarium* je napadán, zejména za vhodných podmínek pro šíření, také ječmen[10,11]. Výskyt fusarióz i mykotoxinů je významně ovlivňován průběhem počasí [112; 113]. Vzhledem k tomu, že počasí je v jednotlivých letech velmi variabilní je i napadení fusariózami velmi rozdílné. U sladovnického ječmene výskyt mykotoxinů může významně ovlivnit posklizňová úprava zrna i způsob skladování. Dalším faktorem, který může významně ovlivnit intenzitu výskytu fusárií je technologie pěstování a náchylnost odrůdy [114; 115]. Pokud jde o agrotechnická opatření, jsou důležité především předplodina a způsob zpracování

[10] BENEŠOVÁ, Karolína, Renata MIKULÍKOVÁ, Sylvie BĚLÁKOVÁ, Zdeněk SVOBODA a Vratislav PSOTA. Identification of substances originating from pathogen - caryopsis interaction and their effect on malt quality. *Kvasny Prumysl*. 2011, **57**(1), 2-7. ISSN 00235830. Dostupné z: doi:10.18832/kp2011001

[11] BENEŠOVÁ, Karolína, Vratislav PSOTA, Renata MIKULÍKOVÁ, Sylvie BĚLÁKOVÁ a Zdeněk SVOBODA. Pathogenic metabolites in barley caryopses and their effect on quality of malting barley and malt. *Kvasny Prumysl*. 2011, **57**(7-8), 215-218. ISSN 00235830. Dostupné z: doi:10.18832/kp2011021

půdy [109; 116]. Výskyt fusarióz může významně ovlivnit také použití fungicidů. Aplikace fungicidu může snížit napadení, ale současně vést ke stresu patogenu a tak může dojít ke zvýšení produkce mykotoxinů [109]. Vzhledem k tomu, že účinnost stávajících fungicidů není vždy uspokojivá, jsou neustále hledány nové postupy, kterými by bylo možno výskyt fusarióz omezit.

Na základě těchto skutečností byl proveden v letech 2012–2017 monitoring obsahu fusariových mykotoxinů (DON, ZON, T-2 a HT-2 toxin) u 592 vzorků zrna sladovnického ječmene imunochemickou metodou ELISA. Obsah mykotoxinů nepřekročil maximální limit EU pro nezpracované obiloviny [98]. Výsledky této dlouhodobé studie byly publikovány[12] a jsou uvedeny v **Příloze č. 4** .

Některé druhy rodu *Fusarium* jsou producenty vysoce toxických mykotoxinů, především deoxynivalenolu (DON), nivalenolu (NIV), HT-2, T-2 toxinu a zearalenonu (ZON) [117; 118]. V poslední době se také hodně diskutuje o významném metabolitu deoxynivalenolu-3-glukosidu (DON-3-Glc) [119; 120] . Fusariové mykotoxiny jsou relativně stabilní sloučeniny a ve většině případů nepodléhají významné degradaci v průběhu běžných technologických operací a mohou přecházet do výsledných produktů určených k lidské spotřebě. Toxiny navíc pronikají difuzí z povrchově rostoucí plísně do vnitřní části potravin a surovin a oblast jejich produkce překrývá značnou část růstové křivky plísně.

Významnými producenty mykotoxinů jsou i některé další druhy hub, které se příležitostně vyskytují na obilovinách. Např. *Aspergillus flavus*, *A. parasiticus* a *A. nomius* produkují zejména aflatoxiny. Ochratoxin A je nejčastěji produkován vláknitými houbami rodu *Asperigillus* a *Penicillium*.

3.1 Trichotecenové mykotoxiny

Skupina trichothecenových mykotoxinů patří mezi nejznámější toxiny produkované převážně vláknitými houbami rodu *Fusarium*. Produkce trichothecenů byla prokázána i u některých hub rodu *Myrothecium, Trichoderma, Trichothecium, Cylinrocarpon, Phomopsis, Verticimonosporium* a *Stachybotrytys*.

Kontaminace organismu trichotheceny má za následek širokou paletu projevů [121]. Může docházet k rozličným syndromům, například ke snižování příjmu nebo totálnímu odmítání potravy, k podráždění kůže a k dermálním nekrózám, zvracení, průjmům a krvácivosti. Trichotheceny jsou popsány i jako imunosupresory a inhibitory syntézy proteinů a DNA. Bez výjimky všechny trichotheceny vykazují pro živočichy větší či menší stupeň toxicity, vykazují i insekticidní efekt. Byla popsána i fytotoxická aktivita. Toxicita je vysvětlována především přítomností epoxyskupiny [122]. Podle charakteristických chemických vlastností se rozlišují čtyři podskupiny trichothecenů, a to trichotheceny typu A, B, C a D.

[12] SVOBODA, Zdeněk, Renata MIKULÍKOVÁ, Karolína BENEŠOVÁ a Sylvie BĚLÁKOVÁ. The occurrence of the selected Fusarium mycotoxins in Czech malting barley, harvested in 2012–2017. *Czech Journal of Food Sciences*. 2019, **37**(6), 439-445. ISSN 12121800. Dostupné z: doi:10.17221/317/2018-CJFS

3.1.1 Trichotecenové toxiny T-2 a HT-2 toxin

Patří mezi trichotheceny typu A, které obecně vykazují vyšší akutní toxicitu než trichoteceny typu B. T-2 toxin byl izolován v roce 1968 z kultury *Fusarium sporotrichioides*. Je považován za jednoho z nejpravděpodobnějších původců mykotoxikózy – alimentární toxické aleukie. V obilninách se běžně vyskytují toxiny T-2 a HT-2 současně, jelikož jeden přechází v druhý. HT-2 je deacetylovaná forma T-2 toxinu [123]. T-2 a HT-2 se vyskytují v obilninách (pšenice, oves, ječmen), v kukuřici, rýži, fazolích a sóji, dále také v některých cereálních výrobcích. T-2 a HT-2 toxiny se obvykle nenacházejí v zrnu obilnin při sklizni, ale objeví se až v důsledku nadměrné vlhkosti v průběhu skladování [123].

T-2 toxin má dermatotoxické (způsobující nekrózu kůže) a emetické (způsobující zvracení nebo dávení) účinky, byly popsány také účinky imunosupresivní, genotoxické a na základě prováděných experimentů se zvířaty se T-2 považuje za potenciální karcinogen a mutagenní sloučeninu [123].

3.1.2 Deoxynivalenol

Patří k trichothecenům typu B. V roce 1973 byl v USA izolován deoxynivalenol z kukuřice napadené mikromycetou *Fusarium graminearum*. Při zkrmování deoxynivalenolem (DON) kontaminované kukuřice bylo u prasat pozorováno zvracení (vomitus). Na základě toho byl odvozen triviální název tohoto mykotoxinu – vomitoxin [124]. DON byl nalezen v následujících potravinách: obilniny (ječmen, pšenice, triticale, proso, žito) a výrobky z nich, dětská výživa z obilnin, různé výrobky z kukuřice, rýže, proso, čirok, otruby, chleba, špagety, müsli, nudle, pivo, chili prášek, koriandr, zázvor, sojové boby, česnek, brambory. DON je velmi stabilní a jeho koncentrace se nemění ani po technologickém zpracování vstupní suroviny do finálního výrobku. V krmivech se nacházejí poměrně vysoké koncentrace deoxynivalenolu, což souvisí s obsahem lepku a vlhkostí zrna. Přenos deoxynivalenolu na dojnice podáváním kontaminovaného krmiva je možný, ale v mléce jsou nálezy DON extrémně nízké (< 4 mg.l^{-1}). Přechod reziduí DON do masa, mléka nebo vajec je také zanedbatelný. Při toxikologickém hodnocení byly u zvířat prokázány kožní změny, gastrointestinální onemocnění, hematologické změny, imunosupresivní a teratogenní účinky [125]. DON je z hlediska praktického výskytu trichothecenů považován za hlavní kontaminant potravin. Je indikátorem možné kontaminace dalšími trichothecenovými mykotoxiny. DON se často vyskytuje v obilovinách společně s nivalenolem, diacetoxyscirpenolem a T-2 toxinem. Jde však naštěstí o jeden z nejméně toxických trichothecenů [125]. DON je prekursorem 3-acetyldeoxynivalenolu (3-ADON) a 15-acetyldeoxynivalenolu (15-ADON) a spolu s ním jsou někdy detekovány v obilninách [124].

3.1.3 Deoxynivalenol – 3 glukosid (DON-3-glc)

V poslední době jsou často diskutovány i tzv. maskované mykotoxiny. Jedná se o konjugované mykotoxiny, ve kterých se toxin obvykle váže na polárnější látku, například glukosu [126]. Jednou z těchto látek je konjugát DON-3-β-D-glukopyranosid (DON-3-glucosid), významný metabolit DON [127]. Některé studie ukázaly, že u fermentovaných výrobků je možné pozorovat významný nárůst hladin některých konjugátů mykotoxinů, přestože u výchozí suroviny nebyla prokázána významná kontaminace [128; 129]. Stále

diskutovanou otázkou zůstává i toxicita těchto látek. V trávicím traktu člověka, a obdobně je tomu prvděpodobně i u hospodářských zvířat, může docházet k uvolňování mykotoxinů z vazeb na komponenty matrice a de facto tak k navýšení toxické zátěže organismu [130].

3.1.4 Nivalenol (NIV)

Patří k trichothecenům typu B. Tento přirozeně se vyskytující toxin je produkován toxinogenními kmeny rodu *Fusarium (F. sporotrichioides*) a byl poprvé izolován z *F. nivale* [125]. V posledních letech je stále častěji detekován v různých obilovinách [107]. IARC (International Agency for Research on Cancer) v r. 1993 došla k závěru, že existují dostatečné důkazy karcinogenních účinků nivalenolu na pokusných zvířatech. U lidí nebyly k dispozici žádné údaje. Celkový závěr byl, že stupeň kancerogenity nebyl určen (skupina 3). U myší má NIV embryotoxický a ferotoxický účinek, teratogenní účinek nebyl prokázán. NIV vykazuje antivirový a imunotoxický účinek. V závislosti na dávce a expozici může mít účinek imunosupresivní a imunostimulační [125]. NIV je jedním z nejméně akutně toxických trichothecenů, na buněčné úrovni je jeho hlavním toxickým účinkem inhibice proteosyntézy prostřednictvím vazby na ribozom.

3.2 Zearalenon

Mezi nejvýznamnějšími producenty zearalenonu náleží *Fusarium graminearum* a *F. semitectum*. Zearalenon byl nalezen v následujících potravinách: obilniny a výrobky z nich (včetně ječmene, sladu a piva), kukuřice, rýže, čirok, proso, boby, ořechy, banány a v některých druzích koření. Jedná se o relativně lipofilní sloučeninu. Obsah zearalenonu často při technologickém zpracování obilovin významně klesá. Akutní toxicita zearalenonu je nízká, nicméně jeho příjem stravou může vyvolat (díky strukturní podobnosti se steroidními hormony estrogeny) hyperestrogenní syndrom. S ohledem na své estrogenní účinky se zearalenon někdy označuje jako mykoestrogen. Je pokládán za vhodný indikátor přítomnosti dalších fusariových mykotoxinů v cereáliích, jako jsou např. trichotheceny DON a nivalenol [125]. U zearalenonu byla prokázána hepatotoxicita, hematotoxicita, imunotoxicita a genotoxicita. Není významně akutně toxický, spolu se svými deriváty však vykazuje významné estrogenní a anabolické účinky [125].

3.3 Ochratoxiny

Ochratoxin A (OTA) je produkován zejména vláknitými mikroskopickými houbami rodu *Aspergilus* (*A. ochraceus, A. melleus*) a *Penicillium* (*P. verrucosum, P. nordicum*). Z chemického hlediska patří OTA do skupiny ochratoxinů. Lze ho obecně charakterizovat jako derivát 7-izokumarinu vázaný na aminoskupinu L-β-fenylalaninu [131].

Ochratoxin A má nefrotoxické, mutagenní, teratogenní, kancerogenní a neurotoxické účinky. Patří mezi významné neurotoxické mykotoxiny a je spojován s nádory ledvin a nádorovým onemocněním varlat [132; 133].

V potravinách byl OTA poprvé stanoven ve víně a hroznové šťávě [134]. Výskyt ochratoxinu A a koncentrace ve vínech jsou vysoce ovlivněny zeměpisnými a klimatickými podmínkami. Vyšší obsahy OTA a vyšší počet kontaminovaných vzorků byly opakovaně zjištěny ve vínech ze Středomoří a dalších jižních vinařských oblastí [135]. Na základě těchto informací jsme provedli stanovení obsahu ochratoxinu A ve vzorcích vína z moravské vinařské oblasti a v zahraničních vínech. Výsledky potvrdily předchozí závěry, že riziko kontaminace vína ochratoxinem A je v jihomoravské vinařské oblasti velmi nízké. Tato studie byla publikována[13] a je uvedena v **Příloze č. 5.**

Ochratoxin A se může také nacházet v různých potravinách rostlinného [136] a živočišného původu, mimo jiné v obilninách a výrobcích z nich – ječmen, slad, pivo. Přítomnost OTA v pivu závisí na kontaminaci pivovarských materiálů, tj. sladovnického ječmene a sladu, mikromycety *Penicillium verrucosum* sp. *a Aspergillus ochraceus* sp. Byl sledován obsah ochratoxinu A v pivovarských surovinách a v pivu, výsledky byly publikovány[14] a jsou uvedeny v **Příloze č**. **6.**

3.4 Aflatoxiny

Aflatoxiny jsou produkovány zejména vláknitými mikroskopickými houbami rodu *Aspergilus* (zejména *A. flavus* a *A. parasiticus*). Nejvýznamnější aflatoxiny jsou B1, B2, G1, G2 a M1 [137]. Jsou toxické a aflatoxiny skupiny B a G patří mezi prokázané kancerogeny [131]. V těle se metabolizují v játrech na reaktivní meziprodukt, epoxid aflatoxin M1. Vyskytují se v mnoha potravinách rostlinného a živočišného původu, jako jsou zejména ořechy, koření, mléko, sýry, apod [138].

Vzhledem k vysoké toxicitě aflatoxinů a ke skutečnosti, že mohou z přirozeně kontaminovaných surovin přecházet do piva, byl proveden monitoring výskytu aflatoxinů B_1, B_2, G_1 a G_2 v pivovarských surovinách a v pivu. Na základě této studie jsme došli k závěru, že piva vařená z kvalitních a dobře skladovaných surovin nepředstavují pro spotřebitele žádné zdravotní riziko expozice aflatoxinů. Výsledky byly publikovány[15] a publikace je uvedena v **Příloze č. 7.**

[13] MIKULÍKOVÁ, Renata, Sylvie BĚLÁKOVÁ, Karolína BENEŠOVÁ a Zdeněk SVOBODA. Study of ochratoxin A content in South Moravian and foreign wines by the UPLC method with fluorescence detection. *Food Chemistry*. 2012, **133**(1), 55-59. ISSN 03088146. Dostupné z: doi:10.1016/j.foodchem.2011.12.061

[14] BĚLÁKOVÁ, Sylvie, Karolína BENEŠOVÁ, Renata MIKULÍKOVÁ a Zdeněk SVOBODA. Determination of ochratoxin A in brewing materials and beer by ultra performance liquid chromatography with fluorescence detection. *Food Chemistry*. 2011, **126**(1), 321-325. ISSN 03088146. Dostupné z: doi:10.1016/j.foodchem.2010.10.062

[15] BENEŠOVÁ, Karolína, Sylvie BĚLÁKOVÁ, Renata MIKULÍKOVÁ a Zdeněk SVOBODA. Monitoring of selected aflatoxins in brewing materials and beer by liquid chromatography/mass spectrometry. *Food Control*. 2012, **25**(2), 626-630. ISSN 09567135. Dostupné z: doi:10.1016/j.foodcont.2011.11.033

3.5 Metody stanovení mykotoxinů

Analýza mykotoxinů je prováděna různými metodami v závislosti na vybavení laboratoří, které stanovení provádějí. Převážně se jedná o stanovení vysokoúčinou kapalinovou chromatografií s hmotnostní detekcí. Tato metoda byla použita k analýze většiny námi sledovaných mykotoxinů. Validace a optimalizace metody byla publikována[16] a je uvedena v **Příloze č.8**. Dále je možné mykotoxiny stanovovat metodami imunochemické enzymové analýzy (např. ELISA) a radioimunologickými, která jsou spíše vhodnější pro screeningová (orientační) měření [100; 139].

3.6 Význam mykotoxinů v průběhu výroby sladu a piva

Mykotoxiny představují určité bezpečnostní riziko pro pivovarský průmysl[17] [128]. Při sladování kontaminovaného ječmene vznikají výhodné podmínky pro rozvoj toxinogenních hub [140]. Během máčení, klíčení i hvozdění jsou houby stále schopny růstu a tvoření mykotoxinů [140]. Výroba sladu – řízeného klíčení obilných zrn - je komplexní biologický proces, který zahrnuje širokou škálu biochemických a fyziologických reakcí. Výměna máčecí vody sice odstraňuje část mykotoxinů, ale při klíčení může docházet opět k jejich nárůstu [140; 141]. Při hvozdění dochází k poklesu obsahu mykotoxinů, ale ne k jejich vymizení [142; 143; 128]. Houby včetně spór se z infikovaného ječmene a sladu do piva dostat nemohou, nejpozději ve stádiu rmutování jsou usmrceny. Část mykotoxinů díky své tepelné stabilitě tímto procesem projde beze změn [144; 140] a může přejít až do finálního produktu – piva [140].

Jak však vyplývá z výše uvedených výsledků, hodnoty koncentrace mykotoxinů v pivu jsou natolik nízké, že představují pouze minimální riziko pro konzumenty. To však neznamená, že by neměla být věnována stálá pozornost minimalizaci mikrobiální kontaminace ječmene a sladu vláknitými houbami.

[16]BOLECHOVÁ, Martina, Karolína BENEŠOVÁ, Sylvie BĚLÁKOVÁ, Josef ČÁSLAVSKÝ, Markéta POSPÍCHALOVÁ a Renata MIKULÍKOVÁ. Determination of seventeen mycotoxins in barley and malt in the Czech Republic. *Food Control*. 2015, **47**, 108-113. ISSN 09567135. Dostupné z: doi:10.1016/j.foodcont.2014.06.045

[17] KELLNER, Vladimír, Renata MIKULÍKOVÁ a Pavel ČEJKA. 15 Nežádoucí a zdraví škodlivé látky sladu. BASAŘOVÁ, Gabriela, ed. *Sladařství: teorie a praxe výroby sladu*. Praha: Havlíček Brain Team, 2015, s. 395-432. ISBN 978-80-87109-47-2.

4 Senzoricky aktivní látky a jejich prekursory

Mezi senzoricky aktivními látkami ovlivňujícími zásadně kvalitu piva hrají významnou úlohu heterocyklické a sirné sloučeniny, z nichž některé se vyznačují vysokou senzorickou aktivitou i v extrémně nízkých koncentracích. Stopová množství těchto sloučenin, které lze běžně nalézt v potravinách, se spolupodílejí na vytváření jejich aroma a tento vliv lze obecně hodnotit jako příznivý. U sladu, resp. u piva to však platí jen ve velmi omezené míře a přítomnost heterocyklických a sirných látek se v tomto směru hodnotí spíše nepříznivě.

4.1 Sirné látky

Sirné sloučeniny se do piva dostávají buď s výchozími surovinami (slad, chmel), nebo vznikají v průběhu chemických či enzymových reakcí během jednotlivých etap výroby (rmutování, vaření, fermentace, stárnutí). V ječmeni a chmelu může obsah sirných sloučenin záviset nejen na odrůdě, ale i na pěstebním místě, průběhu počasí a použité technologii pěstování. U sladu pak závisí obsah sirných látek především na technologii sladování a eventuální kontaminaci nežádoucími mikroorganismy [145; 146].

Většina sirných sloučenin přítomných v ječmeni, sladu a pivu jsou netěkavé látky (aminokyseliny, bílkoviny, anorganické sírany). Tyto látky nejsou přímo odpovědné za nepříznivé vůně a chuti piva, ale jsou důležité jako prekursory, ze kterých za určitých podmínek mohou vznikat senzoricky aktivní látky. Takto vzniklé sloučeniny jsou ve většině případů těkavé a jejich množství bývá nižší než 1 % z celkového množství látek, které mají ve své molekule síru a jsou obsaženy v pivu. Skutečná množství látek odpovědných za sirné vůně jsou proto extrémně nízká [147; 99].

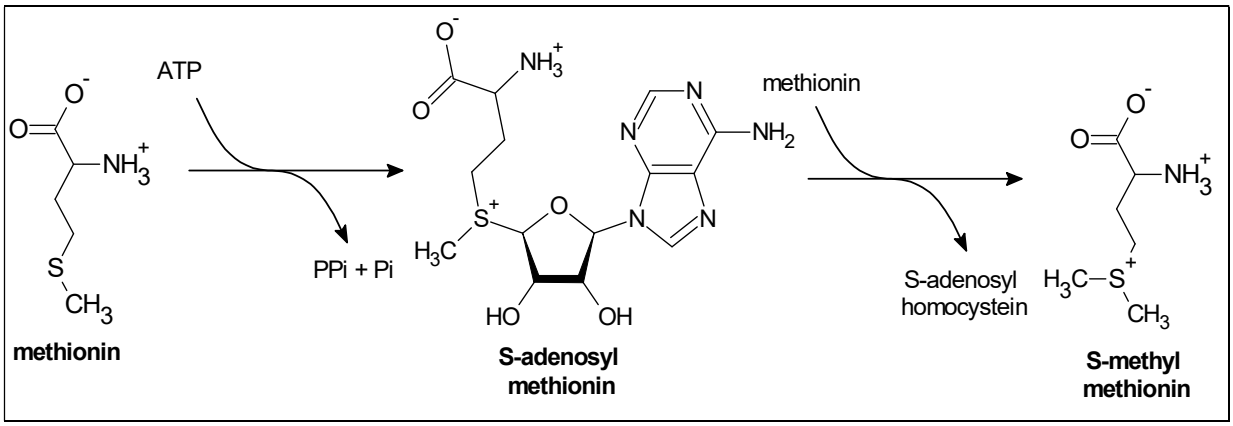
Sirné aminokyseliny jsou přirozenou součástí ječmene, sladu i piva, kde působí jako prekursory těkavých sirných látek. Tyto látky pak mají nezanedbatelnou roli v senzorické kvalitě piva. Těkavé sirné látky mohou nepříznivě ovlivnit chuť piva i ve velmi nízkých koncentracích. Proto je nutné znát nejen obsah jejich prekursorů, ale i možnosti jejich vzniku v průběhu technologie výroby piva.

Vlastní síra se do výchozí suroviny, kterou je ječmen, dostává během vegetace ve formě síranů obsažených v řadě minerálních hnojiv nebo po jejich přeměně i z organických hnojiv, ale také ve formě plynné jako SO_2 [148; 149].

Prvním stupněm asimilace síry je aktivace síranového iontu pomocí ATP-sulfurylasy (EC 2.7.7.4), což vede ke tvorbě adenosin-5'-fosfosulfátu (APS) a pyrofosfátu. Sekundární fosforylací APS (adenylylsulfátkinasa, EC 2.7.1.25) vzniká 3'-fosfoadenosin-5'-fosfosulfát (PAPS). Aktivovaný sulfát z APS, stejně jako z PAPS je přenášen pomocí APS- nebo PAPS-sulfotransferasy (EC 2.8.2.16) do thiolové skupiny přenašeče a vlastní redukce sulfátu na sulfit pak může probíhat vázanou nebo volnou cestou. Sulfity jsou dále redukovány na sulfidy sulfitreduktasou (EC 1.8.99.1) nebo thiosulfatreduktasou (EC 2.8.1.3) [148; 149].

Redukovaná síra vstupuje do metabolismu výhradně ve formě cysteinu a teprve následnou syntézou vzniká další esenciální aminokyselina methionin. Uvedené aminokyseliny jsou prekursory dalších sirných sloučenin [148; 149].

Mezi hlavní meziprodukty vzniku senzoricky aktivních sirných látek během výroby piva patří S-methylmethionin, který vzniká metylací methioninu v cyklu sirných aminokyselin (Obr. 3) [148; 150; 151; 152; 149].



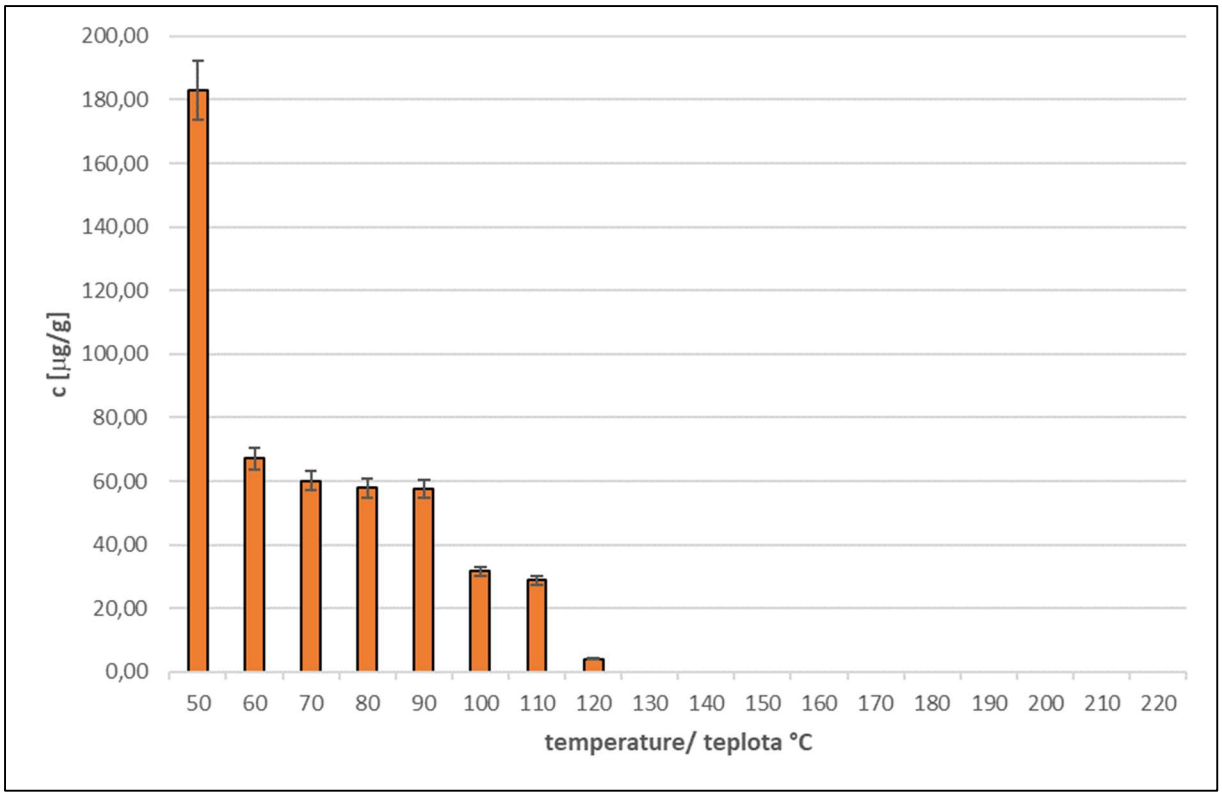
Obr. 3: Vznik S-methylmethioninu [152]

Slady sladované (pražené) při teplotách 50 °C až 220 °C vykazují snižující se koncentraci methioninu se vzrůstající teplotou. Tato závislost je způsobena degradací methioninu na dimethylsulfid. Ten ze sladu uniká a je sledován jako těkavý meziprodukt. V rámci předložené práce byl DMS sledován a výsledky byly publikovány[18,19]. Prokázanou závislost obsahu metioninu na teplotě (Obr.4) lze využít při optimalizaci teploty hvozdění, s cílem snížit obsah metioninu ve vyráběných sladech.

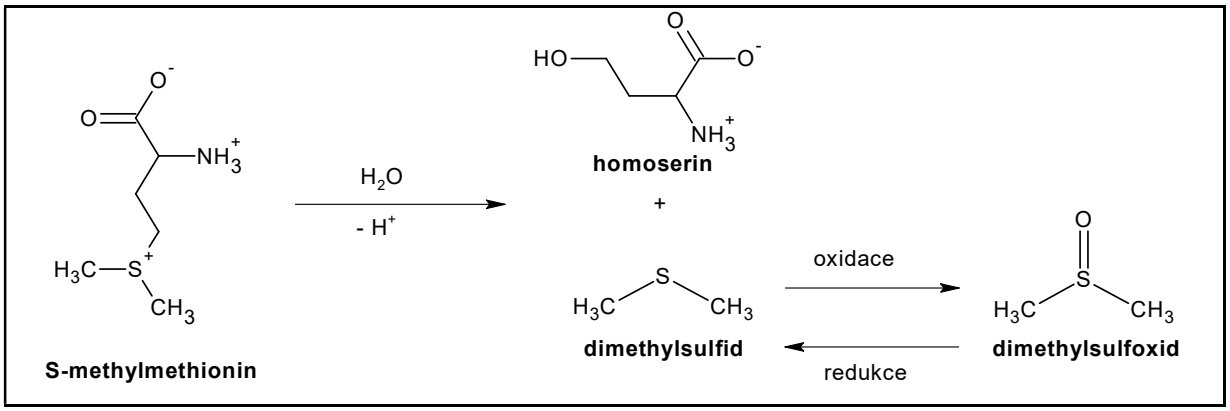
Během hvozdění sladu, když teplota přesáhne 60 °C, je S-methylmethionin degradován na homoserin a dimethylsulfid, takže nezanedbatelná část může být ztracena stržením do plynu. Degradace a syntéza S-methylmethioninu je závislá na vlhkosti a teplotě zrna. Oxidací uvolněného dimethysulfidu vzniká dimethylsulfoxid (Obr.5). Rychlost oxidace roste s teplotou hvozdění. Rozsáhlejší oxidace dimethylsulfidu vede ke vzniku dimethylsulfonu (methylsulfonylmethan – MSM), který není kvasinkami metabolizován [149].

[18] MIKULÍKOVÁ, Renata, Zdeněk SVOBODA, Karolína BENEŠOVÁ a Sylvie BĚLÁKOVÁ. Determination of methionine in malt. *Kvasny Prumysl*. 2009, **55**(11-12), 310-314. ISSN 00235830. Dostupné z: doi:10.18832/kp2009025

[19] KELLNER, Vladimír, Renata MIKULÍKOVÁ a Pavel ČEJKA. 15 Nežádoucí a zdraví škodlivé látky sladu. BASAŘOVÁ, Gabriela, ed. *Sladařství: teorie a praxe výroby sladu*. Praha: Havlíček Brain Team, 2015, s. 395-432. ISBN 978-80-87109-47-2.



Obr. 4: Závislost obsahu methioninu na teplotě hvozdění (pražení) sladu[20]



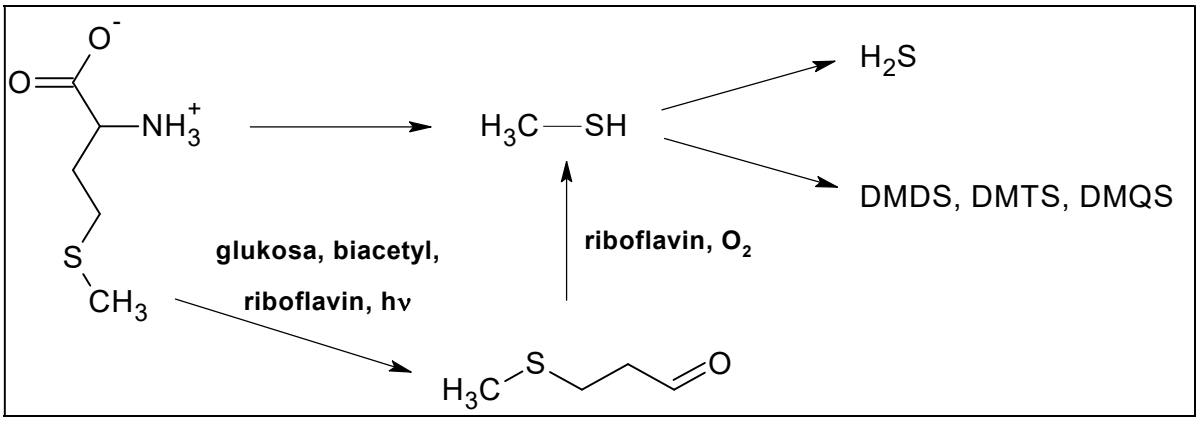
Obr. 5: Vzájemná závislost cest vzniku dimethylsulfidu v pivu [99]

Další cesta vzniku dimethylsulfidu zahrnuje rozklad methioninu vzájemnou reakcí s redukujícími cukry. Hlavním produktem této degradace je methional nebo od něj odvozený methionol. Dalšími dvěma produkty jsou dimethylsulfid a dimethylsulfoxid. Rozkladem methionalu může vznikat ehylmethylsulfid. Termickým rozkladem cysteinu a cystinu vzniká sulfan [99; 146].

[20] MIKULÍKOVÁ, Renata, Zdeněk SVOBODA, Karolína BENEŠOVÁ a Sylvie BĚLÁKOVÁ. Determination of methionine in malt. *Kvasny Prumysl*. 2009, **55**(11-12), 310-314. ISSN 00235830. Dostupné z: doi:10.18832/kp2009025

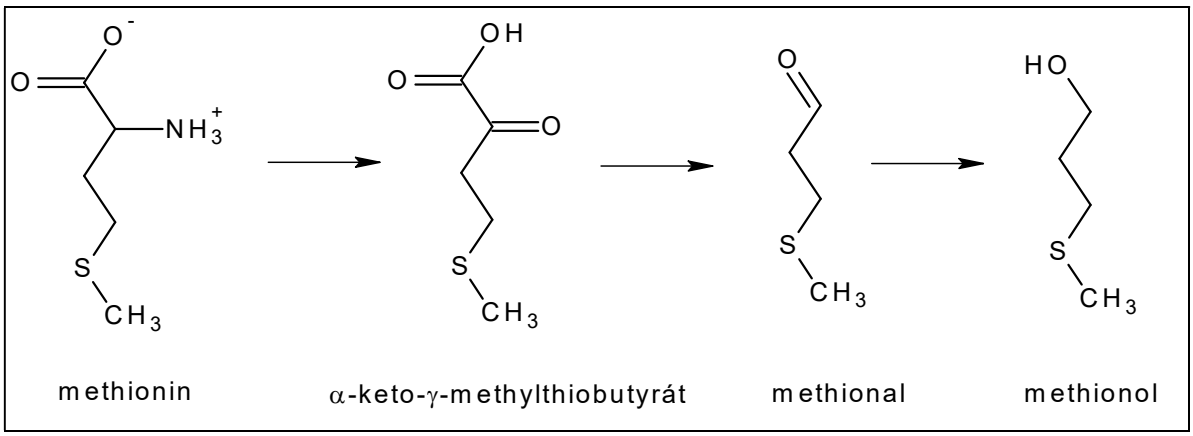
Během rmutování přechází S-methylmethionin, vzniklý při sladování, do roztoku, kde probíhá jeho rozklad. Vznikající dimethylsulfid je strháván vroucími parami. Rychlost vypařování dimethylsulfidu je v této fázi rychlejší než jeho syntéza. Po vaření dochází v chladnoucí mladině stále k degradaci S-methylmethioninu, ale již nedochází k odpařování dimethylsulfidu [146].

Během výroby piva mohu vznikat také různé alkylpolysulfidy. Prekursory těchto alkylpolysulfidů jsou trioly, S-alkylcystein sulfoxid, methional, thiosulfináty a methionin – sulfoxid (Obr.6) [99; 146].

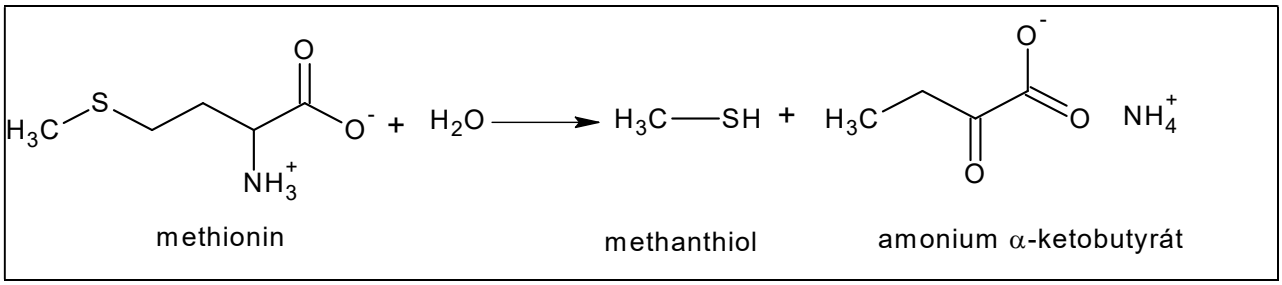


Obr. 6: Možný mechanismus sysntézy polysulfidů z methioninu během rmutování sladu [146]

Během fermentace piva jsou pivovarské kvasinky (*Saccharomyces cerevisiae a S. pastorianus*) schopné metabolizovat sirné aminokyseliny na jednodušší sloučeniny, mimo jiné i na těkavé sirné látky [153].



(a)



(b)

Obr. 7: Pravděpodobná cesta degradace methioninu pivovarskými kvasinkami (Saccharomyces cerevisiae a S. pastorianus): (a) Ehrlichova dráha; (b) enzym methionin γ lyasa (EC 4.4.1.11) [146]

Při kvašení mladiny jsou pivovarské kvasinky schopné metabolizovat S-methylmethionin, ale nejsou schopny ho degradovat na dimethylsulfid. Pravděpodobně ho přeměňují na methionin účinkem methyltransferasy (EC 2.1.1.12), který dále metabolizují (Obr. 7) [146]. Během fermentace pivovarské kvasinky produkují sulfan, a to převážně z cysteinu, v menší míře ze síranů a zanedbatelně z methioninu.

Pivovarské kvasinky jsou schopny během kvašení mladiny produkovat dimethylsulfid rovněž enzymatickou redukcí dimethylsulfoxidu. Tato produkce dimethylsulfidu je nicméně kompenzována jeho ztrátou strháváním s CO_2. Produkovány mohou být i polysulfidy, jako dimethyldisulfid a dimethyltrisulfid [154].

Pivovarské kvasinky během života přijímají organickou i anorganickou síru z prostředí, aby ji použily v metabolismu aminokyselin. Prekursory metabolizovány pivovarskými kvasinkami jsou sírany, siřičitany, methionin, cystein, cystin, thiamin a glutathion [154].

V ležáckých pivech se nachází dimethyltrisulfid, který vzniká degradací methionalu a methionolu v čerstvém pivu. Methional pochází především ze Streckerovy eliminace methioninu při sladování ječmene [155; 156].

4.2 Stanovení sirných látek

Stanovení sirných senzoricky aktivních látek je velmi náročné, protože se nacházejí v analyzovaných matricích (slad, pivo) ve velmi nízkých koncentracích ($\mu g.kg^{-1}$ – $ng.kg^{-1}$). Před vlastní analýzou je třeba analyty extrahovat z matrice a zakoncentrovat. Pro zakoncentrování těkavých látek se používá destilace s vodní parou, headspace metody (extrakce plynem) a mikroextrakce tuhou fází (SPME) [157; 158; 159; 160]. K analýze sirných těkavých látek bývá nejčastěji využívána plynová chromatografie ve spojení se selektivními detektory (např. plamenový fotometrický detektor) [161; 162].

Těkavé látky lze z kapalných i rozmělněných pevných vzorků izolovat šetrnou extrakcí plynem, tedy s využitím tzv. headspace techniky (HS). Podstatou těchto metod je analýza plynné fáze, která byla v kontaktu s extrahovaným materiálem, v ideálním případě až do ustavení rovnovážné distribuce těkavých látek mezi plynnou a kondenzovanou (kapalnou nebo pevnou) fází, která je popsána distribuční konstantou jednotlivých složek v dané soustavě [163; 164].

Rozlišují se dva způsoby uspořádání headspace analýzy [164]:

- Statická headspace

V tomto uspořádání se analyzuje vzorek plynu odebraný z prostoru nad kondenzovanou fází ve statickém uzavřeném systému.

- Dynamická headspace

Kondenzovaná fáze se kontinuálně extrahuje proudem inertního plynu (tzv. stripování), z něhož jsou vynášené páry těkavých látek vhodným způsobem zachycovány.

Statická HS technika je většinou používaná pro stanovení těkavých látek ve vzorcích, které jsou obtížně analyzovatelné běžným chromatografickým postupem. V kombinaci s plynovou chromatografií (HS-GC) umožní nepřímo stanovit těkavé látky v kapalných nebo pevných matricích analýzou plynné fáze, která je v termodynamické rovnováze se vzorkem v uzavřeném systému. Tato technika je většinou doporučovaná pro kapalné vzorky, jejichž matrice může při přímém nástřiku, poškodit chromatografickou kolonu nebo vyžaduje intenzivní čištění vzorku před analýzou [165].

Metodu HS extrakce ve spojení s plynovou chromatografií s FPD detektorem jsme použili pro stanovení DMS v mladině a pivu. Výsledky stanovení a optimalizace metody byly publikovány[21] . Byla optimalizována doba extrakce v závisloti na teplotě. Nejvyšší výtěžnost HS extrakce byla při 50 °C po dobu 30 min. Tyto podmínky byly použity pro stanovení DMS ve vzorcích mladiny a piva. Obsahy DMS v analyzovaných mladinách se pohybovaly od 9,6 do 59,6 $\mu g.l^{-1}$, v pivech od 6,1 do 34,9 $\mu g.l^{-1}$ což koreluje s výsledky uváděnými v literatuře [166].

Obsahy volného DMS ve sladu se pohybují v rozmezí 1–20 $mg.kg^{-1}$, hladiny volného DMS v pivu a mladině jsou řádově nižší pohybují se v rozmezí 2–100 μg^{-1} [99].

Dynamická HS našla široké uplatnění při izolaci a zakoncentrování těkavých látek z kapaných matric [167; 168; 169].

V prvním kroku této metody jsou analyzované látky stripovány z kapalné fáze a mohou být zachyceny třemi způsoby: a) sorpcí na termální sorbent, b) vymražováním c) kryofokusací na začátku chromatografické kolony [164].

Ze sloupce sorbentu jsou analyzované látky uvolňovány extrakcí organickým rozpouštědlem nebo záhřevem za průchodu nosného plynu vedoucího přímo do kolony plynového chromatografu (termická desorpce) [164; 170]. Pro zakoncentrování sirných těkavých látek se používají různé typy sorbentů. Např. pro zachycení thiolů a disulfidů z kapalných matric se používá Carbopack X, Porapak Q a Tenax GC. Molekulární síto 5A a Tenax GC byly popsány pro záchyt dimethylsulfidu, dimethyldisulfidu, sirouhlíku, sirovodíku [171; 172; 173; 174; 175; 176].

[21] SVOBODA, Zdeněk, Renata MIKULÍKOVÁ, Sylvie BĚLÁKOVÁ a Karolína BENEŠOVÁ. Optimization of Determination of Dimethyl Sulfide in Wort and Beer. *Kvasny Prumysl*. 2017, **63**(3), 121-125. ISSN 00235830. Dostupné z: doi:10.18832/kp201714

V posledních letech se stává často používanou technikou mikroextrakce tuhou fází (SPME). SPME je bezrozpouštědlová metoda přípravy vzorku. Tuto metodu vyvinul v polovině 90. let minulého století J. Pawliszyn na universitě Waterloo Ontario, Kanada. Metoda nevyžaduje složitou instrumentaci a je založena na sorpci analytu malým množstvím extrakční fáze na povrchu křemenného vlákna. Analyty se sorbují do dosažení rovnovážného stavu. Množství extrahovaného analytu závisí na hodnotě rozdělovacího koeficientu analyt – vlákno [177; 178].

Křemenné vlákno pokryté polymerem je spojeno s ocelovým pístem a umístěno v duté ocelové jehle. Vlákno je zataženo dovnitř jehly, která propíchne septum v zátce vialky [179].

Vlákno se vysune přímo do vzorku (DI-SPME) nebo do prostoru nad jeho hladinou (HS–SPME). Analyt se sorbuje na povrch vlákna. Po dosažení rovnováhy (2-30 min) se vlákno zasune opět do jehly, která je pak zavedena do injektoru plynového chromatografu, kde je vlákno opětovně vysunuto a po tepelné desorpci je analyt z povrchu vlákna unášen nosným plynem na GC kolonu [178; 179].

Při použití metody SPME, množství extrahovaného analytu závisí, jednak na polaritě a tloušťce vlákna, jednak také na extrakčním čase a koncentraci analytu ve vzorku. Účinnost extrakce lze zvýšit mícháním, přídavkem soli do vzorku, změnou pH nebo teploty [178].

Existuje několik typů vláken vhodných pro extrakci těkavých látek. Afinita vlákna vůči analytu závisí na polaritě stacionární fáze a na vlastnostech daného analytu. Například nepolární polydimethylsiloxane (PDMS) vlákno je preferováno pro extrakci nepolárních analytů, kterými jsou i některé těkavé aromatické látky. Více polární polyakrylátové (PA) je vhodnější pro více polární látky, jako jsou fenoly nebo alkoholy. Smíšená vlákna, obsahující divinylbenzen (DVB) nebo Carboxen (CAR), zvyšují retenční kapacitu [179]. Pro analýzu těkavých aromatických látek mohou být použita i kombinovaná vlákna jako PDMS/DVB, CAR/PDMS, CW/PDMS.

Přestože SPME má maximální citlivost při dosažení rovnováhy, není úplná rovnováha nezbytnou podmínkou pro dosažení přesných a správných výsledků analýzy, a to vhledem k lineárnímu vztahu mezi analytem adsorbovaným na SPME vlákno a jeho vnitřní koncentrací ve vzorku za nerovnovážných podmínek. Přičemž nikdy není dosaženo rovnovážného stavu pro všechny analyty ve vzorku. Většinou je doba extrakce kompromis mezi dobou analýzy a požadovaným detekčním limitem. [178; 179]

Extrakční teplota řídí difúzi analytu na povrch vlákna. Zvýšení teploty má za následek zvýšení difúze a extrakce analytu, ale zároveň snížení hodnoty distribuční konstanty. Pro přesné a správné výsledky, je výběr extrakční teploty v souladu s ostatními parametry SPME klíčovým problémem.

V publikaci[22] byly experimentálně porovnávány automatizované analytické metody SPDE (dynamická mikroextrakce na pevnou fázi) a TDAS (automatizovaná termická desorpce). Pro vlastní analýzu sirných těkavých látek byla použita plynová chromatografie ve spojení s plamenofotometrickým detektorem. Byly sledovány tyto těkavé sirné látky: dimethylsulfid,

[22] MIKULÍKOVÁ, Renata, Zdeněk SVOBODA, Karolína BENEŠOVÁ a Sylvie BĚLÁKOVÁ. Use of modern analytical SPDE and TDAS methods for the analysis of sulphur volatile flavors. *Kvasny Prumysl*. 2011, **57**(7-8), 231-235. ISSN 00235830. Dostupné z: doi:10.18832/kp2011024

dimethyldisulfid, dimethyltrisulfid, sirouhlík, ethylsulfid, diethyldisulfid, methionol, 3-methylthiofen, ethylthioacetát, 2-methyl-1-buthanthiol.

Pro metodu HS-SPDE byly testovány dva typy jehel s různými stacionárními fázemi (PDMS a kombinovaná stacionární fáze PDMS/aktivní uhlí). I když obě stacionární fáze jsou vhodné pro stanovení těkavých látek, měla větší výtěžnost pro stanovované analyty kombinovaná stacionární fáze PDMS/AU. U dynamické head-space metody byly testovány dva typy sorbentů – Tenax TA a Carbotrap. Vhodnější byl sorbent Tenax TA. Jako nejvhodnější metoda pro stanovení sirných těkavých látek je na základě dosažených výsledků metoda dynamické head-space ve spojení TDAS se sorbentem Tenax TA. Metoda SPDE má velmi dobrou výtěžnost pro sledované sirné látky, ale musí být ovládána autosamplerem. Tento způsob extrakce není možno provádět ručně, protože by se při ruční manipulaci nedosáhlo potřebné opakovatelnosti. V každém případě je tato metoda robustnější než metoda SPME.

Pro analýzu sirných těkavých látek bývá nejčastěji využívána plynová chromatografie ve spojení se selektivními detektory [180; 157]. Selektivní detektory jsou obzvláště výhodné při analýzách rozmanitých sirných látek ve složitých matricích. Tyto detektory mohou redukovat časově náročné čištění vzorků, které může být příčinou znečištění vzorků dalšími kontaminanty, nebo dokonce ztráty stanovovaných analytů. Stále nejvíce používaným detektorem pro analýzu sirných látek je plamenový fotometrický detektor. Tento detektor sice vykazuje nelineární (exponenciální) odezvu na sirné látky v závislosti na jejich koncentraci, ale je poměrně levný, robusní a postačující pro mnoho aplikací [161; 162]. Alternativou k plamenově fotometrickému detektoru je pulzní plamenově fotometrický detektor (PFPD). Detektor využívá zdroje plynu plamene v dávkování tak, aby plamen nebyl trvalý. Pulzní zapalování je viditelné a plamen sám zhasíná. Tento cyklus se opakuje 2–4krát za sekundu. Selektivita je zabezpečena vhodným filtrem a časovou dimenzitou [157].

Kombinace plynové chromatografie s hmotnostní spektrometrií je vhodná při identifikaci těkavých sirných látek v různých matricích.

Pro analýzu těkavých sirných látek metodou plynové chromatografie se používají kolony s polární stacionární fází polyethylenglykolu nebo s mírně polární fází 5 % - phenyl-95 % - dimethylpolysiloxanu [181; 182; 183].

Poznatky získané při optimalizaci techniky SPME byly využity k analýzám vín a ovocných destilátů a byly publikovány[23,24] viz **Příloha č.9**.

[23] MIKULÍKOVÁ, Renata, Jan GOLIÁŠ, Vladimíra MRÁZOVÁ. SPME-GC-MS analysis of volatile compounds in Czech white wines from five grape varieties. *Mitteilungen Klosterneuburg.* 2009, 59 (3), pp. 159-165, ISSN 0007-5922.

[24]WINTEROVÁ, R., R. MIKULÍKOVÁ, J. MAZÁČ a P. HAVELEC. Assessment of the authenticity of fruit spirits by gas chromatography and stable isotope ratio analyses. *Czech Journal of Food Sciences*. 2008, **26**(5), 368-375. ISSN 12121800. Dostupné z: doi:10.17221/1610-CJFS

4.3 Lipidy

Obilky ječmene obsahují dva typy lipidů: zásobní a funkční. Zásobní lipidy, speciálně triacylglyceroly slouží jako zásobárna energie při mobilizaci specifických enzymů při poškození, nákaze a dalších stresujících faktorech nebo při klíčení. Když jsou zrna ječmene poškozena nesprávným skladováním nebo jsou vystavena určitým mikroorganismům, může dojít k degradačním reakcím lipidů. Tyto reakce mohou být katalyzovány vlastními endogenními enzymy zrna nebo enzymy mikroorganismů v závislosti na enviromentálních podmínkách nebo poškození. Lipasa (EC 3.1.1.-) a lipoxygenasa (EC 1.13.11.12) jsou dva hlavní enzymy ovlivňující degradaci lipidů v zrnech ječmene [184; 185; 146].

Hydrolýza triglyceridů je katalyzována lipasami, které jsou v zrnu vždy přítomné. Mezi nepříznivé efekty jejich aktivity patří především změny v chuti a aroma potravin, rostoucí acidita olejů a uvolnění nenasycených mastných kyselin, které jsou oxidovány lipoxygenasami [146; 184].

Specifickým substrátem lipoxygenasy je cis,cis-1,4-pentadienová struktura, kterou je možné nalézt u mastných kyselin jako je linolová, linolenová nebo arachidonová kyselina. Lipoxygenasa je degraduje buď na volné kyseliny, triglyceridy nebo methyl- (ethyl-) estery. Primární produkty jsou opticky aktivní cis-trans-konjugované hydroperoxidy. Tyto hydroperoxidy jsou tvořeny radikálovým mechanismem a jsou buď rozloženy, nebo dále oxidovány na sekundární produkty jako jsou alkoholy, kyseliny, ketony nebo aldehydy, které mohou nepříznivě ovlivnit nutriční hodnotu, aroma, chuť a kvalitu potraviny [184; 185].

V uskladněném pivu je základní složkou podílející se na chuti žluklého másla aldehyd trans-2-nonenal[25] [186; 187; 187]. Mechanismus tvorby trans-2-nonenalu v pivu je enzymatická nebo nenezymatická oxidace a oxidace volných mastných kyselin, kde svou roli sehrává právě lipoxygenasa[26] [184].

Jelikož mastné kyseliny obsažené v obilce ječmene a následně ve sladu mohou být zdrojem mnohých senzoricky aktiv1ních látek v pivu, bylo nutné zavést a optimalizovat stanovení tuků a mastných kyselin ve výchozích surovinách. Obsah lipidů byl stanoven pomocí moderní metody extrakce na fluidním loži. Pro analýzu zastoupení mastných kyselin byly porovnávány dvě polární kapilární kolony Supelcowax a SLB-IL 1[27].

Vzhledem k tomu, že aldehyd trans-2-nonenal je základní složkou podílející se na změnách chuti ve skladovaném pivu, byla optimalizována metoda automatické HS-SPME-GC pro

[25] SVOBODA, Zdeněk, Renata MIKULÍKOVÁ, Sylvie BĚLÁKOVÁ, Karolína BENEŠOVÁ, Ivana MÁROVÁ a Zdeněk NESVADBA. Determination of Trans-2-Nonenal in Barley Grain, Malt and Beer. *Kvasny Prumysl*. 2010, **56**(11-12), 428-432. ISSN 00235830. Dostupné z: doi:10.18832/kp2010044

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[27] SVOBODA, Zdeněk, Renata MIKULÍKOVÁ, Sylvie BĚLÁKOVÁ, Karolína BENEŠOVÁ a Zdeněk NESVADBA. Determination of lipid content and fatty acid representation in barley caryopses and malt. *Kvasny Prumysl*. 2009, **55**(11-12), 315-320. ISSN 00235830. Dostupné z: doi:10.18832/kp2009026

stanovení této látky v pivu a pivovarských surovinách. Optimalizace metody byla publikována[28] viz **Příloha č. 10.** Pro extrakci HS-SPME bylo porovnáno pět typů vláken: 100 µm PDMS, 65 µm PDMS/DVB, 85 µm CAR/PDMS, 50/30 µm DVB/CAR/PDMS, 85 µm PA) [188; 189]. Nejvyšší výtěžnosti pro extrakci HS-SPME bylo dosaženo vláknem PDMS/DVB, doba extrakce 20 minut při teplotě 60 °C s přídavkem 1,5 g NaCl. Identifikace trans-2-nonenalu byla provedena metodou HS-SPME-GC-MS, vlastní analýza vzorků byla provedena automatizovanou metodou HS-SPME-GC-FID.

[28] SVOBODA, Zdenek, Renata MIKULÍKOVÁ, Sylvie BĚLÁKOVÁ, Karolína BENEŠOVÁ, Ivana MÁROVÁ a Zdenek NESVADBA. Optimization of Modern Analytical SPME and SPDE Methods for Determination of Trans-2-nonenal in Barley, Malt and Beer. *Chromatographia*. 2011, **73**(S1), 157-161. ISSN 0009-5893. Dostupné z: doi:10.1007/s10337-011-1958-x

5 Alergenní látky

Pro některé spotřebitele mohou představovat určité riziko sladové proteiny. Gluten (lepek – z latinského "lepidlo") je složený protein, který se nachází v potravinách zpracovaných z pšenice a příbuzných druhů, včetně ječmene a žita. Lepek je složen z gliadinů (dříve nazývaných prolaminů) a glutelinů. Jejich triviální názvy vycházející z latinských názvů rostlin. Pro prolaminy se nyní obecně používá název gliadiny (odvozený název od pšeničných prolaminů). Gliadiny pšenice (nazývané gliadiny), žita (nazývané sekaliny), a ječmene (nazývané hordeiny) jsou toxické pro jedince trpící alergií na lepek – tzv. celiakií.

Celiakie patří mezi tzv. autoimunitní onemocnění. Její podstatou je abnormální reakce imunitního systému nemocného jedince na lepek [190]. V jeho střevě vznikají obranné látky namířené proti lepku. Současně poškozují stěnu trávicí trubice (tenkého střeva). Rozvíjí se zánět a poškozená sliznice mění své vlastnosti. Dochází k poruše trávení některých cukrů a nedostatečnému vstřebávání bílkovin, tuků, minerálů a vitamínů. Pro zlepšení kvality života celiatiků se v poslední době rozšiřuje sortiment potravin vhodných pro bezlepkovou dietu. Mezi nové nápoje vhodné pro celiaky patří i bezlepkové pivo. Od 1. ledna 2012 platí nové Nařízení komise (ES) č. 41/2009, které mění označení bezlepkových výrobků. Nařízení uvádí jednotný limit pro bezlepkovou potravinu, a to bez ohledu na to, z jakých surovin byla vyrobena, a to ve výši 20 mg lepku/kg potraviny ve stavu určeném ke spotřebě [191]. Aby se výrobce dostal do této kategorie, musí garantovat plnění podmínek vyhlášky (výrobek musí být laboratorně otestován a na obalu mít označení o bezlepkovosti výrobku).

K analýze obilných proteinů se využívá více metod založených na principu elektroforézy, kapalinové chromatografie i imunochemických reakcí [192; 193]. Při stanovení alergenních účinků prolaminů je nejrozšířenější variantou imunochemický test ELISA. Je to dáno skutečností, že protilátky zaměřené na prokazatelně toxické sekvence gliadinů se v současnosti jeví jako nejracionálnější způsob kontroly potravin z hlediska jejich vhodnosti pro lidi nemocné celiakií.

Pro stanovení toxické sekvence pentapeptidu QQPFP, která se opakovaně vyskytuje v molekulách prolaminu, byl použit test RIDASCREEN® Gliadin competitive. Je to kompetitivní enzymová imunoanalýza pro kvantitativní stanovení peptidových fragmentů prolaminu z pšenice (gliadiny), z rýže (sekaliny) a z ječmene (hordeiny) v pivu, škrobu a škrobovém sirupu. Použitá monoklonální protilátka R5 rozpoznává mezi ostatními toxickou sekvenci QQPFP. Z tohoto důvodu jsme i v naší práci zvolili test ELISA jako základní analytický postup. Výsledky analýz českých a zahraničních piv byly publikovány[29].

Obsahy gliadinu se v českých světlých ležáckých pivech pohybovaly v rozmezí 24,0–108,4 $mg.l^{-1}$, u českých výčepních piv 6,2–108,4 $mg.l^{-1}$. Nejvyšší hodnoty byly naměřeny u českého pšeničného piva 1335,5 $mg.l^{-1}$. Zahraniční piva měla obsahy gliadinu nižší (< 5,0–19,6 $mg.l^{-1}$). Nižší obsahy gliadinu v zahraničních pivech mohou souviset s použitím surogátů (náhražka sladu) při výrobě piva.

[29] MIKULÍKOVÁ, Renata, Zdeněk SVOBODA, Karolína BENEŠOVÁ a Sylvie BĚLÁKOVÁ. Beer and celiac disease. *Kvasny Prumysl*. 2013, **59**(10-11), 321-323. ISSN 00235830. Dostupné z: doi:10.18832/kp2013035

Piva na českém trhu vykazují poměrně široký rozsah koncentrací gliadinu. Při přepočtu na gluten běžně užívaným faktorem 2 jen výjimečně splňují požadavek Codex Alimentarius (mezinárodně platné doporučení Codex Stan 118-1979, čl. 2 Definice a čl.5 Analytické metody, jehož zásady byly plně převzaty do komunitárního Nařízení (ES) č.41/2009/ES z 20.ledna 2009) pro označení „gluten- free“ [191]. Pro označení „gluten- free“ by z analyzovaných českých piv vyhovovalo pouze bezlepkové pivo vyrobené speciální technologií.

6 Závěr

Předložená habilitační práce představuje ucelený soubor výsledků výzkumné činnosti uchazečky v oblasti sledování kontaminujících a škodlivých látek v různých potravinářských matricích, zejména v ječmeni, sladu a pivu pomocí moderních instrumentálních metod.

Většina experimentů byla realizována na VÚPS a.s., pracoviště Sladařský ústav Brno, což je instituce zaměřená na komerční analýzy, ječmene, sladu a některé speciální analýzy piva pro české i zahraniční zákazníky a současně na výzkumnou činnost. Specializované akreditované laboratoře VÚPS, které uchazečka vedla v letech 2001 – 2018 měly jako hlavní náplň vývoj sofistikovaných instrumentálních metod k monitoringu kontaminantů v pivovarských surovinách a finálním produktu – pivu.

Výsledky jsou uspořádány do kapitol podle povahy sledovaných kontaminantů a též s ohledem na technologický postup výroby sladu a piva. V první skupině výsledků byly sledovány procesní kontaminanty – akrylamid a 3-MPCD, které patří k nebezpečným látkám kontaminujícím řadu potravin i potravinářských surovin. Obě látky byly analyzovány ve sladu a pivu a zjištěny ve sladu. Nejvyšší obsahy těchto kontaminantů byly nalezeny ve speciálních a barvicích sladech, které se používají při výrobě speciálních a tmavých piv a také cereálních výrobků. Bylo zjištěno, že obsah těchto kontaminantů je ovlivňován především teplotou a dobou hvozdění. K analýze byly využity metody plynové chromatografie ve spojení s hmotnostním detektorem, použité metody byly validovány a akreditovány.

Další skupinou sledovaných látek kontaminujících suroviny, meziprodukty i finální produkty v průběhu celé technologie piva jsou mykotoxiny. Primárně vznikají v průběhu mikrobiální (plísňové) kontaminace skladovaného ječmene nebo sladu a poněvadž jsou rezistentní a termostabilní, přecházejí až do piva. Mykotoxiny představují určité bezpečnostní riziko pro pivovarský průmysl, proto jsou v pivu i surovinách průběžně monitorovány a je důležité vyvíjet citlivé metody pro jejich stanovení. Výroba sladu z kontaminovaného ječmene nevylučuje kontaminaci vláknitými houbami. Rovněž během máčení, klíčení i hvozdění jsou houby stále schopny růstu a tvoření mykotoxinů. Houby včetně spór se z infikovaného ječmene a sladu do piva dostat nemohou a nejpozději ve stádiu rmutování jsou usmrceny. Část mykotoxinů však díky své tepelné stabilitě tímto procesem projde beze změn a může přejít až do finálního produktu – piva. Jak však vyplývá z výsledků předložené práce, hodnoty koncentrace mykotoxinů v pivu jsou natolik nízké, že představují pouze minimální riziko pro konzumenty. To však neznamená, že by neměla být věnována stálá pozornost minimalizaci mikrobiální kontaminace ječmene a sladu vláknitými houbami.

Další sledovanou skupinou kontaminantů piva a surovin byly senzoricky aktivní heterocyklické a sirné sloučeniny, z nichž některé se vyznačují vysokou senzorickou aktivitou i v extrémně nízkých koncentracích. U sladu, resp. u piva se přítomnost těchto látek hodnotí spíše nepříznivě. Stanovení sirných těkavých látek je metodicky velmi náročné, protože jsou přítomny v nízké koncentraci. K analýze byla v předložené práci vyvinuta metoda HS extrakce ve spojení s plynovou chromatografií s FPD detektorem pro stanovení DMS v mladině a pivu. V rámci práce byly mimo jiné experimentálně porovnávány automatizované analytické metody SPDE (dynamická mikroextrakce na pevnou fázi) a TDAS (automatizovaná termická

desorpce). Byly sledovány tyto těkavé sirné látky: dimethylsulfid, dimethyldisulfid, dimethyltrisulfid, sirouhlík, ethylsulfid, diethyldisulfid, methionol, 3-methylthiofen, ethylthioacetát, 2-methyl-1-buthanthiol. Nejlepších výsledků při stanovení těchto látek bylo dosaženo metodou dynamické head-space ve spojení TDAS se sorbentem Tenax TA.

Z dalších těkavých senzoricky aktivních látek sledovaných v rámci předložené práce je třeba zmínit trans-2-nonenal, který je základní složkou podílející se na změnách chuti ve skladovaném pivu. V práci byla optimalizována metoda automatické HS-SPME-GC pro stanovení této látky v pivu a pivovarských surovinách. Optimalizovanou metodou byl nejvyšší obsah trans-2-nonenalu zjištěn v nealkoholických a nejnižší obsah ve výčepních pivech.

Poslední sledovanou skupinou v pivu a surovinách byly potenciální alergeny. Pro některé spotřebitele mohou představovat určité riziko sladové proteiny. Gluten (lepek) se nachází v potravinách zpracovaných z pšenice a příbuzných druhů, včetně ječmene a žita. V ječmeni patří k lepkovým proteinům hordeiny. V rámci předložené práce byla pro stanovení toxické sekvence pentapeptidu QQPFP využita kompetitivní enzymová imunoanalýza pro kvantitativní stanovení peptidových fragmentů hordeinů z ječmene v pivu. Piva na českém trhu vykazují poměrně široký rozsah koncentrací gliadinu. Při přepočtu na gluten běžně užívaným faktorem 2 česká piva jen výjimečně splňují požadavek Codex Alimentarius pro označení „gluten- free“. Tomuto označení by z analyzovaných českých piv vyhovovalo pouze bezlepkové pivo vyrobené speciální technologií.

Pivovarství patří k historicky i ekonomicky nejvýznamnějším odvětvím potravinářského průmyslu v ČR s významným mezinárodním přesahem. Sledování kvality piva a surovin je proto v centru pozornosti výrobců i konzumentů. Důležité je zejména udržet vysokou kvalitu a bezpečnost „českého piva“ jakožto nositele regionální ochranné známky. Předložená práce komplexně dokumentuje problémy spojené s výskytem různých typů kontaminujících látek doprovázejících jednotlivé kroky v rámci technologie výroby sladu a piva. V rámci odborných aktivit byly vyvinuty metody pro stanovení kontaminantů často ve stopových koncentracích. Bylo provedeno stanovení celé řady kontaminantů a posouzen vliv podmínek technologického zpracování, skladování, odrůdy a dalších faktorů na jejich obsah. Současně byly diskutovány postupy možné minimalizace obsahu procesních kontaminantů zejména v pivu jakožto finálním konzumovaném produkt. V rámci práce byly získány cenné výsledky, které významně přispívají k souboru poznatků nutných k účinné kontrole pivovarských surovin a produktů v průmyslové praxi a tím podporuje dobré jméno českého piva a jeho mezinárodní uznání a prestiž.

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8 Seznam symbolů a zkratek

AU ..... aktivní uhlí

CAR ..... Carboxen

CW ..... Carbowax

DCP ..... dichlorpropanol

DMS ..... dimethylsulfid

DON ..... deoxynivalenol

DVB ..... divinylbenzen

EFSA ..... European Food Safety Authority

ELISA ..... Enzyme-Linked ImmunoSorbent Assay

ES ..... Evropské společenství

EU ..... Evropská unie

FID ..... plamenově ionizační detektor

FPD ..... plamenově fotometrický detektor

GC ..... plynová chromatografie

HS ..... headspace

MS ..... hmotnostní spektrometrie

NIV ..... nivalenol

OTA ..... ochratoxin A

PA ..... polyakrylát

PDMS ..... polydimethylsiloxan

SPDE ..... Solid-phase dynamic extraction

SPME ..... Solid Phase Microextraction

SZPI ..... Státní zemědělská a potravinářská inspekce

TDAS ..... Thermal Desorption Autosampler

ZON ..... zearalenon

9 Seznam příloh

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Short communication

Determination of Acrylamide in Malt with GC/MS

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Received: 26-09-2006

Paper based on a presentation at the 12th International Symposium on Separation Sciences, Lipica, Slovenia, September 27–29, 2006.

Abstract

In food, acrylamide is produced in the course of Maillard reaction and its precursors are reducing saccharides and amino acid asparagine. Acrylamide formation in food depends on food composition and processing conditions. Significant quantities are formed during heat treatment above 120 °C, mostly at 150–180 °C, while at still higher temperatures the extent of formation decreases.

Barley is the raw material for malt production. It is a crop with high content of nitrogen compounds and a high content of starch. During malting, enzymatic activity leads to an increase in the content of reducing saccharides in malt, and during kilning, biochemical changes lead to melanoidin production and these conditions are favourable for acrylamide formation. These processes were studied in several malts using gas chromatography-mass spectrometry. Acrylamide was determined in significant amounts from several μg kg^{-1} – mg kg^{-1}.

Keywords: Acrylamide, gas chromatography, mass spectrometry, malt

1. Introduction

In 2002 the detection of acrylamide (Figure 1), neurotoxic and potentially carcinogenic substance, in food processed at temperatures >120 °C was reported.[1–3] High acrylamide content was found especially in food with a high starch content, such as food from potatoes and cereals.[4]

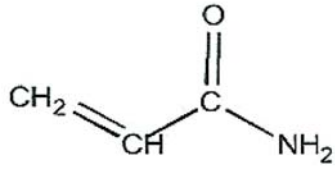

Figure 1. Structural formula of acrylamide (C_3H_5NO, CAS 79-06-1).

Acrylamide originates in the Maillard reaction and its precursors are reducing sugars and asparagine. The mechanisms leading to acrylamide formation in food depend on food composition and processing conditions.[5,6] There are three reaction phases:[7,8]

– production of glycosylamine, followed by Amadori rearrangement,
– dehydration and fragmentation of saccharides accompanying Strecker degradation of amino acids occur,
– Strecker aldehyde undergoes further reduction and dehydration reactions and acrylamide is created.

Besides this, studies of model systems have shown that under certain conditions acrolein and acrylic acid also participate in acrylamide formation after reaction with asparagine in foods rich in lipids.[8,9] Further research in model systems of glucose and asparagine showed the effect of temperature and time of heating. Acrylamide formation was low within the temperature range of 120–140 °C; if increased to 160–180 °C, the content increased dramatically. After reaching 180 °C, acrylamide production declines. The decline in acrylamide formation with higher temperatures can be explained by the fact that acrylamide as an intermediate product of Maillard reaction decomposes into other products.[9,10] Certain processes during production of malt are also favourable for acrylamide formation.[11]

The most frequently used methods for acrylamide determination are[12] HPLC-MS and GC-MS. In the latter case, acrylamide is derivatised by bromation.[13,14]

The advantage of acrylamide bromation is that the product is more volatile and less polar. The resulting derivate is readily extracted from aqueous solutions and it can be more easily detected with GC-MS. Conversion of acrylamide to 2,3-dibromopropionamide is usually performed by an addition of anhydrous potassium bromide, hydro-

bromic acid and a saturated solution of bromine in water. By adding trietylamine, non-stable 2,3-dibromopropionamide is converted to more the stable derivate 2-bromopropenamid.[14]

2. Materials and Methods

2.1. Chemicals

The following substances were used as standards: acrylamide (1 mg mL^{-1}) in methanol (Absolute Standards, USA), ($^{13}C_3$) acrylamide (1 mg mL^{-1}) in methanol (Cambridge Isotope Laboratories, USA), 2,3-dibromopropionamide (1 mg mL^{-1}) in methanol (Absolute Standards, USA). Bromine, potassium bromide, hydrobromic acid, sodium thiosulphate and trietylamine were all obtained from Merck (Germany). Methanol, ethylacetate (for HPLC, Chromservis, Czech Republic) were used.

2.2. Malt Samples

Samples of barley malt for the determination of acrylamide were taken in the course of malting (kilning). The first sample was taken at 60 °C and the last one at 210 °C, sampling was done in intervals of 10 °C. In addition, samples of special and coloured malts were taken (Table 1).

Table 1. The samples of special and coloured malts used in this study.

Pale malt	Pilsener Munich
Special malt	Smoked Melanoidin
Caramel Malt	CARAPILS® CARAHELL® CARARED® CARAAMBER® CARAMUNICH® CARAAROMA®
Roasted malt	CARAFA® CAAFA®SPECIAL
Wheat malt	Wheat – pale Wheat – dark CARA – WHEAT Wheat – roasted
Rye malt	Rye – pale Rye – caramel Rye – roasted

2.3. Sample preparation

10 µl of internal standard ($^{13}C_3$ acrylamide) and 50 mL of distilled water (60 °C) were added to the ground sample (5 g). After sonication (20 min) the homogenate was transferred quantitatively into centrifuge tubes and centrifuged at 8000 rpm^{-1} for 30 min. 2 g of KBr and HBr acid (pH 0–1) were added to 5 mL of supernatant. After cooling, 2 mL of bromine water was added. Contents in the flask were stirred and the flask was placed in a container with crushed ice for 10 h into the refrigerator. After bromination the excess bromine was titrated with $Na_2S_2O_3$ (1 mol L^{-1} solution) until discoloration occurred. The content of the flasks was transferred to teflon centrifuge tubes, 5 mL of ethylacetate was added to each tube and the content was shaken for 3 min and then centrifuged at 5000 rpm^{-1} for 5 min. After centrifugation, 1 mL of the organic phase was delivered with a pipette to a plastic microtube and triethylamine (0.2 mL) was added. Microtubes were shaken and after 15 min, they were centrifuged at 5000 rpm^{-1} for 5 min. After centrifugation, the tube content was delivered to glass vials and analyzed using GC/MSD.

2.4. Instrumentation

Gas chromatograph Trace GC Ultra Finnigan with mass detector Trace DSQ Thermo Finnigan was used with the DB-WAX capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific, USA). The column was held at 50 °C (1 min), then programmed at 15 °C min^{-1} to 150 °C (5 min). The temperature of the PTV injector was 200 °C in splitless mode (1 min). Transfer line temperature was 200 °C. The mass selective detector operated in selected ion monitoring (SIM) mode with positive electron impact (EI) ionization. The carrier gas was helium with a flow 1.5 mL min^{-1}. Under these conditions, the retention time of acrylamide and ($^{13}C_3$) acrylamide derivatives was 16.34 min.

Identification of acrylamide was conducted on the basis of retention time and for 2-bromopropenamide of specific ions *m/z* 149 and 151, quantification was performed using a calibration curve. To attain reliable results and maximum selectivity, the isotopically marked ($^{13}C_3$) acrylamide (*m/z* 152.154 for 2-bromo($^{13}C_3$)propenamide) was used as an internal standard.

3. Results and Discussion

The calibration curve was linear in the range from 30 to 620 µg kg^{-1} (acrylamide content in a real sample) with the correlation coefficient of 0.9985, LOQ was 25 µg kg^{-1}. Recovery was confirmed by samples spiked with isotopically marked ($^{13}C_3$) acrylamide and it ranged from 72%–86%.

Figure 2 shows the results of the dependence of acrylamide formation on kilning temperature. Maillard reactions occur at two main stages in the malting process; during wort boiling and in the production of speciality malts. Therefore, there is a strong possibility that acrylamide could be found in beer.

During kilning, acrylamide is already created from 60 °C. Formation of acrylamide at temperatures from 60–100 °C can be explained by long heating at temperatures above 60 °C. Malt is pre-dried for even 10 h at temperatures of 55–65 °C, after which kilning at temperatures follows, which is specific for the given type of malt (to 225 °C), held from 90 min to 2.5 h. Maximal acrylamide formation was found in the temperature interval 150–170 °C. Then, a decrease in acrylamide formation follows. The decrease may be explained by the fact that acrylamide as an intermediate product of Maillard reaction.[7,8]

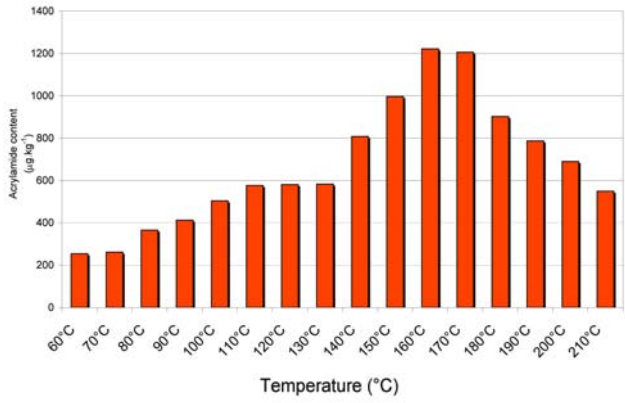

Figure 2. Dependence of acrylamide formation on kilning temperature.

Figure 3 shows acrylamide content in special and coloured malts Coloured and special malts are typically prepared by roasting kilned malt, a process comparable to coffee roasting.[15] In pale malts, acrylamide content was in the range of 630–660 μg kg^{-1}. In special melanoidin malt its content was 2210 μg kg^{-1}. Melanoidin malt has the same acrylamide content as the malt sampled in the course of kilning at 130 °C. This high value of acrylamide corresponds to the melanoidin malt preparation conditions, at which the Maillard reaction is promoted. Rye malts exhibit lower acrylamide contents in caramel and roasted malts than barley malts prepared at the same temperatures. Lower acrylamide content is caused by different contents of asparagines and reducing sugars in modified rye malt. In a similar way, the lower acrylamide content in wheat malts can be explained.

In roasted barley malts, the highest acrylamide content is in the malt CARAFA® SPECIAL. The highest acrylamide content of all the malts analyzed was determined in malts of the type CARAMÜNICH (3084 μg kg^{-1}). This corresponds to the method of caramel malt production (kilning temperature 150–170 °C) and conforms with the described dependence of acrylamide formation on temperature during kilning.

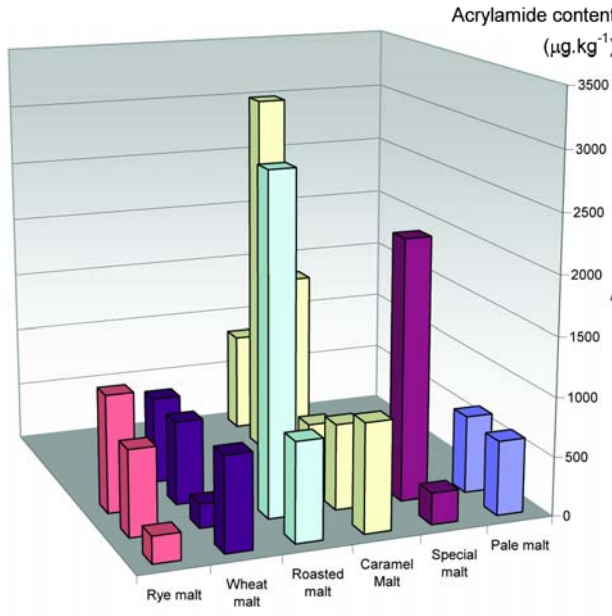

Figure 3 Acrylamide content in special malts.

4. Conclusion

Malt analyses proved the dependence of acrylamide content on kilning temperature. With the increasing temperature, acrylamide content in malt increased until 180 °C, after which it starts to decrease, due to further reactions of non-enzymatic browning. Higher acrylamide contents were found in special and coloured malts. This fact is connected with higher temperatures during kilning of these malts.

5. Acknowledgement

The results presented in this study were acquired in the framework of Research Plan of MSM 6019369701.

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Povzetek

Tvorba akrilamida v hrani je posledica Maillardove reakcije. Prekurzorji so reducirajoči sladkorji in aminokislina asparagin. Na tvorbo akrilamida vpliva tudi temperatura, pospešena je pri temperaturah nad 120 °C, zlasti pa pri 150–180 °C, medtem ko pri še večjih temperaturah akrilamid sodeluje v nadaljnjih reakcijah in se njegova vsebnost zmanjša. Ječmen je surovina, iz katere pridelujemo slad. Vsebuje veliko dušikovih spojin in škroba. Med pridelavo sladu se vsebnost reducirajočih sladkorjev povečuje zaradi encimskih reakcij. Biokemijske reakcije med nadaljnjimi procesi vodijo to tvorbe melanoidina. Nadaljnji nastanek akrilamida smo preučevali v več vzorcih sladu z uporabo plinske kromatografije z masnospektrometrično detekcijo. Določili smo ga v intervalu med μg kg^{-1} in mg kg^{-1}.

Food Chemistry 206 (2016) 124–130

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Rapid assignment of malting barley varieties by matrix-assisted laser desorption–ionisation – Time-of-flight mass spectrometry

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ARTICLE INFO

Article history:
Received 29 September 2015
Received in revised form 29 February 2016
Accepted 16 March 2016
Available online 17 March 2016

Keywords:
Barley
Malting
MALDI-TOF MS
Proteins

ABSTRACT

A method for discriminating malting barley varieties based on direct matrix-assisted laser desorption–ionisation – time-of-flight mass spectrometry (MALDI-TOF MS) fingerprinting of proteins was developed. Signals corresponding to hordeins were obtained by simple mixing of powdered barley grain with a MALDI matrix solution containing 12.5 mg mL^{-1} of ferulic acid in an acetonitrile:water:formic acid 50:33:17 *v*/*v*/*v* mixture. Compared to previous attempts at MALDI-TOF mass spectrometric analysis of barley proteins, the extraction and fractionation steps were practically omitted, resulting in a significant reduction in analytical time and costs. The discriminatory power was examined on twenty malting barley varieties and the practicability of the method was tested on sixty barley samples acquired from Pilsner Urquell Brewery. The method is proposed as a rapid tool for variety assignment and purity determination of malting barley that may replace gel electrophoresis currently used for this purpose.

1. Introduction

Due to their influence on malting quality, the identity of malting barley varieties and their purity (i. e. the affiliation of the grains within the entire batch to a single variety) is a fundamental step preceding the process of beer production (Newton, Swanston, Guy, & Ellis, 1998). Routinely, the identity and purity of malting barley varieties are determined by means of one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) of the hordein fraction (Almgard & Landegre, 1974). In this approach, which has been used for several decades as a standard method in breweries, hordeins are extracted from crushed barley grains overnight, followed by electrophoretic separation on the second day and visualisation of protein bands on the third day. Variety identification and purity determination are based on a comparison of protein band patterns with those of standard malting barley varieties or comparisons between several grains from the same batch, respectively.

As well as involving time-consuming analytical steps, this standard protocol is complicated by safety issues, due to the use of toxic chemicals (such as acrylamide, *N*,*N*-methylenebisacrylamide or mercaptoethanol). In addition, it has been shown that some barley varieties are not distinguishable by means of 1D-PAGE (Almeida & Cavalli-Molina, 2000).

Matrix-assisted laser desorption–ionisation – time-of-flight mass spectrometry (MALDI-TOF MS) caused a revolution in microbial diagnostics (Bizzini & Greub, 2010; Clark, Kaleta, Arora, & Wolk, 2013; Welker & Moore, 2011; Šedo, Sedláček, & Zdráhal, 2011). Due to its rapidity and high discriminatory power, it has become the method of choice for identification of bacterial pathogens. Aside from bacteria and fungi, other types of materials can also be subjected to MALDI-TOF MS profiling analysis.

The concept of cereal protein profiling by MALDI-TOF MS, with the aim of discriminating different cereal varieties, has already been investigated. Specific gliadin signals obtained by MALDI-TOF MS after a 20 min extraction of milled grains enabled the identification of wheat varieties with the aid of artificial neural networks (Bloch, Kesmir, Petersen, Jacobsen, & Sondergaard, 1999). A similar approach was also demonstrated on rye and barley (Bloch et al., 2001), while data treatment based on multivariate analysis was used for this purpose (Gottlieb et al., 2002).

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http://dx.doi.org/10.1016/j.foodchem.2016.03.056
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Discrimination between malting barley varieties on the basis of MALDI-TOF mass spectral comparison of hordeins was achieved for the first time by Šalplachta and Bobáľová (2009) after optimisation of the extraction procedure. MALDI-TOF MS also enabled brewing process monitoring, as was shown by MALDI-TOF MS profiling of water-soluble proteins (Laštovičková, Mazanec, Benkovská, & Bobáľová, 2010). A fundamentally different approach for identification of malting barley varieties was developed by Pattermore, Rice, Marshall, Waugh, and Henry (2010), who employed MALDI-TOF MS profiling of DNA fragments in a multiplexed single nucleotide polymorphism (SNP) assay.

In the present paper, we introduce a rapid and easy method for discrimination between malting barley varieties based on MALDI-TOF MS analysis of proteins obtained directly from the barley material.

2. Materials and methods

2.1. Barley samples

Certified standards of registered malting barley varieties were obtained from Oseva Pro (Prague, Czech Republic; varieties Akcent, Bojos, Laudis, Malz, Marthe, Tipple, and Wintmalt), from Bio-Rad spol s.r.o. (Prague, Czech Republic; varieties Ebson and Esterel), and Central Institute for Supervising and Testing in Agriculture (Brno, Czech Republic; varieties Advent, Aksamit, Aktiv, Blaník, Delphi, Despina, Kangoo, Radegast, Sebastian, Tolar, and Xanadu). Standards for Malz and Wintmalt varieties were obtained after being grown at two different locations in the Czech Republic (samples labelled "CZ") and Slovakia (samples labelled "SK").

Sixty samples of malting barley selected for evaluation of the method were delivered to Pilsner Urquell Brewery from different producers over a two-year period (2013–2014). The sampling was carried out by acquiring eight small sub-samples from different parts of each batch.

2.2. Chemicals

Ferulic acid (FerA) and 6-aza-2-thiothymine (ATT) were obtained from Sigma–Aldrich (Steinheim, Germany). Trifluoroacetic acid (TFA) and acetonitrile (ACN) were from Merck (Darmstadt, Germany). Formic acid (FA) was from Riedel de Haën (Seelse, Germany). α-Cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), and sinapinic acid (SA) were obtained from Bruker Daltonik (Leipzig, Germany). All chemicals were of analytical grade purity. Water was prepared using a Milli-Q plus 185 apparatus (Millipore, Billerica, MA).

2.3. Sample preparation

A single barley grain per sample was crushed using a mortar and pestle. The certified malting varieties were prepared in

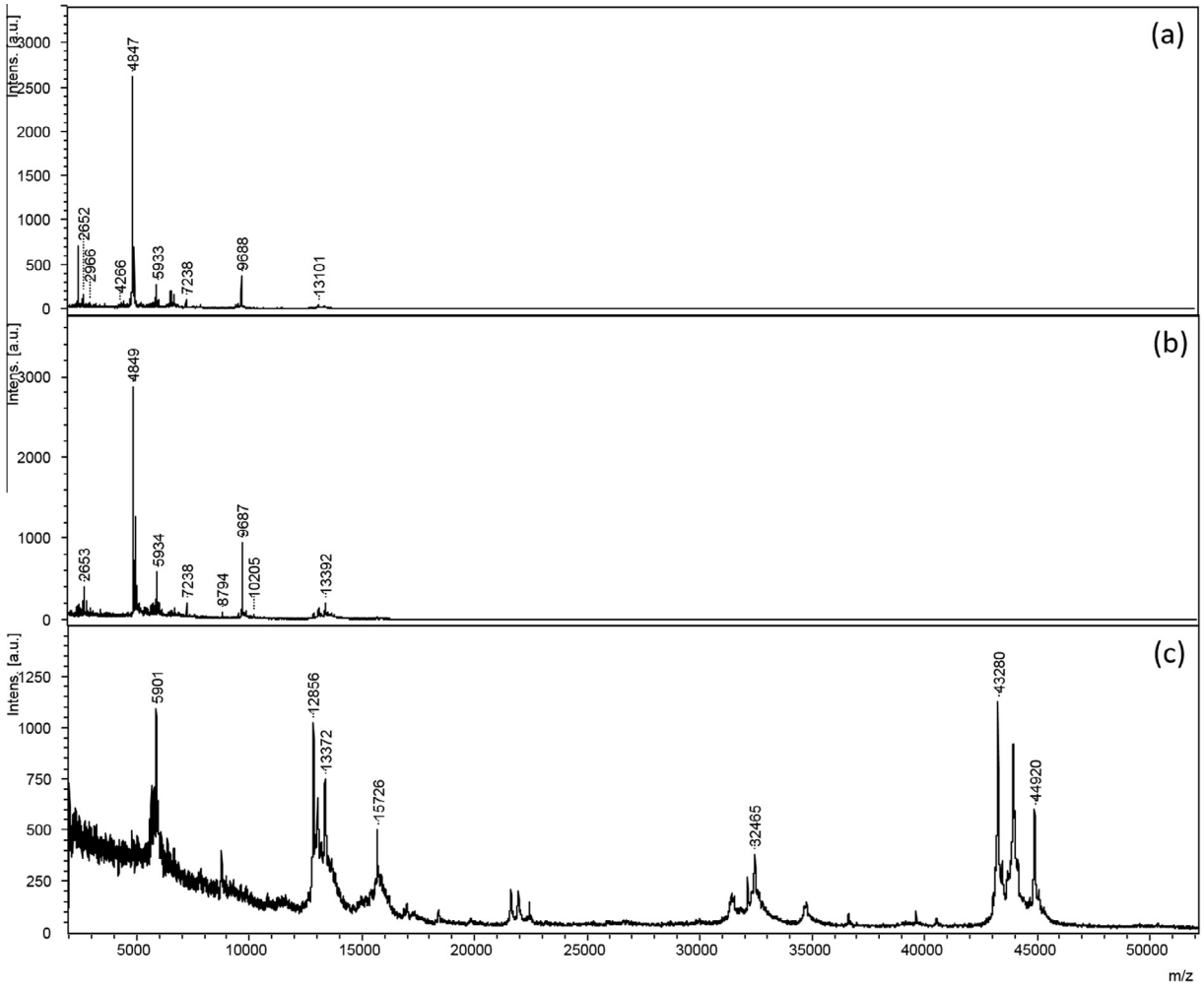

Fig. 1. MALDI-TOF mass spectra of Bojos standard by using (a) CHCA, (b) ferulic acid with 0.1% TFA, and (c) strongly acidified ferulic acid (17% FA).

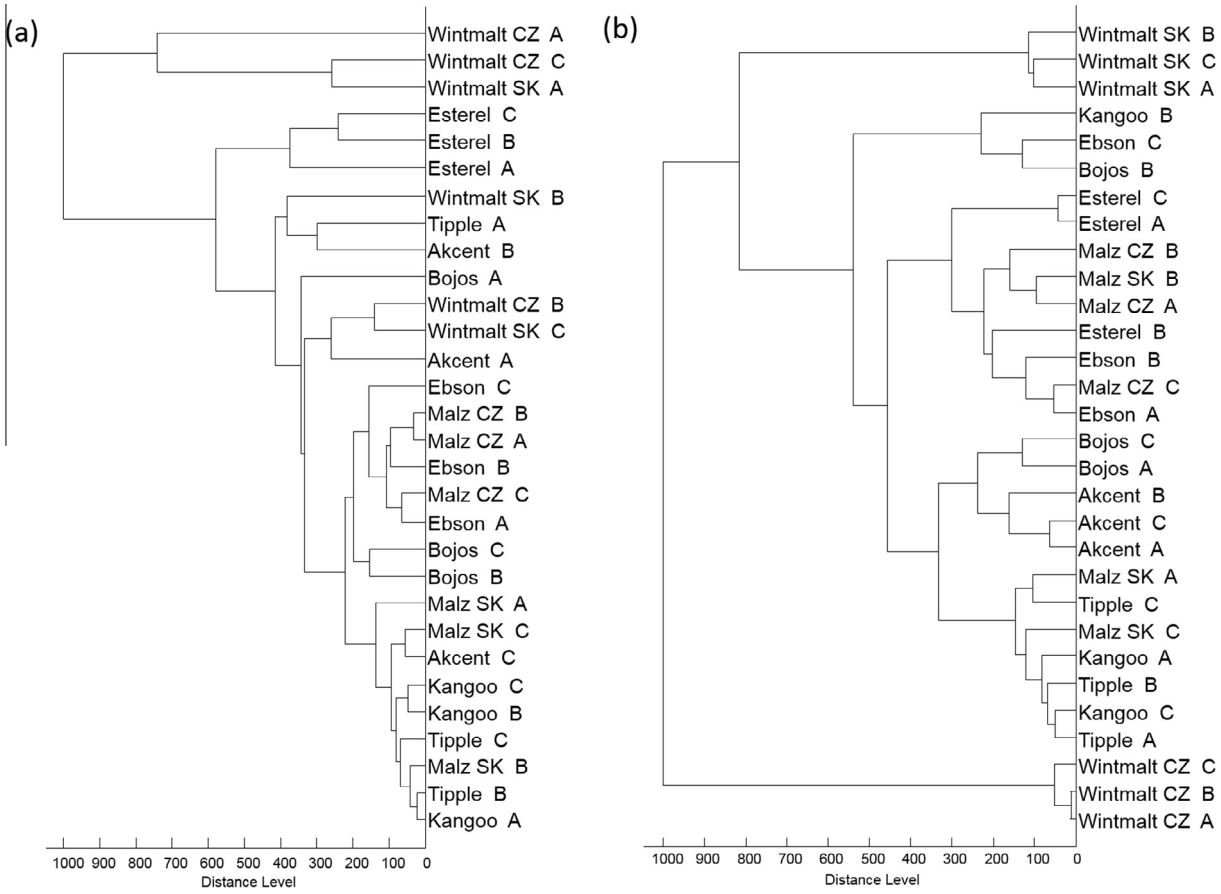

Fig. 2. Discrimination between eight malting barley varieties by cluster analysis based on their MALDI-TOF mass spectra obtained with (a) CHCA and (b) ferulic acid with 0.1% TFA. Different grains are labelled "A", "B", and "C". Standards from different resources are labelled "CZ" and "SK".

triplicate to test the discriminatory power of the method. The optimised MALDI matrix consisted of a solution of 12.5 mg mL^{-1} ferulic acid in an acetonitrile:water:formic acid (50:33:17, *v/v/v*) mixture. A minimum visible amount of powdered material was transferred to a 200-μL Eppendorf tube containing 20 μL of the matrix solution) and mixed by vortexing (Biosan V-1 plus; Biosan SIA, Riga, Latvia) at 3000 rpm for 10 s. The mixture was left standing for 10 s (centrifugation was found to be unnecessary), the extract was deposited onto three sample spots of a stainless steel MALDI target and allowed to air dry at room temperature. Other variations in the matrix compound and solvent composition are described in the Section 3.

2.4. MALDI-TOF mass spectrometric analysis

MALDI-TOF mass spectra measurements were carried out using an Ultraflextreme instrument (Bruker Daltonik, Bremen, Germany) operated in linear positive mode under FlexControl 3.4 software. External calibration of the mass spectra was performed using protein calibration mixture II and *Escherichia coli* DH5 alpha standard (both obtained from Bruker Daltonik) for the high-mass range and low-mass range, respectively. The ion source voltage settings were different in relation to the mass range of detected compounds. For low-mass/high-mass detection, the values were: ion source I 20.00 kV/20.00 kV, ion source II 18.70 kV/18.45 kV, lens 6.00 kV/6.50 kV, and pulsed ion extraction 130 ns/480 ns (optimum values should be adjusted by experienced technicians as they may differ between individual instruments). The laser was set to 120% of the threshold laser power (i. e. the laser power at which peaks start to appear) for a particular type of sample. Three independent spectra, each comprising 1000 laser shots, were acquired from each of the sample spots. Within an individual sample spot, the laser was directed manually, where a minimum of 200 and a maximum of 400 shots were obtained from each position on the sample spot. Mass spectra were processed using Flex Analysis (version 3.4; Bruker Daltonik) and BioTyper software (version 3.1; Bruker Daltonik). The mass spectra were smoothed, subjected to baseline subtraction and normalisation to the most intense signal under default settings of the Biotyper software. Within the mass range 20–50 kDa a maximum of 100 peaks per spectrum with signal-to-noise ratio greater than 3 were further considered. As input data for cluster analysis, signals present in at least three out of the total of nine replicate analyses of each barley sample were taken into account (in a format of Biotyper MSPs – main spectra projections). The MALDI-TOF mass spectra-based dendrogram was generated using a correlation distance measure (one minus Pearson's correlation coefficient calculated from the sequences of values) with the average linkage algorithm (unweighted average distance – UPGMA) settings, available within the Biotyper software.

2.5. SDS–PAGE

Hordein-enriched fraction of barley grains was prepared by extracting wholemeal flour (from one grain) twice in 0.5 mL of 60% (v/v) ethanol, 2% (w/v) dithiothreitol. Extraction was carried out in a thermomixer for 30 min at room temperature. Alcohol-soluble extract was dried down in a SpeedVac vacuum concentra-

Table 1
Optimization of the MALDI matrix solution.

Matrix/TFA conc.	*S/N* peak 4850 Da	Resolution peak 4850 Da	*S/N* peak 9687 Da	Resolution peak 9687 Da	Peak area ratio 9687/ 4850 Da	Number of 100% reproducible peaks	Number of 80–100% reproducible peaks
ATT/0.1	190 ± 21	593 ± 13	160 ± 38	933 ± 19	0.57 ± 0.05	16	39
ATT/1	347 ± 62	611 ± 19	85 ± 24	906 ± 31	0.26 ± 0.06	19	37
DHB/0.1	132 ± 43	528 ± 20	52 ± 20	739 ± 31	0.45 ± 0.05	25	49
DHB/1	164 ± 15	580 ± 18	50 ± 9	813 ± 40	0.30 ± 0.03	15	37
FerA/0.1	286 ± 25	639 ± 14	212 ± 33	1020 ± 16	0.66 ± 0.07	30	54
FerA/1	247 ± 57	635 ± 14	106 ± 18	1004 ± 12	0.48 ± 0.02	28	46
HCCA/0.1	230 ± 41	391 ± 12	57 ± 12	486 ± 42	0.25 ± 0.02	21	43
HCCA/1	246 ± 26	361 ± 8	85 ± 12	415 ± 30	0.34 ± 0.05	19	49
SA/0.1	130 ± 21	556 ± 18	32 ± 12	826 ± 37	0.20 ± 0.05	16	28
SA/1	325 ± 60	533 ± 20	107 ± 31	800 ± 49	0.28 ± 0.05	10	38

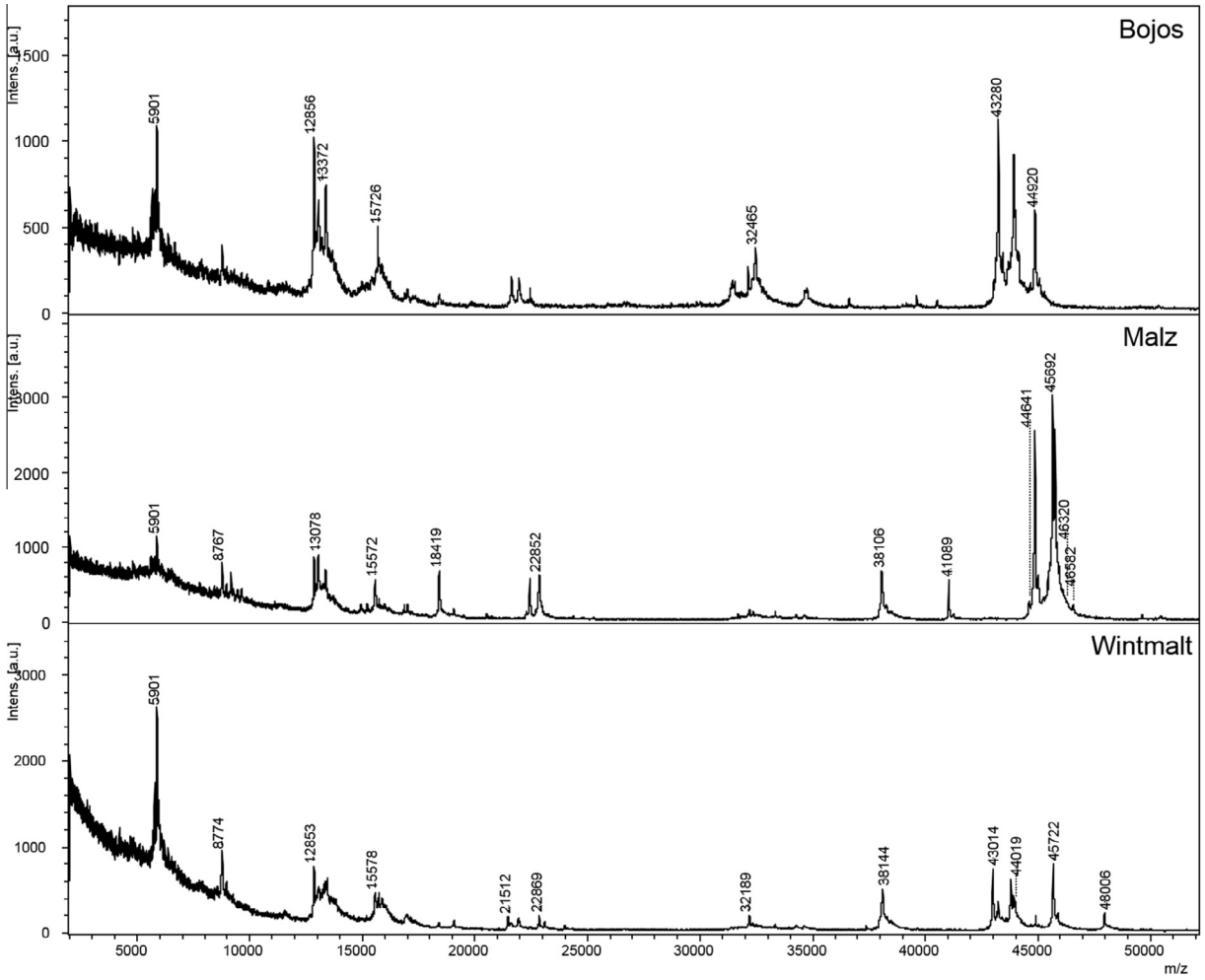

Fig. 3. MALDI-TOF mass spectra of three standards of malting barley varieties obtained by using strongly acidified ferulic acid as a MALDI matrix.

tor. The dry powder was dissolved in 50 μL of Laemmli sample buffer, incubated for 5 min at 95 °C, and centrifuged for 10 min at 15,000*g*. The supernatant (4 μL) was loaded onto 18-well Bio-Rad 4–15% SDS–PAGE gradient gel together with two Bio-Rad ladders: PrecisionPlus and Dual Xtra. The gel ran at a constant voltage of 200 V in a Criterion cell (Bio-Rad). Protein staining was performed with BioSafe Coomassie Blue G-250 (Bio-Rad).

3. Results and discussion

Barley protein signals were initially obtained after direct application of powdered grains onto the MALDI target which was overlaid with a MALDI matrix solution. However, cross-contamination using this simple approach was apparent. For that reason, subsequent analyses were conducted in the manner described in Section 2.3, employing direct and rapid extraction of the barley proteins into the matrix solution. Primarily, we examined the same MALDI matrix solution as is routinely used for MALDI-TOF MS profiling of bacteria (saturated solution of CHCA in an acetonitrile:water:TFA mixture, 50:47.5:2.5, *v/v/v*). By extracting and ionising proteins from barley by CHCA, the mass range of detected compounds in the resulting mass spectra was 2–14 kDa (Fig. 1a). Ten standards of eight malting barley varieties were analysed with the aim of testing if their discrimination was

possible on the basis of the signals obtained. To examine the relevance of the method, two standards (varieties Wintmalt and Malz) were obtained from two independent resources. As shown in the dendrogram in Fig. 2a, almost no discrimination was achieved between all ten varieties. For that reason, we optimised the matrix solution composition. This included testing five different MALDI matrices, which were selected due to their ability to assist in protein ionisation in MALDI. Each of these matrices was prepared in two solvents, differing in acidity (two levels of TFA concentration were tested). Powdered grain of the Bojos standard was subjected to extraction and fifteen analyses using each of these matrix solutions. As a platform with the potential to show the highest discriminatory power, we sought a method that could provide spectra containing a high number of well-resolved and reproducible signals. The following mass spectral features were monitored: signal-to-noise ratio, mass spectral resolution, and the number of reproducible signals (detected in all fifteen (100%) or at least twelve (80–100%) of the analyses). We also noted that detection of intense signals around 5 kDa was achieved using all of the matrices, while compounds around 10 kDa were ionised with variable efficiencies. For that reason, relative intensity of these signals was selected as another parameter for optimisation. The summary of results is given in Table 1. Ferulic acid containing 0.1% TFA was found to be the most suitable matrix, showing the highest *S*/*N* and resolution for peaks around 10 kDa, and the highest number of reproducible signals.

The mass spectra obtained using this optimised matrix solution are displayed in Fig. 1b. Compared to CHCA (Fig. 1a), more signals were gained in the mass range of 8–13 kDa. Unfortunately, the cluster analysis based on mass spectra obtained using ferulic acid containing 0.1% TFA again showed poor discrimination between the malting barley varieties (Fig. 2b). Detailed visual inspection of the mass spectra indicated some candidates for peaks around 13 kDa characteristic of some of the varieties; however, their specificity was not found to be sufficient for reliable discrimination.

As hordeins represent the class of proteins showing the highest variability among barley varieties (Dai et al., 2014), a MALDI matrix capable of ionisation of compounds with relatively higher molecular weights from complex protein mixtures has the potential to enhance the discriminatory power of the method. We thus examined strongly acidified ferulic acid, which modulates signal suppression effects connected to MALDI in favour of high-mass proteins (Madonna et al., 2000; Šedo, Nemec, Křížová, Kačalová, & Zdráhal, 2013). As seen in Fig. 1c, the detection of signals in a significantly wider mass range was achieved using this matrix. Importantly, different malting barley varieties were found to show visibly distinct signals above 20 kDa, as demonstrated over Fig. 3 (the mass spectra of the remaining seventeen malting barley varieties tested are shown in Supplementary Fig. 1). The possibility of discriminating between twenty malting barley varieties was demonstrated by means of cluster analysis constructed on the basis of MALDI-TOF mass spectra obtained from three grains per standard variety analysed individually (Fig. 4). In addition, ten of the standards were re-analysed after two months by a less experienced operator to verify the robustness of the method. The clustering proved to be independent of the mass spectral quality achieved by the operator. The only exception was observed in the case of variety Esterel; however, the clustering of separated groups of samples A–C (operator 1) and D–F (operator 2) was very close. Nevertheless, limits in the discriminatory power of the method

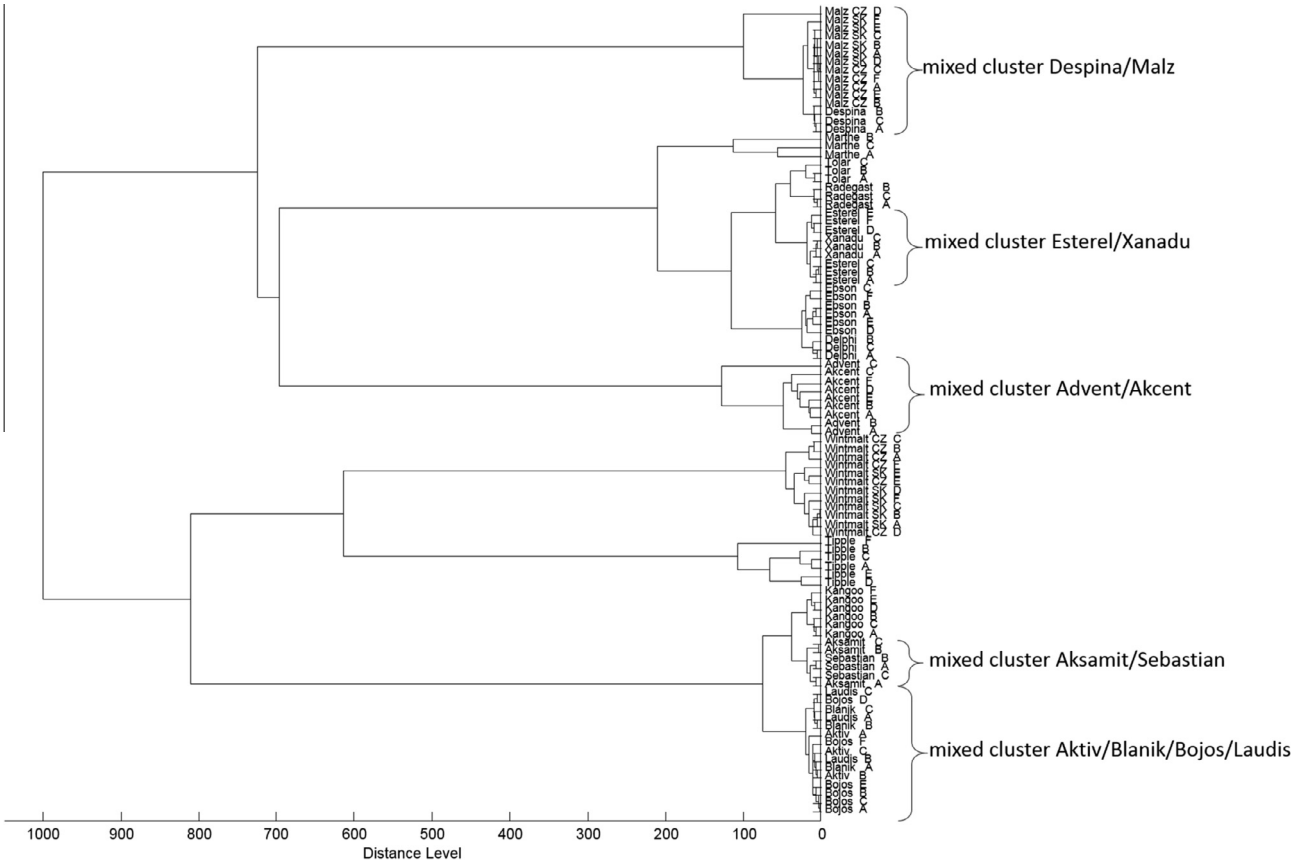

Fig. 4. Discrimination between twenty malting barley varieties by cluster analysis based on their MALDI-TOF mass spectra. Different grains analysed by operator 1 are labelled "A", "B", and "C", grains analysed by operator 2 are labelled "D", "E", and "F". Standards from different resources are labelled "CZ" and "SK".

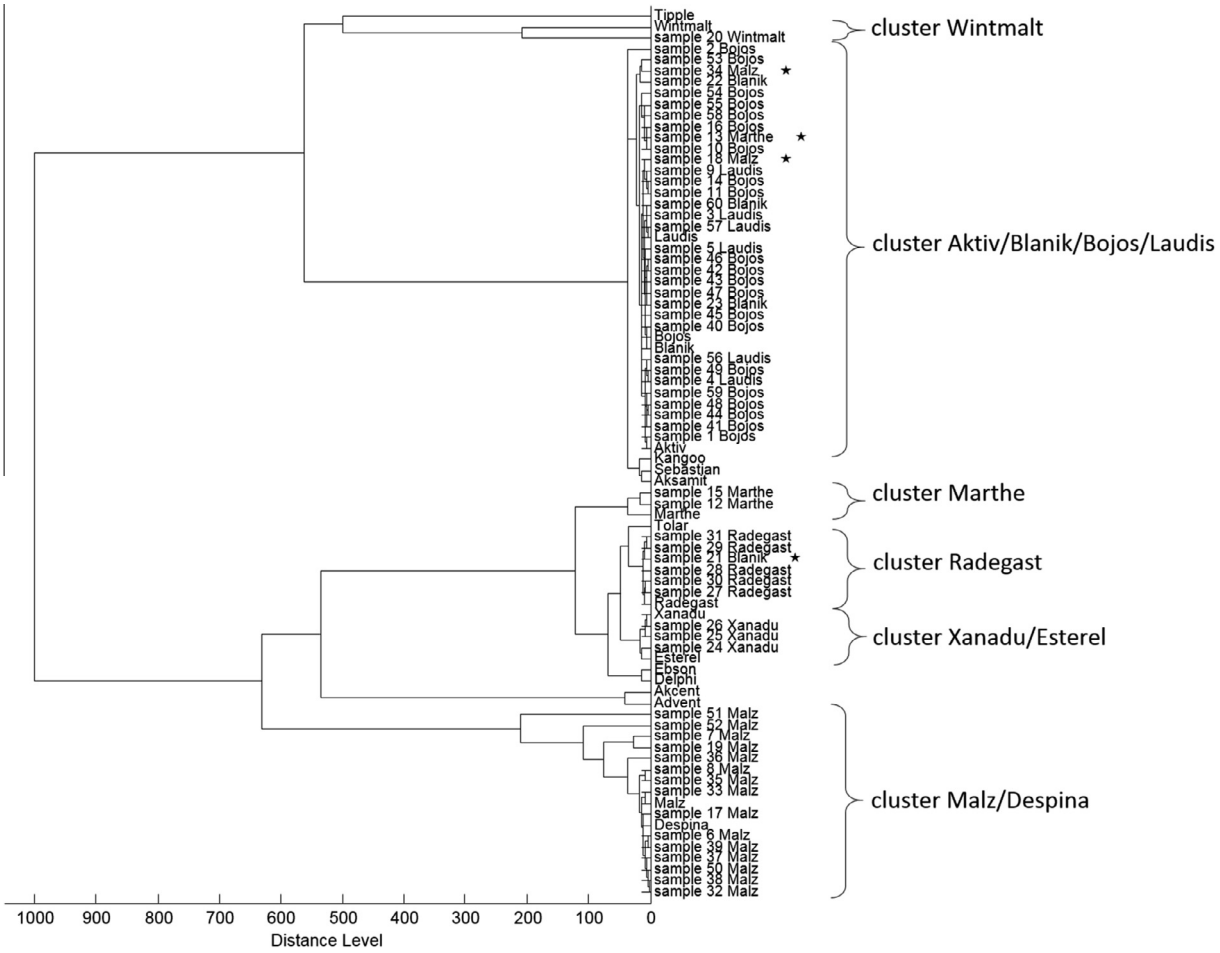

Fig. 5. Assignment of sixty malting barley samples to standards by cluster analysis based on their MALDI-TOF mass spectra. Samples showing disagreement between their original designation and analysis results are highlighted with an asterisk.

were revealed by the existence of clusters containing unseparated or very closely grouped varieties. This indicates that under our experimental set-up, some of the samples could not be identified unambiguously but they could be assigned to particular groups of indistinguishable varieties. Among these problematic groups, at least a close relationship between varieties Aktiv, Blaník, Bojos and Laudis was found to be supported by their origin, as they were created by hybridisation from the variety Madonna. Similarly to MALDI-TOF MS, these varieties were not distinguishable by means of 1D-SDS PAGE, as they did not show any specific bands allowing their discrimination (see Supplementary Fig. 2).

Similar problems were not encountered by Šalplachta and Bobáľová (2009), who demonstrated distinguishing a different set of thirteen malting barley varieties, overlapping with our set only in four cases, based on MALDI-TOF mass spectra of extracted hordeins. However, the authors evaluated the discriminatory power of the method only on the basis of visual comparison of a single MALDI-TOF mass spectrum per variety.

To examine the practicability of the method, sixty samples of barley delivered to Pilsner Urquell Brewery from different producers over a two-year period were subjected to MALDI-TOF mass spectrometric analysis using the proposed method. These samples were designated to individual varieties by the producers and their purity and identity was checked independently by means of SDS–PAGE. As seen from Fig. 5, MALDI-TOF mass spectrometric analysis was capable of verifying variety identity. Out of the total of 60 samples, only four were not clustered with a corresponding standard or group in the dendrogram. In addition to cluster analysis, the assignment of samples was carried out by a common Biotyper scoring algorithm. Results equivalent to those obtained by means of cluster analysis were achieved for all 60 samples, as the highest identification log(score) always corresponded to MSPs of standards grouped together with the samples by cluster analysis.

Several advantages of MALDI-TOF MS over SDS–PAGE are indisputable. Compared with the standard procedure, for analysis of 14 grains from one batch for purity control, a 2-fold decrease in analytical costs and an 18-fold reduction in time was achieved by switching from SDS–PAGE to MALDI-TOF MS. Importantly, costs of SDS–PAGE are even higher if one considers specific safety issues and toxic waste handling connected with acrylamide use. A frequently discussed concern related to MALDI-TOF mass spectrometric profiling is connected to repeatability of the method. In the case of our novel method, this issue is considerable also, due to the necessity of manual mass spectra acquisition which involves the "sweet-spot" tracing. The results of clustering outputs in our study, conducted over a period of several months by two MALDI MS operators (Fig. 4), proved the potential of the method to provide robust identification results. Another effect accompanying MALDI-TOF mass spectrometric analysis is the mutual suppression of ionisation among individual components of the analysed mixture. In the course of our study using individual barley grains, we did not encounter suppression effects that impaired the detection of hordein signals significantly. For barley purity determination, the method should be used in a manner analogous to SDS–PAGE,

where comparison of parallel analyses of individual barley grains from one batch is conducted. In the case of a mixture of grains from blended varieties analysed directly, both methods would provide unspecific profiles not enabling determination of a particular variety.

MALDI-TOF MS analysis can give uncertain outputs in cases concerning indistinguishable varieties, although this is also the case using the established SDS–PAGE method. This is, particularly, important for Czech breweries in the case of two varieties Bojos and Laudis that are used for malting currently. The introduction of MALDI-TOF MS for barley profiling should always be accompanied with adequate testing of samples relevant to the individual brewery, as breweries world-wide, or even on the level of particular regions, are supplied with different malting barley varieties. The limits in discriminatory power should always be thoroughly examined on a set of relevant malting barley varieties to avoid generating misleading results.

4. Conclusions

MALDI-TOF mass spectra obtained after short mixing of powdered material with strongly acidified ferulic acid solution show high-mass proteins that are characteristic of individual malting barley varieties or their groups. Thanks to its rapidity and simplicity, the method has the potential to replace SDS–PAGE that is routinely used in the brewing industry for recognition of malting barley varieties and purity control of barley samples. Together with MALDI-TOF MS identification of spoilage bacteria from brewery bottling halls and beers (Vávrová, Matoulková, Balážová, & Šedo, 2014; Wieme et al., 2014) and direct beer fingerprinting applications for technology and authenticity control (Šedo, Márová, & Zdráhal, 2012), assignment of malting barley varieties represents another effective application of MALDI-TOF MS in the brewing industry.

Acknowledgements

This work was carried out with the support of Proteomics Core Facility of CEITEC – Central European Institute of Technology under CIISB project, ID number LM2015043, funded by the Ministry of Education, Youth and Sports of the Czech Republic; supported by projects CEITEC (CZ.1.05/1.1.00/02.0068), financed from European Regional Development Fund, the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Program II. O.Š. and Z.Z. thank for the support of Czech Science Foundation (project No. P206/12/G151). Hana Konečná and Danuše Fridrichová from Core Facility of CEITEC are gratefully acknowledged for SDS–PAGE analyses.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.03.056.

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Research Article

Received: 11 February 2016 Revised: 12 May 2016 Accepted article published: 20 May 2016 Published online in Wiley Online Library: 14 June 2016

(wileyonlinelibrary.com) DOI 10.1002/jsfa.7811

A combination of additives can synergically decrease acrylamide content in gingerbread without compromising sensory quality

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Abstract

BACKGROUND: The present study tested whether replacement of the leavening agent ammonium carbonate by sodium hydrogen carbonate in combination with calcium cation and acidifying agent will synergically decrease acrylamide (AA) content in gingerbread.

RESULTS: The type of leavening agent and the presence of Ca^{2+} and citric acid accounted for 33.6%, 13.2% and 53.2% of the explained variability of the AA content, respectively ($P < 0.01$). The AA content in gingerbread produced with $(NH_4)_2CO_3$ alone was 186.5 μg kg^{-1}. Irrespective of other tested additives, $NaHCO_3$ decreased ($P < 0.05$) AA content to 42% compared to $(NH_4)_2CO_3$. Combination of $NaHCO_3 + CaCl_2$ + citric acid in dough reduced ($P < 0.05$) AA content below the limit of detection (25 μg kg^{-1}). The AA content in gingerbread (y; μg kg^{-1}) decreased with an increasing number of additives used (x) according to the equation $y = 158.8 - 47.94x$ ($r^2 = 0.42$; $P < 0.0001$). A comprehensive sensory analysis did not indicate any significant deterioration ($P > 0.05$) in the organoleptic quality of gingerbread produced using calcium cation and citric acid.

CONCLUSION: The present study demonstrates that the combination of additives $NaHCO_3$/Ca^{2+}/citric acid synergically decreases AA content in gingerbread without compromising the sensory quality.

Keywords: acrylamide; gingerbread; honey; food safety; additives; sensory analysis

INTRODUCTION

Gingerbread belongs to the risky food because of the presence of acrylamide (AA). AA is a neurotoxin that is listed by the International Agency for Research on Cancer as a probable human carcinogen.[1] Although epidemiological associations have not indicated that AA is a human carcinogen, the margins of exposure indicate concern with respect to neoplastic effects based on animal evidence.[2] Based on the AA content in foods monitored from 2007 to 2010, the most risky foods are instant coffee and potato crisps (mean AA levels in the year 2010 of 1123 and 675 mg kg^{-1}, respectively).[3] In a more recent scientific opinion reported by the EFSA CONTAM Panel,[2] the highest AA levels were in solid coffee substitutes and coffee, as well as in potato fried products, with an estimated dietary AA exposure across survey and age groups of 0.4–1.9 μg kg^{-1} body weight (BW) day^{-1} and 0.6–3.4 μg kg^{-1} BW day^{-1}, respectively. The Panel[2] concurrently selected the bench mark dose lower limit ($BMDL_{10}$) value of 0.17 μg kg^{-1} BW day^{-1} for neoplastic effects in mice.

Despite a relatively low AA content in soft bread (mean value 30 mg kg^{-1}),[3] this food item contributed between 20% and 50% to the overall AA intake in Europe.[4] In this context, AA content in gingerbread is not insignificant (mean AA content: 415 mg kg^{-1}; 90th percentile: 1187 mg kg^{-1}).[3]

Moreover, gingerbreads are usually produced with ammonium carbonate as a leavening agent. Another indispensable component is honey, with a high content of reducing sugars, including fructose; these are two significant risk factors for production of AA in thermally processed foods.[5–7]

The main route of AA formation in the above-mentioned products is a Maillard-type reaction of amino acid asparagine with reducing sugars via a Schiff base intermediate at temperatures above 120 °C.[3] Acrylamide formation depends, amongst other factors, on the overall thermal input (superposition of temperature and a baking time);[8] the greatest amount of AA is formed in the surface layer of the thermally treated product. This accounts for the high risk levels associated with some small-sized products (such as a white pepper-gingerbread tested in the present study) as a result of a high (i.e. unfavourable) surface/volume ratio.

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In addition, considering the fact that white pepper-gingerbread is baked at 180 °C, a relatively high AA content can be expected. Nevertheless, data regarding AA content in this particular type of gingerbread have not yet been reported in the literature.

Regarding the possible carcinogenicity of AA, several mitigation measures were proposed regarding the relevant foods. These measures are based on altering the four main factors affecting the Maillard reaction: precursor content, temperature, water activity and pH.[9] The European Food and Drink Industry (CIAA) summarized the parameters used for mitigating AA burden for consumers in the 'CIAA Acrylamide Toolbox'.[10] These parameters include agronomical factors (sugars, asparagine), recipe (ammonium carbonate, sodium bicarbonate, pH, minor ingredients), processing (thermal input) and final preparation.

In particular, the certain additives were tested in this context: ions (Na^+, Mg^{2+}, Ca^{2+}), organic acids and amino acids,[11–14] spice antioxidants,[15] and asparaginase.[16]

However, when changing the recipe or the temperature regime, the organoleptic acceptability of the product by consumers should be considered. Instrumental parameters such as colour (trichromatic parameters L*, b*, a*) are usually measured;[17] evaluation by a sensory panel is reported only exceptionally.[7]

The objectives of the present study were: (1) to determine AA content in a white pepper-gingerbread produced according to the original recipe; (2) to compare AA levels in this 'classically' produced white pepper-gingerbread with white pepper-gingerbreads produced using alternative additives (sodium bicarbonate, calcium ions, acidifying agent), including their combinations with possible synergic effects; and (3) to evaluate the organoleptic acceptability of the white pepper-gingerbread produced according to both the alternative and 'classical' recipes. The ultimate goal was to propose a recipe leading to a safe product.

MATERIALS AND METHODS

Gingerbread preparation

The white pepper-gingerbread was tested. The main ingredients (g kg^{-1} product) consisted of wheat flour (mineral content 0.6%) 550; honey (linden flower-honeydew; electrical conductivity 75 mS m^{-1}; water content 18.5%) 360; eggs (common supermarket) 82; and freshly grounded spices: cinnamon 3.61, cloves 1.31, nutmeg 0.33, ginger 0.33, cardamom 0.33, coriander 0.25, allspice (*Pimentadioica*) 0.25, white pepper 1.56 and NaCl 0.25.

The addition of the certain other substances into the gingerbread was also tested: ammonium carbonate, E503, $(NH_4)_2CO_3$ (at an amount comprising 84 mmol kg^{-1} product); sodium bicarbonate, E500, $NaHCO_3$ (154 mmol kg^{-1}); calcium chloride, E509, $CaCl_2$ (83 mmol kg^{-1}); and citric acid, E330, $C_6H_5O_7$ (38 mmol kg^{-1}). The presence of these substances (including their combinations) in the gingerbread samples and the corresponding labelling of the samples are both reported in Table 1.

For preparation of gingerbread, honey was heated to 30 °C to decrease its viscosity and facilitate homogenization with eggs and spices. Next, one-quarter of the flour dose, the leavening agent (ammonium carbonate dissolved in 5 g of water or sodium bicarbonate without water) and a tested additive (if used) (Table 1) was added (calcium chloride was dissolved in 5 g of water; citric acid was added directly to the dough without water). Then, the remaining three-quarters of the wheat flour dose were added. Dough was processed into cylinders (width 1.5–2.0 cm), which were divided into 1-cm portions to produce single ball-shaped gingerbreads.

The balls (weighing approximately 10 g) were placed on a baking foil and the foil with gingerbreads was carried over to the pre-heated baking metal plate. Gingerbreads were baked at 180 °C in an electric oven with simultaneous bottom and upper baking. The total baking time was 7 min 30 s; the metal plate with gingerbreads was rotated by 180° after 3 min 45 s to ensure a uniform effect of temperature. After the baking was finished, gingerbreads on the baking foil were immediately placed on a marble board for cooling (with an instantaneous decrease of baking temperature). After cooling down, gingerbreads were put into sample containers at 50 pieces each.

Eight batches of gingerbread corresponding to the tested additives (their combinations are shown in Table 1) were prepared within a given experiment.

Acrylamide determination

Within each batch, four gingerbreads were analyzed for AA content in duplicate ($n = 4$). The whole procedure was repeated four times.

The standards and chemicals used were: AA (1 mg mL^{-1}) in methanol (Absolute Standards, Hamden, CT, USA); ($^{13}C_3$) acrylamide (1 mg mL^{-1}) in methanol (Cambridge Isotope Lab, Inc., Tewksbury, MA, USA); 2,3-dibromopropionamide (1 mg mL^{-1}) in methanol (Absolute Standards); bromine, potassium bromide, hydrobromic acid, saturated bromine water, sodium thiosulphate and trietylamine (all from Merck, Darmstadt, Germany); methanol and ethylacetate (purity for high-performance liquid chromatography; Sigma-Aldrich, Praha, Czech Republic).

The sample was prepared and AA was determined according to Mikulíková and Sobotová.[18] Ground sample (10 g) was placed into a 50-mL volumetric flask, 10 μL of internal standard [($^{13}C_3$) acrylamide] was added, and the volume was levelled up with distilled water at 60 °C. After sonication (20 min) in an ultrasonic bath, the homogenate was transferred quantitatively into a centrifugal tube and centrifuged at 5000 × *g* for 30 minutes. Five microlitres of supernatant was mixed with 2 g of potassium bromide and hydrobromic acid was added to achieve pH 0–1.

After cooling, 2 mL of bromine water was added. The content of the flask was stirred and the flask was placed into a refrigerator in a container with crushed ice for 10 h. After bromination, the excess bromine was titrated with sodium thiosulphate (1 mol L^{-1} solution) to discoloration. Flask content was transferred into a Teflon centrifugal tube, 5 mL of ethylacetate was added and the contents were shaken for 3 min and then centrifuged at 2000 × *g* for 5 min. After centrifugation, 1 mL of organic phase was pipetted onto a plastic microtube and 0.2 mL of triethylamine was added. The microtube was shaken and, after 15 min, it was centrifuged at 2000 × g for an additional 5 min. After centrifugation, the tube content was delivered to a glass vial and AA content was determined using gas chromatography/mass spectrometry (GC/MS).

Acrylamide was determined using the gas chromatograph Trace GC Ultra Finnigen with DB-WAX capillary column (30 m × 0.25 mm × 0.25 µm; J&W Scientific, Folsom, CA, USA) and the mass selective detector Trace DSQ ThermoFinnigan (Arcade, NY, USA). The temperature of the PTV injector working in the splitless mode was 200 °C (1 min). The column temperature programme was: 50 °C (1 min) → 15 °C min^{-1} to 150 °C (5 min). The GC/MS interface transfer line temperature was held at 200 °C. The mass selective detector operated in the selected ion monitoring mode with positive electron impact ionization. The carrier gas was

Table 1. Sample labelling based on the presence of the tested additives in gingerbread

	Additives				
	Leavening agent		Cation	Acidifying agent	
	Ammonium carbonate (A) $(NH_4)_2CO_3$	Sodium bicarbonate (S) $NaHCO_3$	Calcium chloride (Ca) $CaCl_2$	Citric acid (C) $C_6H_8O_7$	Sample labelling
Presence in the sample	+	–	–	–	A
	+	–	–	+	AC
	–	+	–	–	S
	–	+	–	+	SC
	+	–	+	–	ACa
	+	–	+	+	ACaC
	–	+	+	–	SCa
	–	+	+	+	SCaC

helium with a flow rate of 1.5 mL min^{-1}. Under these conditions, the retention time of the brominated AA and ($^{13}C_3$)acrylamide derivatives was 14.80 min.

Acrylamide was identified based on the retention time and the presence of the two specific ions of 2-bromopropenamide: *m/z* 149 $[C_3H_4{}^{79}BrNO]^+$ and *m/z* 151 $[C_3H_4{}^{81}BrNO]^+$. Quantification was performed using a calibration curve. Isotopically marked ($^{13}C_3$)acrylamide was used as an internal standard; molecular ions of 2-bromo($^{13}C_3$)propenamide *m/z* 152 and *m/z* 154, respectively were selected.

Physical parameters

The colour of the gingerbread samples was determined as reflectance values based on the L*a*b* system (lightness, redness, yellowness)[19] using a spectrophotometer CM-3500d (Konica Minolta, Osaka, Japan) containing an integrated spectral component, a D65 illuminator and a 10° observer.

Samples were measured directly (without a foil or a Petri dish) at room temperature. The L*a*b* values were determined at the top and the bottom surface and at the cross-cut section; the average value from these three determinations was used in the statistical evaluation. Three samples of each gingerbread variant were measured in duplicate ($n = 3$).

Firmness was measured using a TIRATEST 27025 testing machine (TIRA Maschinenbau GmbH, Schalkau, Germany). A puncture test was carried out with a cylindrical probe (diameter 3 mm) at a velocityof 50 mm s^{-1}. Eight samples of each gingerbread variant were measured ($n = 8$).

Sensory analysis

Sensory analysis was performed in a sensory laboratory equipped according to the standard ČSN ISO 8589 (1989) by a panel of nine trained assessors in three consecutive sessions (carried out on three different days; $n = 27$) and, subsequently, by 20 real consumers (two sessions in 2 days with different consumers each day were performed; $n = 20$).

A 100-mm unstructured line scale was used to evaluate of each of the nine descriptors (including anchor points placed at the very ends of a particular line scale): shape (regular → irregular); colour (light → dark), surface (intact → wrinkled; blistered); odour (typical, distinctive → bland, unimpressive); porosity (porous → non-porous); firmness (soft, pliable → tough); spice taste (distinctive → unimpressive); flavour (characteristic → bland; off-flavour, including specification); overall impression (excellent → inferior).

Statistical analysis

The differences between the gingerbread variants were evaluated using a one-way analysis of the variance ratio test, including post-hoc Tukey's test. The effects of the additives on the total and explained variability in AA content in gingerbread were tested by a generalized linear model of the main effects analysis of variance (ANOVA). All above-mentioned tests including correlation analysis were performed using STATISTICA, version 12 (StatSoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

The calibration curve regarding AA determination was linear and in the range 25–1540 μg kg^{-1} (AA concentration in real samples), with a correlation coefficient of 0.9989. Relative SD, limit of detection and limit of quantification (LOQ) were 10%, 10 μg kg^{-1} and 25 μg kg^{-1}, respectively. Recovery [determined using samples spiked with isotopically marked ($^{13}C_3$)acrylamide] ranged from 68% to 75%.

Labelling of gingerbread variants based on the presence of the tested additives is shown in Table 1. As shown in Table 2, all tested additives had a significant effect ($P < 0.01$) on AA content in gingerbread and, together, they explained more than 70% of total variability in the content of this toxin. The most important factor was the acidifying agent (citric acid), for which the presence/absence in dough accounted for more than half of the explained variability in AA content in gingerbread. Leavening agent explained more than one-third of AA content variability. The least important (but nevertheless still highly significant, $P < 0.01$) factor was the calcium cation.

The above-mentioned data are ilustrated in more detail in Fig. 1. Regarding the leavening agent, sodium bicarbonate decreased ($P < 0.05$) AA content in gingerbread to less than one-half (from 88.4 to 37.5 mg kg^{-1}) compared to ammonium carbonate. The presence of citric acid decreased ($P < 0.05$) AA content by more than three-fold compared to an unacidified sample. Despite the fact that the addition of calcium cation to dough had no statistically significant effect ($P = 0.093$), AA content in gingerbread produced with $CaCl_2$ was only 60% of the value established in gingerbread produced without the cation.

Several conclusions can be drawn based on the testing of the single gingerbread samples produced with different additives or their combinations (Fig. 2).

Table 2. Effect of the tested additives on the total and explained variability in AA content in gingerbread; generalized linear model of the main effects ANOVA

Effect	*F*-value	*P*-value	Ratio of the Total variability	Ratio of the Explained variability
Leavening agent[a]	21.9	0.0001	23.6	33.6
Cation[b]	8.6	0.0067	9.3	13.2
Acidifying agent[c]	34.9	0.0000	37.4	53.2

[a] $(NH_4)_2CO_3$ (84 mmol kg^{-1}) or $NaHCO_3$ (154 mmol kg^{-1}).
[b] Presence (83 mmol kg^{-1}) or absence of calcium chloride ($CaCl_2$).
[c] Presence (38 mmol kg^{-1}) or absence of citric acid ($C_6H_8O_7$).

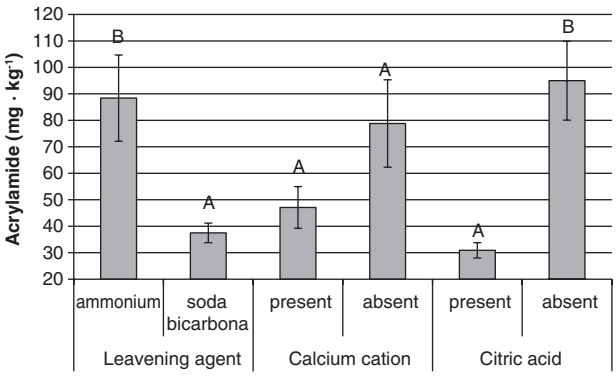

Figure 1. Effect of the tested variability factors on AA content in gingerbread. Ammonium carbonate: $(NH_4)_2CO_3$ (84 mmol kg^{-1}); sodium bicarbonate: $NaHCO_3$ (154 mmol kg^{-1}); calcium cation: $CaCl_2$ (83 mmol kg^{-1}); citric acid: $C_6H_8O_7$ (38 mmol kg^{-1}). A, B: means with different uppercase letters within a given factor (irrespective of other tested factors) indicate a statistically significant difference ($P < 0.05$; one-way ANOVA with *post-hoc* Tukey's test).

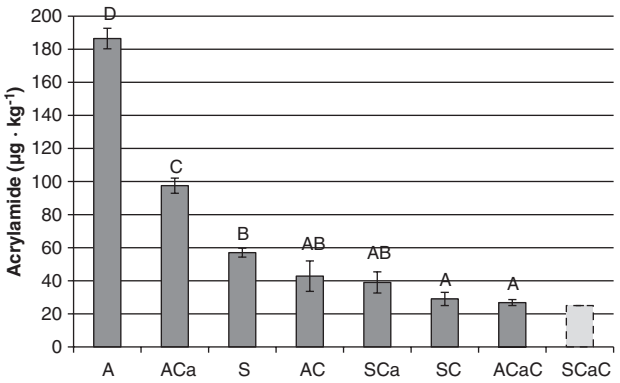

Figure 2. Acrylamide content in gingerbread prepared with ammonium carbonate [A; $(NH_4)_2CO_3$] or sodium bicarbonate [S; $NaHCO_3$] as leavening agent; with calcium chloride [Ca; $CaCl_2$] or without this form of calcium cation; with citric acid [C; $C_6H_8O_7$] or without this acidifying agent; including various combinations of the additives. A, gingerbread with ammonium carbonate [$(NH_4)_2CO_3$; 84 mmol kg^{-1}], without calcium cation, without citric acid; ACa, gingerbread with ammonium, with calcium cation [$CaCl_2$; 83 mmol kg^{-1}], without citric acid; S, gingerbread with sodium bicarbonate [$NaHCO_3$; 154 mmol kg^{-1}], without calcium cation, without citric acid; AC, gingerbread with ammonium, without calcium cation, with citric acid [$C_6H_8O_7$; 38 mmol kg^{-1}]; SCa gingerbread with sodium bicarbonate, with calcium cation, without citric acid; SC, gingerbread with sodium bicarbonate, without calcium cation, with citric acid; ACaC, gingerbread with ammonium, with calcium cation, with citric acid; SCaC, gingerbread with sodium bicarbonate, with calcium cation, with citric acid; mean ± SEM; SCaC sample has no error bar because the AA content in all four experiments was under the limit of detection (< 25 μg kg^{-1}); A–D: means with different uppercase letters indicate a statistically significant difference ($P < 0.05$; one-way ANOVA with *post-hoc* Tukey's test).

First, compared to the original recipe with ammonium carbonate as the only additive (sample A), AA content in all other samples was substantially reduced ($P < 0.05$): to 52% in gingerbread with ammonium carbonate+calcium chloride (ACa) and down to 14% in the sample containing ammonium carbonate with calcium chloride and citric acid (ACaC; AA content in the SCaC sample was below the limit of detection of 25 μg kg^{-1}).

Second, a synergic effect of the tested additives can be presumed from a trend in the decrease of AA content in the gingerbread samples with an increase of the additive number (Fig. 2). When AA content in gingerbread (y; μg kg^{-1}) was expressed as a function of the number of additives used (y), the regression was: $y = 158.8 - 47.94x$ ($r^2 = 0.42$; $P < 0.001$).

Sensory traits of the white pepper-gingerbread samples, including instrumentally measured colour parameters (L*a*b* values) and firmness, are reported in Table 3.

Based on the sensory analysis, the lightest ($P < 0.05$) gingerbread variants were those containing both citric acid and calcium ions (SCaC and ACaC). The darkest ($P < 0.05$) was the AC-variant. These data corresponded to the instrumentally measured L* values (Table 3). It follows from these results that citric acid maintained the dark coloration of the sample caused by the Maillard reaction. At the same time, however, it decreased ($P < 0.05$) the AA content to 23% of the control variant A (Fig. 2).

The relationship between the AA content and gingerbread lightness was stronger in the case of the L* value compared to colour evaluated by sensory analysis: $r = -0.49$ and $r = +0.31$, respectively ($P < 0.01$ in both cases).

The highest level ($P < 0.05$) of instrumentally measured firmness (TIRATEST) was established in a ACaC sample (5.38 N) (Table 3); the correlation coefficient between physically and subjectively measured firmness was $r = 0.54$ ($P < 0.01$).

When evaluated by the trained assessors, the best overall impression ($P < 0.05$) was established in the S-sample. However, the SCaC-variant was no different ($P < 0.05$) in this parameter compared to either the S- or the control A gingerbread based on the analysis of the real consumers. Moreover, the low (i.e. favourable) values reported for most of the sensory parameters of the SCaC-sample (Table 3) suitably complement a synergic effect of the tested additives on AA reduction.

The LOQ established in the present study (25 μg kg^{-1}) is well within the limit recommended for the given analytical method for gingerbread (50 μg kg^{-1}) by the European Food Safety Authority.[20] An LOQ of 50 μg kg^{-1} was also reported by Geng *et al.*[21] for various fried products. However, it is also possible to decrease LOQ by one order of magnitude, as established in an experiment by Troise *et al.*[22] for three different kinds of food.

By contrast, AA recoveries found in the present study (68–75%) are somewhat lower compared to the values reported by Geng *et al.*[21] (90–102%), as well as by Pittet *et al.*[23] for wheat semolina samples (93–104%) and Troise *et al.*[22] for French fries, ground coffee and cookies (90–97%).

AA content in white pepper-gingerbread produced using the original recipe with ammonium carbonate (187 μg kg^{-1}; sample A) (Fig. 2) was lower than indicated by most of the literature

Table 3. Parameters of the sensory analysis of the gingerbread samples, including instrumentally measured traits

Trait		Sample (mean ± SEM)[a] A	AC	S	SC	ACa	ACaC	SCa	SCaC
trained assessors ($n = 27$)	Shape[b]	40 ± 5^{A}	40 ± 6^{A}	25 ± 4^{A}	26 ± 4^{A}	22 ± 4^{A}	28 ± 5^{A}	26 ± 6^{A}	23 ± 5^{A}
	Colour[b]	61 ± 4^{D}	80 ± 3^{E}	47 ± 4^{C}	32 ± 3^{B}	37 ± 3^{BC}	13 ± 2^{A}	26 ± 3^{B}	7 ± 1^{A}
	Surface[b]	42 ± 5^{C}	41 ± 6^{C}	33 ± 5^{BC}	32 ± 4^{ABC}	39 ± 5^{C}	18 ± 4^{AB}	27 ± 4^{ABC}	12 ± 3^{A}
	Odour[b]	55 ± 5^{B}	56 ± 5^{B}	21 ± 4^{A}	45 ± 5^{B}	41 ± 5^{AB}	55 ± 6^{B}	40 ± 5^{AB}	36 ± 6^{AB}
	Porosity[b]	31 ± 4^{A}	32 ± 5^{A}	44 ± 5^{ABC}	56 ± 4^{BC}	36 ± 5^{AB}	44 ± 5^{ABC}	58 ± 6^{C}	45 ± 6^{ABC}
	Firmness[b]	56 ± 4^{BC}	74 ± 4^{C}	9 ± 2^{A}	50 ± 5^{B}	19 ± 2^{A}	62 ± 5^{BC}	55 ± 5^{BC}	61 ± 6^{BC}
	Spice taste[b]	40 ± 5^{A}	47 ± 6^{A}	40 ± 4^{A}	38 ± 5^{A}	26 ± 5^{A}	38 ± 6^{A}	45 ± 5^{A}	37 ± 5^{A}
	Flavour[b]	49 ± 6^{BC}	66 ± 5^{C}	24 ± 5^{A}	46 ± 5^{BC}	50 ± 6^{BC}	68 ± 5^{C}	51 ± 5^{BC}	38 ± 5^{AB}
	Overall impression[b]	53 ± 5^{BCD}	71 ± 4^{D}	23 ± 5^{A}	46 ± 4^{B}	50 ± 5^{BC}	68 ± 4^{CD}	50 ± 4^{BC}	46 ± 5^{B}
Real consumers (n = 20)	Shape[b]	40 ± 5^{A}	35 ± 3^{A}	30 ± 3^{A}	37 ± 4^{A}	29 ± 2^{A}	33 ± 4^{A}	34 ± 5^{A}	24 ± 3^{A}
	Colour[b]	56 ± 3^{D}	74 ± 4^{E}	57 ± 3^{D}	51 ± 4^{CD}	34 ± 2^{B}	26 ± 4^{AB}	38 ± 2^{BC}	18 ± 2^{A}
	Surface[b]	66 ± 4^{D}	54 ± 6^{CD}	51 ± 5^{CD}	42 ± 5^{ABC}	53 ± 5^{CD}	49 ± 5^{BCD}	30 ± 3^{AB}	25 ± 4^{A}
	Odour[b]	49 ± 5^{A}	59 ± 5^{A}	45 ± 5^{A}	55 ± 5^{A}	56 ± 5^{A}	60 ± 6^{A}	57 ± 6^{A}	51 ± 4^{A}
	Porosity[b]	25 ± 3^{A}	38 ± 5^{AB}	36 ± 4^{AB}	44 ± 6^{ABC}	46 ± 4^{BC}	45 ± 5^{BC}	61 ± 5^{C}	55 ± 5^{BC}
	Firmness[b]	33 ± 4^{A}	70 ± 3^{B}	28 ± 3^{A}	38 ± 4^{A}	35 ± 4^{A}	74 ± 3^{B}	63 ± 5^{B}	72 ± 4^{B}
	Spice taste[b]	41 ± 4^{A}	31 ± 4^{A}	47 ± 4^{A}	43 ± 4^{A}	38 ± 4^{A}	46 ± 4^{A}	43 ± 4^{A}	43 ± 4^{A}
	Flavour[b]	49 ± 3^{A}	51 ± 4^{A}	48 ± 4^{A}	46 ± 5^{A}	52 ± 4^{A}	61 ± 4^{A}	49 ± 4^{A}	49 ± 4^{A}
	Overall impression[b]	51 ± 4^{A}	59 ± 3^{AB}	53 ± 4^{AB}	53 ± 5^{AB}	59 ± 5^{AB}	68 ± 3^{B}	51 ± 5^{A}	50 ± 3^{A}
Firmness (N)[c]		3.1 ± 0.03^{C}	4.73 ± 0.11^{E}	1.78 ± 0.02^{A}	2.84 ± 0.06^{C}	2.66 ± 0.03^{B}	5.38 ± 0.13^{F}	4.40 ± 0.06^{D}	4.67 ± 0.09^{DE}
L*[d]		54 ± 0.07^{C}	49 ± 0.15^{A}	57 ± 0.20^{D}	61 ± 0.18^{G}	52 ± 0.16^{B}	60 ± 0.31^{F}	59 ± 0.12^{E}	65 ± 0.19^{H}
a*[e]		12 ± 0.04^{D}	12 ± 0.08^{E}	13 ± 0.07^{F}	10 ± 0.12^{B}	12 ± 0.07^{D}	10 ± 0.10^{B}	11 ± 0.04^{C}	9 ± 0.07^{A}
b*[f]		29 ± 0.13^{C}	27 ± 0.17^{A}	37 ± 0.06^{G}	32 ± 0.06^{E}	28 ± 0.09^{B}	29 ± 0.27^{C}	34 ± 0.07^{F}	31 ± 0.11^{D}

[a] The substances (including their combinations) added to the dough were: A, ammonium, $(NH_4)_2CO_3$; S, sodium bicarbonate, $NaHCO_3$; Ca, calcium chloride, $CaCl_2$; C, citric acid, $C_6H_8O_7$.
[b] Points on an unstructured 100-mm scale with defined anchor points; evaluated by nine trained assessors in three sessions ($n = 27$) and by 20 real consumers (10 different consumers in each of two sessions; $n = 20$), respectively; descriptor anchor points: shape (regular → irregular); colour (light → dark), surface (intact → wrinkled; blistered); odour (typical, distinctive → bland, unimpressive); porosity (porous → non-porous); firmness (soft, pliable → tough); spice taste (distinctive → unimpressive); flavour (characteristic → bland; off-flavour, including specification); overall impression (excellent → inferior) .
[c] Measured by a TIRATEST machine.
[d] Lightness, [e] redness and [f] yellowness (as measured by a Konica Minolta CM-3500d spectrophotometer; Centre Internationale de l'Eclairage, 1976).
[A–H] Means with different superscript uppercase letters within a line differ at $P < 0.05$ (*post-hoc* Tukey's test).

data regarding AA content in a 'common' gingerbread (mean values, μg kg^{-1}): 415,[3] 890,[24] 481,[6] 303 (median; reporting European Union database on AA levels in food)[25] and 415.[20] A lower value (46 μg kg^{-1}) was reported by Papoušek *et al.*[26]

As far as the above-mentioned AA levels are concerned, gingerbread can be classified somewhere in the middle of the range of the relevant foods, from cereal-based baby food (31 μg kg^{-1})[3] to cocktail snacks (1060 μg kg^{-1}) or potato crisps (1249 μg kg^{-1})[24] to coffee substitutes (1350 μg kg^{-1}).[20]

An AA-promoting effect of the ammonium carbonate as a baking agent $(NH_4)_2CO_3$ found in the present study (Figs 1 and 2) confirms the results of previous experiments investigating the AA content in foods.[5,7,27] The N atom from the ammonium carbonate is not incorporated into AA and the amidation of acrylic acid by ammonia does not contribute to the AA content in gingerbread;[6] the promoting effect of ammonium carbonate is indirect as a result of the provision of more reactive carbonyls (glyoxal) originating from the reaction of ammonia with reducing sugars.

Substituting ammonium carbonate by sodium bicarbonate decreased ($P < 0.05$) AA content in the white pepper-gingerbread to 42% in the present study (Fig. 1). Similar results were reported by Amrein *et al.*[27] AA content in gingerbread decreased by 60%. $NaHCO_3$ reduced the AA content in gingerbread to one-third compared to $(NH_4)_2CO_3$ in an experiment by Amrein *et al.*[6] On the other hand, adding $NaHCO_3$ to a dough formulation resulted in only a weak decrease (13%) in AA content in fried fine bakery products.[16]

Divalent cations (Ca^{2+}) added to the dough can markedly reduce AA content in foods[28] by interacting with asparagine and consequently preventing Schiff base intermediate formation.[29] When calcium cation was added to the dough in the present study, the AA content in the white pepper-gingerbread was only 60% of the value established in the variants produced without Ca^{2+} (Fig. 1). Salazar *et al.*[30] similarly reported a reduction of AA in tortilla chips made of flours prepared from corn nixtamalized with $Ca(OH)_2$.

The substantial effect of adding citric acid into the dough on the reduction of the AA content in white pepper-gingerbread in the present study (with a more than three-fold decrease) (Fig. 1) was likely a result of the Maillard reaction being pH dependent: at a lower pH, the reactive amino group of the precursor amino acids (asparagine) is protonated to the non-reactive NH_3^+ group[3] and it is therefore unavailable for further reactions leading to AA formation.[9] Amrein *et al.*[27] reported a decrease of AA in gingerbread from 500 to 120 μg kg^{-1} when 500 g of citric acid per 100 kg of dough was added. Similarly, 0.5 and 5.0 g of citric acid per 100 g of dough decreased the AA content in gingerbread by a factor 4 and 40, respectively, in an experiment reported by Amrein *et al.*[6] This effect also applies to other thermally treated foods: soaking or

blanching potatoes in a citric acid solution reduced AA in potato crisps by 50%.[31]

The first requirement when deciding on the possible presence of synergistic effects is to define the null interaction in the joint action of two or more agents using concentration–time response models.[32] The derivation of the mathematical kinetic models testing the synergistic action of more additives on the decrease of production of any toxicant (including AA) is not described in available literature (in contrast, for example, to synergy of an effect of more toxicants on living systems[33]). Nevertheless, based on the logistic-exponential model of Zhang and Zhang,[34] the chemistry of the joint effect of additives tested in the present study on AA reduction (which likely manifests itself mainly during the formation-predominant kinetic stage, but also partially during the elimination-predominant kinetic stage[34]) can be summarized as described below.

$NaHCO_3$ decreases sugar fragmentation (i.e. less glyoxal is generated from glucose/fructose and consequently less AA is formed).[27] Changes in the ionic strength induced by the positively-charged Na^+ ion affect the rate of the addition reactions of the amino groups of amino acids to the double bonds of conjugated vinyl compounds such as AA; changes in the ionic microenvironment contribute to the mitigating effects of positively-charged metal ions.[35] Mono- and divalent cations (Na^+, Ca^{2+}) interact with asparagine and prevent Schiff base intermediate formation and, consequently, AA generation.[29] $CaCl_2$ ionic and electronic associations with asparagine supress early-stage Maillard reactions.[35]

Moreover, metal cations (Na^+, Ca^{2+}) cause pH reduction; amongst other things, increased acidity reduces AA formation as a result of hydrolysis of the carboxamide group leading to aspartic acid.[28] The beneficial effects of a low pH (caused by the above-mentioned metal cations, but predominantly by organic acids, including the citric acid tested in the present study) result not only from protonation of the reactive free α-NH_2 group of asparagine to the non-reactive α-NH_3^+ form, but also from partial acid-catalyzed hydrolysis of asparagine to aspartic acid and of AA to acrylic acid.[35] The protonation of the α-amino group of asparagine caused by organic acids hinders the formation of the N-substituted glycosylamine.[5]

Regarding sensory evaluation, Pedreschi *et al.*[3] recommend utilizing calcium salts only in a low concentration to avoid an off-taste. This finding was partly confirmed in the present study by a less favourable flavour of the SCa variant ($P < 0.05$) compared to the corresponding sample without calcium salt (51 and 24 points on a 100-mm evaluation scale, respectively) (Table 3). On the other hand, the flavour or the overall impression of the gingerbread variants containing $CaCl_2$ did not differ ($P > 0.05$) from control sample A (Table 3).

The adverse effect of low pH (citric acid) on taste is dependent on the organic acid concentration.[14]The amount of organic acids (citric, tartaric) should be limited to 250 mg per 100 g (i.e. 13 mmol kg^{-1}) of dough to comply with the sensory standards for gingerbread.[27] In the present study, the flavour of the gingerbread variants containing citric acid (38 mmol kg^{-1}) did not differ ($P > 0.05$) from control A (Table 3). On the other hand, citric acid increased ($P < 0.05$) gingerbread browning in the present study (compare A and AC variants) (Table 3), in contrast to the results of Amrein *et al.*,[6] who reported a reduction of browning in gingerbread after adding 52 mmol kg^{-1} of citric acid.

Regarding the leavening agents, Kukurová *et al.*[7] reported that cookies produced with the addition of $(NH_4)_2CO_3$ were the most pleasant, which does not agree with the better ($P < 0.05$) flavour and overall impression of gingerbread variants produced with $NaHCO_3$ compared to $(NH_4)_2CO_3$ found in the present study (Table 3). Moreover, the results of the present study (insignificant differences between A and AC variants with respect to shape and porosity, respectively) (Table 3) do not agree with the data reported in the study by Amrein *et al.*[6], for which the addition of sodium bicarbonate (up to 199 mmol kg^{-1}) resulted in an alkaline taste and insufficient leavening.

Regarding colour parameters, Amrein *et al.*[6] and Haase *et al.*[17] reported an insignificant difference in the L* value for biscuits produced with $(NH_4)_2CO_3$ and $NaHCO_3$, respectively. This finding does not agree with the results of the present study in which there was a higher L* value ($P < 0.05$) for the S-gingerbread compared to the A-sample, as confirmed also by sensory analysis (the colour of the S- and A-gingerbread was 47 and 61 points, respectively; $P < 0.05$) (Table 3).

On the other hand, the higher values for the colour parameters a* and b* with respect to cookies produced with $NaHCO_3$ compared to $(NH_4)_2CO_3$ in an experiment by Kukurová *et al.*[7] agree with our data: both a* and b* values of the S-variants were higher ($P < 0.05$) compared to the A-samples (Table 3).

However, a relationship between the AA content and the colour parameters was not so strong in the present study (although highly significant, correlation coefficients were < 0.5) as that reported by Ciesarová *et al.*[16] for fried fine bakery products (correlation coefficients in the range 0.88–0.98).

Despite the product tested in the present study comprising was a small-volume gingerbread produced using the key substances promoting AA formation (ammonium carbonate, fructose-rich honey), the AA content in the original (control) variant was relatively low (below 200 µg kg^{-1}) compared to more common types of gingerbread (mostly > 400 µg kg^{-1}).

Replacing ammonium carbonate with sodium bicarbonate, adding calcium ions or lowering the pH of the dough using citric acid decreased AA content by 47–86% compared to the original recipe.

An effect of each of the aforementioned additives on AA production is well known. However, the present study contributes to the state of knowledge about AA by demonstrating a synergic effect of the combination of different additives. So far, additive or synergic effects have been studied only marginally in this context: Hanley *et al.*[11] reported a greater AA reduction in fried potatoes using a combination of glutamine (acidic amino acid) and lysine (basic amino acid) compared to treatment with each of these amino acids individually. Similar results were achieved using a combination of citric acid and some amino acids.[12,14]

The highest AA reduction achieved in the present study was an 86% decrease (compare the A and SCaC variants shown in Fig. 2), which is less than the 97% decrease reported by Ciesarová *et al.*[36] using asparaginase. However, in contrast to their experiment,[36] the present study suggests an easy and an inexpensive alternative for the production of gingerbread.

Moreover, the combination of additives proposed in the present study ($NaHCO_3$/Ca^{2+}/citric acid) decreased AA content in gingerbread below the limit of detection without any significant deterioration of the sensory parameters.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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https://doi.org/10.17221/317/2018-CJFS

The occurrence of the selected *Fusarium* mycotoxins in Czech malting barley, harvested in 2012–2017

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Citation: Svoboda Z., Mikulíková R., Benešová K., Běláková S. (2019): The occurrence of the selected *Fusarium* mycotoxins in Czech malting barley, harvested in 2012–2017. Czech J. Food Sci., 37: 439–445.

Abstract: In 2012–2017, the occurrence of deoxynivalenol, zearalenone and T-2 toxin in 592 samples of malting barley from different regions of the Czech Republic was studied using the ELISA immunochemical method. On average, the total content of positive samples was 18.6% for deoxynivalenol (DON), 9.5% for zearalenone (ZEN) and 20.5% for T-2 toxin. The highest values measured were 917 µg/kg for DON (2012), 42 µg/kg for ZEN (2017) and 199 µg/kg for T-2 toxin (2013). The maximal DON and ZEN contents in cereals intended for food production are limited by the Commission Regulation (EC) No. 1881/2006, the EU limit from 2013 applies for the sum of T-2 and HT-2 toxins. Concentrations of any of the mycotoxins studied did not exceed the EU limit in any barley samples.

Keywords: malting barley; *Fusarium;* mycotoxins; ELISA

Mycotoxins are toxic secondary metabolites produced by microscopic filamentous fungi, namely *Fusarium, Aspergillus* and *Penicilium* sp. They are thermostable, low-molecular substances with different chemical structures and a number of negative effects on human and animal health. They commonly contaminate economic crops, food and feed (Krska *et al.* 2007), representing thus a major global economic problem (Ji *et al*. 2016). With the ongoing climate change, an increased risk of the occurrence of mycotoxigenic fungi and mycotoxins is expected due to the adaptation of fungal pathogens to altered conditions (Geisen *et al.* 2017; Medina *et al*. 2017). The spectrum of explored and described mycotoxins is expanding (Václavíková *et al.* 2013; Bolechová *et al.* 2015; Luz *et al.* 2017). Modified forms of mycotoxins represent new research trends (Freire & Sant'ana 2018). Cereals may be simultaneously contaminated with two or more mycotoxins (Běláková *et al.* 2014; Pleadin *et al.* 2013, 2017), detection of one mycotoxin may indicate the presence of another, and this contamination influences the cumulative toxic effect.

Fusarium fungi belong to the most important producers of mycotoxins, at the same time they are important pathogens of agricultural crops (Placinta 1999; Morcia *et al.* 2013). The occurrence of fusarioses and mycotoxins is greatly influenced by the course of weather. The occurrence, quantity and type of mycotoxin may depend on the environment, the type of fungi present, the severity of the infection and the cultivar or crop type. In malting barley, the presence of mycotoxins can be significantly affected by the storage and post-harvest treatment of the grain. Another factor that can significantly affect the intensity of the *Fusarium* occurrence is the technology of growing and susceptibility of the variety, pre-crop, soil cultivation and, also, by the application of fungicides. Relationships and interactions arising among the plant, pathogenes, microscopic fungi, environment, manner of plant protection and treatment are very complex and they significantly affect mycotoxin production and content (Terzi *et al.* 2014).

The most well-known toxins produced predominantly by filamentous *Fusarium* fungi include the group of trichothecene mycotoxins, zearalenones and the group of fumonisins (Wolf-Hall 2007; Capriotti *et al.* 2010).

Supported by the Ministry of Agriculture of the Czech Republic, Project No. RO 1917.

https://doi.org/10.17221/317/2018-CJFS

The contamination of the organism with trichothecenes results in a wide variety of manifestations; various syndromes may include reduced intake or total rejection of food, skin irritation and dermal necrosis, vomiting, diarrhea and bleeding. Trichothecenes have also been described as immunosuppressants and inhibitors of protein and DNA synthesis (Mostrom 2011). All trichothecenes, without exception, exhibit a higher or lower degree of toxicity for the animals; they also exhibit an insecticidal effect. Phytotoxic activity has also been described (Abbas *et al.* 2013). Research into the acute and chronic toxicity of these substances is ongoing (Groopman *et al.* 2013; Escrivá *et al.* 2015).

DON, the most observed trichothecene, is often detected in cereals. Its occurrence in cereals varies from year to year depending mainly on the weather in the given locality, type of pre-crop and resistance of the variety. It is an indicator of a possible contamination with other mycotoxins. DON often co-occurs with the acetylated isomers of 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and other *Fusarium* toxins, such as nivalenol (NIV), zearalenone (ZEN), T-2 toxin (T-2) and HT-2 toxin (HT-2) (Pestka 2007; Bryla *et al.* 2016).

In zearalenone and its metabolites, estrogenic effect, reproduction and developmental toxicity in animals have been proven; they pose hepatotoxic, hematotoxic, immunotoxic and genotoxic effects (Zinedine *et al.* 2007).

Controlling the health safety of consumed food and feed in terms of the presence of mycotoxins is an absolute prerequisite for the health protection of the population. Control programs for monitoring mycotoxins in food and feed have been introduced in many countries, including the European Union (EU). Commission Regulation (EC) No. 1881/2006, supplemented by Commission Regulation (EC) No. 1126/2007, set the Maximum Levels (ML) of selected mycotoxins in cereals and other foodstuffs. The currently valid European legislation stipulates the content of selected *Fusarium* mycotoxins in unprocessed cereals, including malting barley: 1250 µg/kg for DON and 100 µg/kg for ZEN. For the sum of T-2 and HT-2 toxins, the indicative limit of 200 µg/kg is used. The indicative levels are based on the occurrence data available in the EFSA database and they are not feed and food safety levels (EU Commision Recommmendation 2013/165/EU).

This study summarizes the results of six-year (2012–2017) monitoring of the selected trichothecene mycotoxins (DON and T-2 toxin) and ZEN in malting barley from the 14 regions in the Czech Republic.

MATERIAL AND METHODS

Standards and chemicals. Methanol (analytical grade) for the mycotoxin extraction was purchased from Sigma-Aldrich (Germany). Certified reference materials (CRMs) – naturally contaminated wheat (for DON and T-2 toxin) and corn (ZEN) was purchased from Trilogy (USA). ELISA test kits (AgraQuant® Deoxynivalenol Assay 0.25/5.0, AgraQuant® Zearalenone Plus Assay 25/1000 and AgraQuant® T-2 Toxin Assay 20/500) were provided by Romer Labs (Tulln, Austria).

Barley samples. In 2012–2017, totally 592 samples of malting spring barley were analysed (*i.e.* the varieties KWS Ariane, Blanik, Bojos, Francin, Kangoo, KWS Irina, Laudis 550, Malz, Marthe, Overture, Prestige, Radegast, Sebastian, Sunshine, SY Tepee, Wintmalt, Xanadu, Manta, Pionier, RGT Plane), *i.e.* in 2012 (n = 117), 2013 (n = 98), 2014 (n = 116), 2015 (n = 109), 2016 (n = 110) and 2017 (n = 42). The samples were obtained directly from the growers from all regions of the Czech Republic as described at Běláková *et al.* (2014).

Determination of mycotoxins. Ground barley sample (20 g) was extracted with 100 ml of deionized H_2O (for the DON determination) or of 100 ml of MeOH : H_2O 70 : 30 (for ZEN and T-2 toxin), shaken for 50 min, centrifuged at 4000 rpm for 15 min, an aliquot of the supernatant (100 µl) was diluted according to the instructions or used directly for the analysis.

Mycotoxin concentrations (DON, ZEN and T-2 toxin) were determined using competitive ELISA test kits as instructed by the kit manufacturer. Each kit contains a microtiter plate with 96 wells coated with antibodies , standard solutions containing different concentrations of mycotoxins, an enzyme conjugate, anti-antibody, substrate and chromogen solution, stop solution, and washing and dilution buffers. The calibration curve was constructed according to the instructions in the set, repeatability – the relative standard deviation (% RSD) was calculated from 10 determinations. The LOD and LOQ values are given by the manufacturer. The quality control of the method was established using the appropriate CRM**s**. The parameters of the method for targeted mycotoxins are presented in Table 1.

RESULTS AND DISCUSSION

The occurrence of the individual mycotoxins in barley in the individual years. The contents of mycotoxins DON, ZEN and T-2 toxin in barley are summarized in Table 2. The minimal and maximal

https://doi.org/10.17221/317/2018-CJFS

Table 1. Parameters of the ELISA method for targeted mycotoxins

Mycotoxin	LOD (µg/kg)	LO (µg/kg)	RSD (%)	CRM declared (µg/kg)	CRM measured (µg/kg)
DON	200	250	9.5	700 ± 100	859.0 ± 81.6
ZEN	20	25	10.2	454.2 ± 37.6	419.8 ± 42.8
T-2	10	20	7.9	57.1 ± 10.5	60.9 ± 4.8

LOD – limit of detection; LOD – limit of quantification; CRM declared – given by the manufacturer; CRM measured – measured by the laboratory

values and percentage of positive samples in each harvest are given, samples where the mycotoxin content was detected above the quantification limit are considered positive. To calculate the average values in individual years, the mycotoxin content was taken into account only in positive samples. For the evaluation, the basic statistical characteristics were used (means, absolute and relative frequencies). Results are expressed in Figures 1–3.

The level of DON contamination ranged from 4.8% in 2017 to 29.1% in 2012. Since the ELISA immunochemical screening test for DON has a higher detection limit, the samples with any lower DON concentration were not captured. The highest determined DON content (917 µg/kg) was found in harvest 2012. In all other samples tested, DON ranged from 250 to 668 µg/kg and was comparable to harvests in the next years when the average DON content moved around 300 µg/kg.

The lowest level of contamination was evident in mycotoxin ZEN, when in 2012, 2013, and 2015, no positive samples were found; in years 2014, 2016 and 2017 12–30% positive samples with the maximal ZEN content of 42 µg/kg were detected

No sample positive for the presence of T-2 toxin was found in the years 2012 and 2017. In 2013–2016, the level of contamination moved in the scope from 11.8% to 46.9%, with the highest concentration (199 µg/kg) measured in 2013. Average mycotoxin contents are summarized in Figure 1.

The most contaminated harvests were those in 2014 and 2016, when all three monitored mycotoxins were detected in some samples. In 2012, no sample positive for ZEN and T-2 toxin contents was found and 29% of the samples were positive for DON content, including the above mentioned sample with the highest DON content. In 2017, no tested sample was positive for T-2 toxin, 11.9% of the samples contained ZEN above LOQ and only 4.8% of the samples were positive for the DON content.

From Figure 2 which summarizes the total occurrence of mycotoxins in barley in the period of

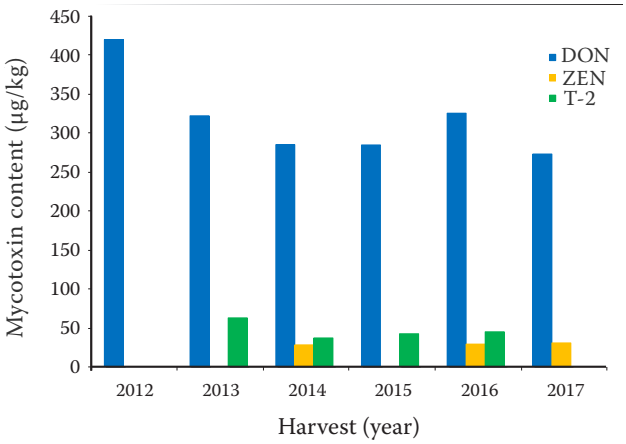

Figure 1. Average content of the studied mycotoxins in the individual years

Table 2. Occurrence of the studied mycotoxins (µg/kg) in barley in the Czech Republic in 2012–2017

Harvest	DON				ZEN				T-2			
	analysed/positive	(%)	min.	max.	analysed/positive	(%)	min.	max.	analysed/positive	%	min.	max.
2012	117/34	29.1	252	917	117/0	0	0	0	117/0	0	0	0
2013	9898/13	13.3	251	568	98/0	0	0	0	98/26	26.5	26	199
2014	116/23	19.8	250	577	116/18	15.2	25	39	116/39	33.6	25	90
2015	109/8	7.3	255	348	109/0	0	0	0	109/43	46.9	26	145
2016	110/30	27.3	251	668	110/33	30.0	26	39	110/13	11.8	25	129
2017	42/2	4.8	260	286	42/5	11.9	28	42	42/0	0	0	0
Total	592/110	18.6			592/56	9.5			592/121	20.4		

https://doi.org/10.17221/317/2018-CJFS

2012–2017, it is evident that on average the total content of the positive samples in the given years was 18.6%, 9.5%, and 20.5% of DON, ZEN, and T-2 toxin, respectively.

Some processes used in beer production act as a decontamination process, although some of the mycotoxins present in barley and malt may pass to the final product, nevertheless, the content found does not pose a significant health hazard to consumers (Wolf-Hall 2007; Karlovsky *et al.* 2016; Pascari *et al.* 2017).

The effect of the variety and previous crop on the mycotoxin content. To evaluate the effect of a variety, only varieties with a sufficient number of samples (> 30) were selected, these were the varieties Bojos, Blaník, Laudis 550, and Malz. The numbers of the mentioned varieties were as follows: Bojos 208 samples, Laudis 550 61 samples, Blaník 39 samples and Malz 152 samples. Figure 3 shows that the variety did not have effect on the DON, ZEN and T-2 toxin content.

Maize, wheat, sugar beet, potatoes, spring barley, and rape were grown as a previous crop before malting barley. Maize affected the DON content most (detected average value 420.5 µg/kg), the adverse effect of maize as a pre-crop on Fusaria contamination and subsequently higher mycotoxin levels has already been confirmed by previous research (Polišenská *et al.* 2012; Qiu *et al.* 2016), further, winter wheat (341.4 µg/kg), rape (324.8 µg/kg) and sugar beet (310.3 µg/kg). The effect of the previous crop on ZEN and T-2 toxin was not confirmed. Figure 3 shows the effect of the variety on mycotoxin content.

Comparison with the available data in the European countries. Barley contamination with *Fusarium* mycotoxins is quite common in the European countries and worldwide. In the period of 2005–2010, Polišenská *et al.* (2012) analysed 327 samples of malting barley for the DON content using the ELISA assay. The level of contamination (when DON was > 40 µg/kg) varied from 63% in 2010 with the highest value of DON 227 µg/kg to 96% in 2009 when the highest measured value was 7050 µg/kg. In 2006, 2007, and 2010, the same authors also analysed ZEN content when in 2006, 100% positive samples were found (> 2 µg/kg) and the maximal detected value was 222 µg/kg. Conversely, in 2007 and 2010, 45%, and 22% of positive samples with a maximum of 48 and 14 µg/kg, respectively, were found (Polišenská *et al.* 2012). The results are practically comparable with Běláková *et al.* (2014), who monitored 325 samples of the malting barley harvested from 2008 to 2011. The maximum measured DON levels varied from 106.1 to 2213.5 µg/kg, ZEN from 21.4 to 59.4 µg/kg and ΣT-2, HT-2 toxins from 53.4 to 145 µg/kg (Běláková *et al.* 2014).

In 2012, monitoring of cereals including 20 samples of barley for the mycotoxin presence was conducted in Ireland (Food Safety 2015). Twelve samples contained DON at low concentrations (to 200 µg/kg), on the other hand, ZEN was found in 11 samples, the highest measured value was 150 µg/kg. T-2 and HT-2 were not found at quantifiable concentrations. The samples were analysed by the method of liquid chromatography with UV, FLD or MS detection.

In Switzerland, in 2013 and 2014, 280 and 160 barley samples for the presence of *Fusarium* mycotoxins were tested by the HPLC/MS method. DON was the prevailing mycotoxin in both harvests with the average concentration of 235 µg/kg in 2013 and 47 µg/kg in 2014. In 2013 the highest determined concentration was 4860 µg/kg, in 2014 of 1725 µg/kg. The average ZEN content was 3.7 µg/kg and 10.2 µg/kg in 2013

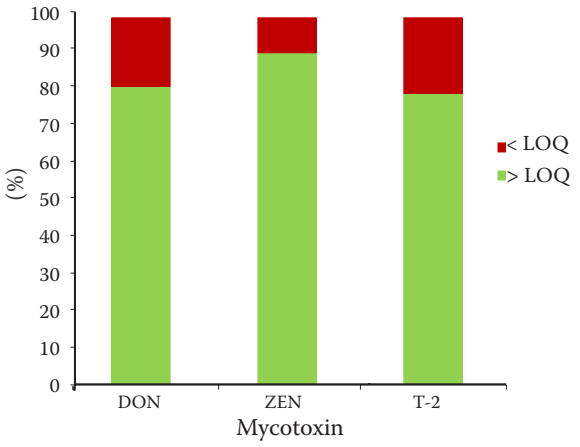

Figure 2. Total mycotoxin content in 2012–2017

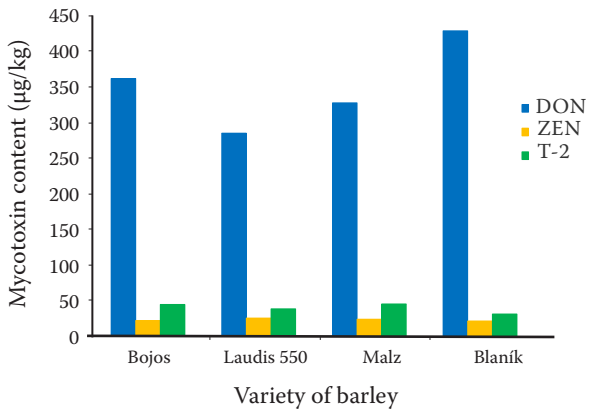

Figure 3. The effect of the variety on mycotoxin content

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and 2014, respectively. The highest concentration of 240 µg/kg was determined in 2014. T-2 and HT-2 toxins were detected in harvest 2013 in 16 (6%) and in harvest 2014 in 10 (6%) samples and the average concentration was 15.4% or 9.7% (Schöneberg *et al.* 2016).

In 2013–2015, research into mycotoxins in cereals in the area of Bosnia and Herzegovina investigated a total of 58 barley samples for the presence of mycotoxins DON, ZEN, and fumonisins by the ELISA method. The published data showed that DON was detected totally in 22 samples (38%) in quantifiable concentrations with the average content of 365 µg/kg and the highest detected values of 578 µg/kg, ZEN was found in 20 samples (34%) with the highest measured concentration of 84 µg/kg (Pleadin *et al.* 2017). We can state that considering different weather conditions and used analytical methods the occurrence of the studied mycotoxins is comparable.

In Poland, Bryla *et al.* (2016) analysed 26 mycotoxins in 147 various grain samples from the harvest 2014 included 8 samples of spring barley and 16 samples of winter barley. Four samples of spring barley contained DON with maximum concentration of 222 µg/kg and six samples contained ZEN with highest concentration of 31 µg/kg, all 16 samples of winter barley contained DON with maximum 1602 µg/kg and ten samples contained ZEN with maximum 19 µg/kg.

Similar data have been also presented in previously published research. For example, in 2001, eight samples harvested in Eastern Slovakia were analysed using LC/UV, five of which were contaminated with DON of the average concentration of 187 µg/kg with the highest concentration found 530 µg/kg (Čonková *et al.* 2006). Edwards (2009) analysed in UK totally 446 barley samples from harvests 2002 to 2005 (approximately 25% of which were collected from organic growers) for the presence of 10 trichothecene mycotoxins by the GC/MS method. DON was found in 57% of the tested samples and only one of them from harvest 2005 exceeded ML EU with concentration of 1416 µg/kg. 12% of samples were T-2 and HT-2 positive of highest concentration of 138 µg/kg. The author stated that no significant differences in contamination between barleys grown in conventional and organic farming were found. Pleadin *et al.* (2013) mapped concentration of *Fusarium* mycotoxins in cereals in six different Croatian regions, including 34 barley samples. They reported 53% samples contaminated by DON with an average content of 228 µg/kg and the highest content of 342 µg/kg, 9% of samples positive for ZEN with the average content of 32 µg/kg and the highest content of 68 µg/kg. 32% of the samples contained T-2 with the average value of 13 µg/kg and the highest content of 26 µg/kg. On the other hand, Tabuc *et al.* (2009) who within their research using ELISA examined totally 21 barley samples harvested in 2002–2004 in south eastern Romania, reported slightly higher concentrations of DON and ZEN. The authors found DON in the range from 0 to 4000 µg/kg and concentrations in 13 (62%) samples exceeded ML EU, and all samples were ZEN contaminated at the average concentration of 133 µg/kg and 71% of them exceeded ML EU.

Some of the cited authors also mapped in their studies the occurrence and representation of the individual pathogens of *Fusarium* species, they described local weather conditions in the given seasons and suggested that a great variability of the occurring pathogen or simultaneous co-occurrence of more pathogens and also occurrence of their metabolites was affected by these conditions. These facts indicate that the natural contamination of cereals, including malting barley by mycotoxins cannot be completely eliminated not even if proper farming practices are maintained.

CONCLUSION

In 2012–2017, screening of totally 592 samples of malting barley for the content of *Fusarium* mycotoxins by immunochemical ELISA method was conducted. On average, the total content of positive samples in all the studied mycotoxins was to 20.5% in the given years. The mycotoxin content was affected by a pre-crop, the least suitable was maize, and the effect of a barley variety was not proven. Mycotoxin contents did not exceed ML EU for unprocessed cereals in DON and ZEN and indicative limits for T-2 and HT-2 toxins in any of the studied samples. As mycotoxins may pass into malt and beer produced from the malting barley, further monitoring of quality of the input materials to protect consumers' health is necessary.

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Received: 2018–11–02
Accepted after corrections: 2019–10–14

Food Chemistry 133 (2012) 55–59

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Study of ochratoxin A content in South Moravian and foreign wines by the UPLC method with fluorescence detection

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ARTICLE INFO

Article history:
Received 26 July 2010
Received in revised form 10 June 2011
Accepted 21 December 2011
Available online 10 January 2012

Keywords:
Ochratoxin A
Wine
UPLC
Immunoaffinity column
Fluorescence

ABSTRACT

In 2009–2010, 72 wine samples of Moravian and foreign origin were analysed for ochratoxin A contamination. A fast analytical method based on immunoaffinity column clean-up and followed by the ultra performance liquid chromatography coupled to fluorescence detection was used. LOD and LOQ values were 0.3 and 1.0 ng/L. Ochratoxin A was detected in 11% of Moravian wines and the detected OTA level ranged from 1.2 to 71.2 ng/L. In foreign wines, OTA level ranged from 1.6 to 227.0 ng/L. The values of OTA in all studied samples were significantly below the maximum allowable limit, 2.0 μg/kg, set by the European Union.

1. Introduction

Ochratoxins, secondary metabolites of toxigenic fungi (microscopic micromycetes), are dangerous contaminants of natural origin. They are produced by *Aspergillus* species (*A. ochraceus*, *A. sulphureus*, *A. sclerotinum*, *A. niger*, and *A. carbonarius*) in tropical and subtropical areas, by *Penicillium* species (*P. verrucosum*, *P. purpurascens*, and *P. commune*) in colder areas (Gupta, 2007). Ochratoxin A (OTA) is the most significant and widespread mycotoxin of the ochratoxin group. The main toxic effects of OTA are nephrotoxicity, hepatotoxicity, immunotoxicity, teratogenicity and neurotoxicity. OTA also has potential mutagenic and carcinogenic effects (Pfohl-Leszkowicz & Manderville, 2007; Ringot, Chango, Schneider, & Larondelle, 2006). It acts as a cumulative poison, with quick absorption and slow elimination. So far, only little information has been available on the combined effects of OTA and other mycotoxins (Ringot et al., 2006).

Ochratoxin A occurs in a number of food commodities both of plant and animal origin. The main OTA sources in food are cereals and cereal products (e.g. Duarte, Pena, & Lino, 2010; Kabak, 2009), followed by wine and wine grape products, including raisins (Aksoy, Eltem, Meyvaci, Altindisli, & Karabat, 2007; Ostrý, Ruprich, & Škarková, 2002). OTA also occurs in green and roasted coffee (e.g. Sugita-Konishi et al., 2006), legumes, spices and green tea. Contamination of brewing materials (Scott, 1996) by OTA is often associated with the presence of OTA in beer (e.g. Běláková, Benešová, Mikulíková, & Svoboda, 2011; Scott & Kanhere, 1995). The main animal sources are pork meat, blood and innards (Gareis & Scheuer, 2000).

OTA in wine and grape juice was first reported in Switzerland in 1996 (Zimmerli & Dick, 1996). Since then a number of studies focusing on OTA content in wine and wine grapes have been carried out. Summary review was given in studies from 2006 to 2007 (Mateo, Medina, Mateo, Mateo, & Jiménez, 2007; Varga & Kozakiewicz, 2006).

Higher OTA content was mainly found in red wines; rosé and white wines followed, OTA content depends on a winemaking process (Meca, Blaiotta, & Ritieni, 2010). The highest OTA content was detected in special and liquor wines (Valero, Marín, Ramos, & Sanchis, 2008). The European Commission set the maximum allowable limits (MALs) for wine to 2.0 μg/kg. (Off. Journal of the European Union, 2006). However, this limit does not apply to liquor or dessert wines with more than 15% alcohol content.

It is well known that the ochratoxin A occurrence and concentration in wines is highly affected by geographical and climatic conditions. Higher OTA content and higher number of contaminated samples was repeatedly detected in wines from the Mediterranean and other southern wine-producing areas. For example, OTA concentration in Italian wines varied from 0.01 to 4.0 μg/L (Brera, Soriano, Debegnach, & Miraglia, 2004), similarly, in Greek wines OTA level ranged from <LOD to 2.82 μg/L (Stefanaki, Foufa, Tsatsou-Dritsa, & Dais, 2003) and the maximum OTA value in Portugal wines was 2.4 μg/L (Pena, Cerejo, Silva, & Lino, 2010). Lower concentrations were measured in Spain, where OTA values

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doi:10.1016/j.foodchem.2011.12.061

varied from 0.06 to 0.316 μg/L (Lopez de Cerain, González-Penas, Jiménez, & Bello, 2002); similarly, OTA values in Turkish wines varied from 0.006 to 0.815 μg/L (Var & Kabak, 2007) and the maximum value in Croatian wines was 21.0 ng/L (Flajs, Domijan, Ivić, Cvjetković, & Peraica, 2009).

In central European wine regions, the OTA occurrence in grapes and wine was significantly lower. OTA was not detected in products from Hungary (Berente et al., 2005) and similarly, OTA was detected only in 1 of 116 wine samples from Austria (Eder, Paar, Edinger, & Lew, 2002). In Slovakia, content of OTA and other mycotoxins was analysed in wine grapes from South Slovakian, Nitrian and Small Carpathian regions with maximal content of 1.03 μg/kg (Mikušová, Ritieni, Santini, Juhasová, & Šrobárová, 2010). In 2005 a pilot study on the OTA occurrence in fresh grape juice, must and wine from South Moravia was performed. OTA content was under the limit of quantification (8 ng/L) in all samples (Ostrý et al., 2007).

The Moravian wine region includes around 97% of the total area under wine in the Czech Republic (Kraus, 2005). The Moravian wine region is divided into four sub-regions: Znojmo, Mikulov, Velké Pavlovice and Slovácko (Fig. 1). The total area of vineyards is 18,512 ha. Soil conditions are various with prevailing stony, skeletal, sandy and also clayey soils. The region has moderate inland climate with the average annual temperature is 9.42 °C, average annual rainfall 510 mm and average annual sunshine is 2244 h. Wet and fresh air blowing from the Atlantic Ocean slows down ripening of wine grapes contributing to the development of various aromatic and spicy substances. All these conditions imprint Moravian wines their unique character.

The aim of this study was to determine ochratoxin A content in wine samples from the Moravian wine production area using a new more sensitive UPLC method and compare the detected OTA levels with those found in foreign wines.

2. Experimental

2.1. Materials and reagents

OTA standard (solution, 10 μg/ml in acetonitrile), analytical and HPLC reagents were obtained from Sigma–Aldrich (Steinheim, Germany). The immunoaffinity columns Ochraprep were purchased from the company R-Biopharm (Germany).

Phosphate buffer (PBS) was prepared by mixing 800 ml of deionised water containing 19.1 ± 0.1 g of disodium hydrogen phosphate and 200 ml of deionised water containing 1.8 ± 0.1 g of potassium dihydrogen phosphate. The pH of the resulted phosphate buffer was adjusted to 7.4 with 2 M NaOH solution.

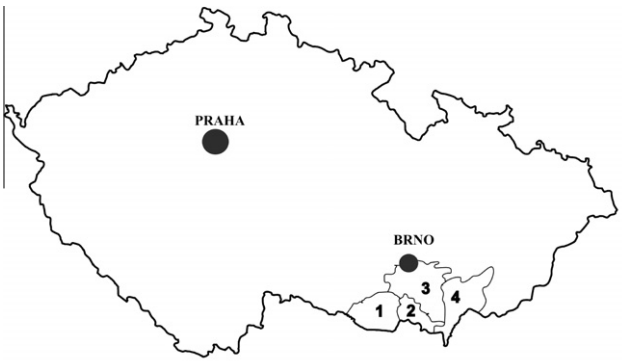

Fig. 1. The Moravian wine regions: 1, Znojmo sub-region; 2, Mikulov sub-region; 3, Velké Pavlovice sub-region; 4, Slovácko sub-region.

Mobile phase was prepared by adjusting 500 ml of deionised water to pH 2.0 with concentrated H_3PO_4. Before use it was filtered through the 0.22 μm nylon filter.

2.2. Samples

Two sets of samples were analysed: The first set included 46 wine samples from all four Moravian subregions. Wines were purchased in retail stores, specialised shops or delivered directly by a producer (wine grower). All of them were quality wines or special wines and archive wines. The effects of weather conditions in the harvest year, the fungicide treatments or production procedures were not determined. The other set contained 25 foreign wine samples (22 samples from Europe, two from Chile and one from South Australia). Wines were randomly purchased in the local stores during 2009.

2.3. Preparation of wine samples

NaOH (2 M) was added to 50 ml of the wine sample and pH was adjusted to 7.2. Then the sample was applied to the immunoaffinity column (OCHRAPREP) and washed with 20 ml of phosphate buffer. Ochratoxin A was eluted repeatedly (3×) with the mixture of 1.5 ml of methanol:acetic acid (98:2, v/v). The obtained eluate was concentrated on the rotary vacuum vaporiser, the residue obtained was transferred to 1 ml of methanol:water (50:50, v/v). The prepared sample was filtered prior to the analysis through a 0.22 μm nylon microfilter.

2.4. Preparation of standard calibration curve

OTA standard (Sigma–Aldrich) concentration was 10 μg/ml in acetonitrile (ACN). Concentration of stock solution prepared from this standard was 100 ng/ml in methanol:water 50:50, v/v. A nine-point calibration curve of methanol:water 50:50, v/v (at concentrations of 0.2, 0.4, 0.5, 1, 2, 4, 6, 8 and 10 ng/ml) was constructed. Fresh calibration solutions were prepared every day. Each solution was injected twice; mean was calculated from two measurements. Calibration curve was constructed as the dependence of the peak area on concentration of the standard. Ochratoxin A was identified by the comparison of retention time of a corresponding peak with the peak of the standard. The external standard method was used for the quantitative evaluation.

2.5. Instrumentation and OTA analyses

OTA standards and samples were analysed on the Acquity UPLC chromatographic system (Waters, USA). The system is equipped with a binary high-pressure gradient pump, vacuum degasser, autosampler with the Rheodyne injector, thermostat of columns and programmable PDA and a fluorescence detector. Data were collected and processed by the Empower software.

Analyses were performed on an Waters Acquity BEH C18 column (100 × 2.1 mm, 1.7 μm particle size) using binary gradient acetonitrile (ACN):water adjusted with H_3PO_4 to pH 2.0 (0 min 40% ACN, 2 min: 60% ACN, 2–2.2 min: 60% ACN, 2.5 min: 40% ACN). The flow rate of the mobile phase was 0.3 ml/min, the column temperature 40 °C, injection volume was 10 μl. The total analysis time was 5 min. The system was equipped with a fluorescence detector set to an excitation wavelength of 335 nm and emission wavelength of 440 nm.

2.6. OTA analysis by Mass Spectrometry

For the identification and confirmation of OTA in some selected samples, the High Performance Liquid Chromatography/Mass

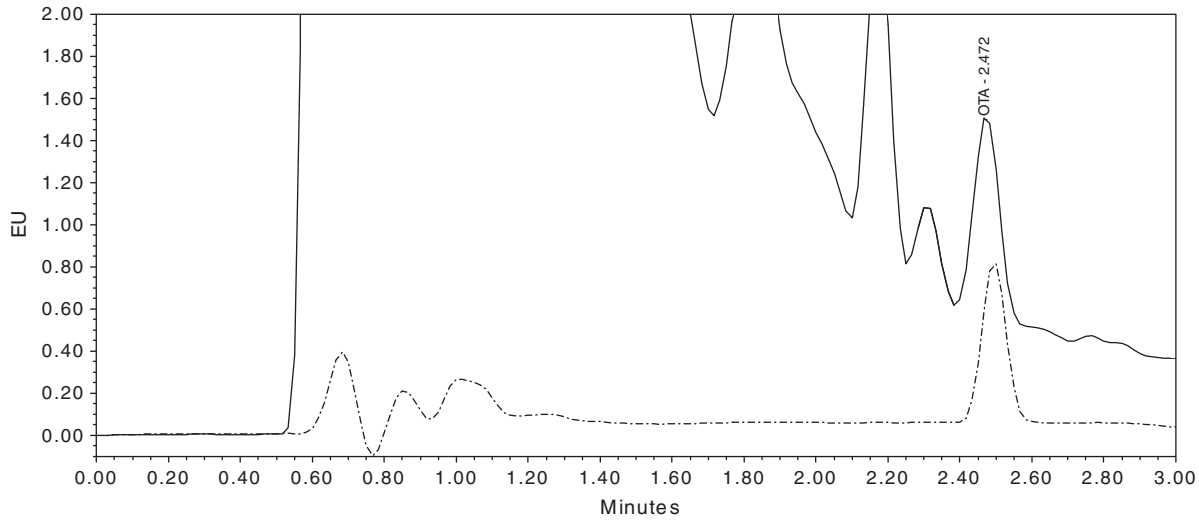

Fig. 2. OTA standard (interrupted line) and wine contaminated with OTA (full line).

Table 1
OTA concentrations in Moravian wines.

Sub-region	Variety	Kind	Description	Year	Content of OTA (ng/L)
Mikulov	Chardonnay	White		2006	<LOQ
Mikulov	Chardonnay	White		2006	<LOQ
Mikulov	Ryzlink rýnský	White	Wine with the attribute late harvest	2008	<LOQ
Mikulov	Ryzlink vlašský	White	Wine with the attribute late harvest	2009	<LOQ
Slovácko	Rulandské bílé	White	Straw wine	2003	<LOQ
Slovácko	Aurelius	White	Botritic harvest	2005	<LOQ
Slovácko	Chardonnay	White		2007	<LOQ
Slovácko	Ryzlink vlašský	White	Quality varietal wine dry	2007	<LOQ
Slovácko	Rulandské bílé	White		2009	<LOQ
Slovácko	Ryzlink vlašský	White		2009	<LOQ
Slovácko	Müller Thurgau	White		2009	<LOQ
Slovácko	Frankovka	Red	Quality varietal wine	2007	<LOQ
Slovácko	André	Red	Wine with the attribute late harvest	2008	<LOQ
Velké Pavlovice	Chardonnay	White			<LOQ
Velké Pavlovice	Chardonnay	White		2004	3.5
Velké Pavlovice	Rulandské bílé	White	Selection of grapes	2005	<LOQ
Velké Pavlovice	Müller Thurgau	White	Dry	2009	<LOQ
Velké Pavlovice	Rulandské bílé	White	Quality varietal wine dry	2009	4.0
Velké Pavlovice	Ryzlink vlašský	White		2009	<LOQ
Velké Pavlovice	Sylvánské + Müller Thurgau	White		2009	<LOQ
Velké Pavlovice	Pálava	White		2009	<LOQ
Velké Pavlovice	Cabernet Sauvignon	Red	Dry wine with the attribute late harvest	2007	71.2
Velké Pavlovice	Svatovavřinecké + Frankovka	Red		2009	<LOQ
Znojmo	Chardonnay	White		2006	17.3
Znojmo	Chardonnay	White	Quality varietal wine dry	2006	<LOQ
Znojmo	Müller Thurgau	White		2008	<LOQ
Znojmo	Chardonnay	White		2008	<LOQ
Znojmo	Veltlínské zelené	White		2008	<LOQ
Znojmo	Rulandské bílé	White		2008	<LOQ
Znojmo	Müller Thurgau	White	Dry	2008	<LOQ
Znojmo	Neuburské	White		2009	<LOQ
Znojmo	Veltlínské zelené	White		2009	<LOQ
Znojmo	Ryzlink rýnský	White		2009	<LOQ
Znojmo	Müller Thurgau	White		2009	<LOQ
Znojmo	Veltlínské zelené	White		2009	<LOQ
Znojmo	Veltlínské zelené	White		2009	<LOQ
Znojmo	Rulandské bílé	White		2009	<LOQ
Znojmo	Modrý Portugal	Red	Quality varietal wine dry	2006	<LOQ
Znojmo	Svatovavřinecké	Red	Quality varietal wine dry	2007	<LOQ
Znojmo	Modrý Portugal	Red		2008	1.2
Znojmo	Zweigeltrebe + Frankovka	Red		2008	<LOQ
Znojmo	Modrý Portugal	Red	Quality varietal wine dry	2009	<LOQ
Znojmo	Zweigeltrebe + Frankovka	Red		2009	<LOQ
Znojmo	Zweigeltrebe	Red		2009	<LOQ
Znojmo	Frankovka	Red		2009	<LOQ
Znojmo	Svatovavřinecké	Red		2009	<LOQ

Spectrometry method was used. The chromatographic system Finnigan Surveyor with ion trap LCQ Advantage (Thermo-Fisher, USA) with atmospheric pressure ionisation controlled by the Excalibur software was used. Conditions were as follows: The Kinetex PFP column (3.0 × 100 mm, 2.6 μm particle size) using isocratic elution with a methanol:10 mM ammonium acetate in water (70:30, v/v) mixture as a mobile phase was employed. The flow rate was 0.3 ml/min and injection volume was 25 μl. The retention time of OTA was 2.5 min. The following MS parameters were employed: For electrospray ionisation with positive ion polarity, the capillary voltage was set to 3.0 kV, the capillary temperature to 250 °C, the sheath gas flow (nitrogen) to 40 L/min. The collision energy was 40% and the fragmentation time was 30 ms. To determine the product ion of OTA, the protonated molecule ($[M+H]^+$) at m/z 404 was isolated, and the fragment ions were detected in a scan range of m/z 300–450. The most intensive product ion was m/z 358 ($[M+H–HCOOH]^+$). OTA was detected in full scan mode of MS^2 404 → 358.

3. Results and discussion

3.1. Analytical methods

OTA can be analysed with the method of direct injection (Berente et al., 2005; Dall'Asta, Galaverna, Dossena, & Marchelli, 2004; Pena et al., 2010). However, the detection limits are high, around 1 μg/L as the analyte is not pre-concentrated.

Therefore, the selective immunoaffinity column was chosen as the most suitable method for obtaining high purity OTA extract with a low content of matrix components that could cause interference in the assay. However, these components must be sufficiently separated from the targeted analyte. Using this method a very low OTA concentration can be detected. This method is very fast, simple and environmentally friendly as no toxic chlorinated solvents are used.

The optimised gradient elution was used for the chromatographic analyses. Five injections of blank (mobile phase) were performed and no peak was detected at the same retention time of OTA. Subsequently five injections with OTA standard at different concentrations were applied. A peak with a gradually increasing area without any interferences was observed. Then five injections of wine (matrix) without OTA contamination were performed, at standard retention time no peak was observed again.

Fig. 2 shows the chromatograms of OTA standard (2.0 ng/ml) and a contaminated red wine sample with OTA content of 2.45 ng/ml (i.e. 0.049 ng/ml in a real sample). Samples were pre-concentrated fifty times.

The calibration curve showed a good linear relationship between peak areas and OTA concentration. Curve was linear in the given range with the regression coefficient $R^2 = 0.9995$. Equation of the calibration curve was $y = 17{,}452 \pm 144x + 1570 \pm 714$. Effi-Validation program was used for calculation of the confidence intervals ($a\langle 17{,}112–17{,}792\rangle$; $b\langle -118.25–3258.20\rangle$).

The limit of detection (LOD) was defined as the concentration at which the signal to noise ratio equals 3. The limit of quantification (LOQ) was defined as the concentration where the signal to noise ratio equals 10. Limit of detection was 0.015 ng/ml and limit of quantification was 0.05 ng/ml, after recalculation to the wine matrix, it was 0.3 and 1.0 ng/L, respectively. RSD for repeatability in wine was 5.3%.

Recovery was calculated using the spiked sample. The sample with zero OTA concentration was selected and spiked with the OTA standard of the given concentration (1.0 ng/ml) and then it was extracted and analysed using the method described above. The value of recovery was 95%. The OTA content in real wine samples is given in ng/L without recalculation for recovery. Relative extended uncertainty of the determination was 9.6%.

3.2. Occurrence of OTA in wine

3.2.1. Moravian wines

The set included 46 samples, 33 white and 13 red wines. OTA was detected in 5 samples, i.e. 11%. Three contaminated wines were from the region of Velké Pavlovice. The highest detected OTA concentration (71.2 ng/L) was in archive Cabernet Sauvignon from 2007. Higher OTA concentration in red wines may depend on

Table 2
OTA concentrations in foreign wines.

Country of origin	Wine description	Kind	Year	Content of OTA (ng/L)
France	Cuvée	White	2004	1.6
France	Cuvée	White	2005	<LOQ
France	Chardonnay	White	2007	17.1
France	Cuvée	Rosé	2007	3.4
France	Cuvée	Rosé	2007	<LOQ
France	Cuvée	Red	2006	<LOQ
France	Cuvée	Red	2007	<LOQ
Italy	Chardonnay	White	2006	<LOQ
Italy	Soave	White	2008	3.3
Italy	Sangiovese	Red	2006	<LOQ
Italy	Cuvée	Red	2007	<LOQ
Hungary	Tokaji	White	2001	<LOQ
Portugal	Port	White		13.6
Portugal	Cuvée	Rosé		2.0
Greece	Cuvée	White		227.0
Greece	Cuvée	Red		109.0
Greece	Merlot	Red	2006	127.3
Spain	Port	White		<LOQ
Spain	Chardonnay	White	2004	1.7
Spain	Chardonnay	White	2005	75.9
Spain	Cuvée	White	2006	<LOQ
Spain	Shiraz	Red	2005	59.5
Spain	Cabernet	Red	2006	16.9
Chile	André	Red	2000	4.6
Chile	Cabernet Sauvignon	Red	2009	49.0
South Australia	Chardonnay	White	2002	5.00

technology used for their production; grapes are allowed to ferment with their skins, which can be highly contaminated with toxigenic fungi. Most wines were from the Znojmo region where OTA was detected in two of 23 wine samples. The variety Chardonnay from 2006 contained 17.3 ng/L and Modrý Portugal from 2008 1.2 ng/L, i.e. trace amounts closely above the limit of quantification. OTA was not detected in 4 wines from the Mikulov region and 9 samples from the Slovácko region. Neither year nor variety affected the OTA content. Enhanced OTA content was not confirmed in wines with higher content of sugar and alcohol (straw wines, special selection of berries). Results are summarised in Table 1.

The present results confirmed previous conclusions (Berente et al., 2005; Eder et al., 2002; Mikušová et al., 2010; Ostrý et al., 2007) that risk of wine contamination with ochratoxin A is very low in the South Moravian wine region.

3.2.2. Foreign wines

This set included 26 wine samples – 13 white, 3 rosé and 10 red wines. OTA was detected in 16 samples, i.e. 64%, average OTA content was 44.0 ng/L. Of 13 white wines eight samples were positive, average OTA content was 43.2 ng/L, of three rosé wines two positive samples were detected, average OTA content was 2.7 ng/L and of 10 red wines six were positive, i.e. 60%, with average OTA content of 61.0 ng/L. Relatively higher OTA content was detected in all wines from Greece (109.0–227.0 ng/L) and Spain (1.7–75.9 ng/L). These results confirm findings of other authors that OTA content strongly depends on the geographical position of a vineyard and climatic conditions, red wines having relatively the highest OTA content. The highest OTA level measured in this study was detected in white dessert wine from Greece. Dessert wines are quite prone to be contaminated with OTA. The high levels of OTA can be due to the raisining (Valero et al., 2008). OTA contents detected in this set of samples of wines imported from the Mediterranean region were substantially lower compared to data given in foreign/other studies (Brera et al., 2004; Lopez de Cerain et al., 2002; Pena et al., 2010; Stefanaki et al., 2003), limiting factor can be a small size of the set. Of the oversea wines, the highest OTA content was found in Cabernet Sauvignon from Chile. The detected OTA content in all the analysed samples was under the maximum allowable limit (MAL) set by the EU, 2.0 μg/kg. Results are summarised in Table 2.

4. Conclusion

The method for the extraction of ochratoxin A from wine was optimised and the analytical UPLC method with fluorometric detection was developed and validated. This method enables to obtain results in a substantially shorter period compared to the classical HPLC method. LOD and LOQ values were 0.3 and 1.0 ng/L. The method is simple and fast and it is suitable for monitoring the OTA occurrence in wine.

During the 3-year period (2008–2010) this method was used for the analysis of 72 wine samples of Moravian and foreign origin. OTA was detected in 11% of Moravian wine samples and the detected levels ranged from 1.2 to 71.2 ng/L. OTA was detected in 64% of foreign wine samples and it ranged from 1.6 to 227.0 ng/L. The results confirmed our presumption that risk of ochratoxin A occurrence in wines from the South Moravian region is very low. The values of OTA in all studied samples were significantly below the maximum allowable limit.

Acknowledgement

Results were achieved in the framework of the Research Plan of the MEYS 6019369701.

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Food Chemistry 126 (2011) 321–325

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Determination of ochratoxin A in brewing materials and beer by ultra performance liquid chromatography with fluorescence detection

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ARTICLE INFO

Article history:
Received 1 March 2010
Received in revised form 5 October 2010
Accepted 13 October 2010

Keywords:
Ochratoxin A
UPLC
Fluorescence
Brewing
Barley
Malt
Beer

ABSTRACT

In 2008–2009 the total set of 237 samples of malting barley, malt, hop, wort, and beer was analysed for ochratoxin A (OTA) contamination using the ultra performance liquid chromatography (UPLC) coupled to fluorescence detection (FLD). The UPLC method is a fast technique with low limits of detection and quantification (LOD and LOQ) compared to other methods used routinely. LOD and LOQ values were 0.0003 and 0.001 ng/ml for beer, 0.05 and 0.2 μg/kg for barley and malt, 0.16 and 0.5 μg/kg for hop, respectively.

Ochratoxin A was detected in one barley sample (0.3 μg/kg), one malt (0.7 μg/kg) and one hop sample (0.6 μg/kg). OTA content was also determined during the brewing process. In addition, samples of both domestic and foreign beers, obtained from local stores, were analysed. OTA content was determined in 39% of beer samples, the detected OTA level ranged from 0.001 to 0.0544 ng/ml. Only one beer sample contained 0.2438 ng/ml OTA.

1. Introduction

Mycotoxins, products of secondary metabolism of filamentous micromycetes, belong to the most serious contaminants of natural origin. These highly dangerous chemical substances are known for their toxic effects. Ochratoxins comprise a group of mycotoxins produced in tropical and subtropical areas, namely by *Aspergillus* species (*A. ochraceus, A. sulphureus, A. sclerotinum, A. niger, A. carbonarius*), in colder areas by *Penicillium* species (*P. verrucosum, P. purpurascens, P. commune*). Chemically, they can be characterised as derivatives of 7-isocumarin linked to amino acid L-β-phenylalanin. In 1992 three new mycotoxins, analogues of ochratoxin A, containing hydroxyprolin, serin or lysine instead of phenylalanin, were isolated from micromycetes of *A. ochraceus* (Hadidane et al., 1992).

Ochratoxin A (OTA) is the most significant and widespread mycotoxin of the ochratoxin group. For the first time it was isolated in the laboratory research from *A. ochraceus* species in the Republic of South Africa in 1965 (van der Merwe, Steyn, & Fourie, 1965) and as a naturally occurring contaminant it was found in maize in 1969 in the USA (Shotwell, Hesseltine, & Goulden, 1969).

The main toxic effects of OTA are nephrotoxicity, immunotoxicity, mutagenicity, carcinogenicity, teratogenicity and neurotoxicity. These effects were confirmed experimentally in animals and can be thus assumed in humans as well (Pfohl-Leszkowicz & Manderville, 2007). Study on the mechanisms of OTA effects on living organisms has proved the inhibition of protein synthesis, increase in lipid peroxidation, damage to saccharide and calcium metabolism and damage to mitochondrial functions (Dirheimer & Creppy, 1991). OTA acts as a cumulative poison with quick absorption and slow elimination. Toxicokinetic profile is animal species dependent. In humans, OTA is metabolised very slowly with a half-life of more than 30 days. Recent studies have demonstrated that exposition to this toxin can represent a world wide problem (Pena, Seifrtová, Lino, Silveira, & Solich, 2006; Ueno et al., 1998). Ochratoxin A, similarly as other mycotoxins, is heat-resistant (Bullerman & Bianchini, 2007) and neither freezing can eliminate it from food. Prevention is of utmost importance and therefore high attention has been devoted to food and feed protection already in the phase of growing, e.g. treatment with appropriate fungicides (Amézqueta, González-Peñas, Murillo-Arbizu, & López de Cerain, 2009).

Ochratoxin A occurs in a number of commodities both of plant and animal origin. The main OTA sources in food are cereals, mainly barley, rye, oats, wheat, rice and maize, and cereal products (Kabak, 2009; Sugita-Konishi et al., 2006). Wine was identified as the second biggest source of OTA. OTA was also detected in wine juice and wine vinegar (Varga & Kozakiewicz, 2006), in pork meat, blood, innards (livers, kidneys, products from blood), bovine milk (Sørensen & Elbæk, 2005), in coffee (Sugita-Konishi et al., 2006), beer (Reinsch, Töpfer, Lehmann, Nehls, & Panne, 2007) legumes, spices, green tea and dried fruits, such as figs. (Karbancioglu-Güler

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0308-8146/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2010.10.062

& Heperkan, 2008) or raisins (Sugita-Konishi et al., 2006). OTA may also represent a potential airborne hazard, in water-damaged buildings, or occupational contamination, in workplaces with high mould exposure, such as agricultural, farm and alimentary industries (Duarte, Pena, & Lino, 2009). The European Commission set the new maximum allowable limits (MAL) for some food commodities in 2006 (Official Journal of the European Union, 2006).

The presence of OTA in beer depends on contamination of brewing materials, i.e. malting barley and malt, with micromycetes of *Penicillium verrucosum* sp. and *Aspergillus ochraceus* sp. Several studies of brewing materials have been performed. OTA content in barley ranged from 0.1–2.7 μg/kg (Thelman & Weber, 1997), 0.01–0.495 μg/kg (Wolf, 2000) and 0.53–12 μg/kg (Gumus, Arici, & Demirci, 2004). OTA content in malt ranged from 0.1–0.92 μg/kg (Thelman & Weber, 1997) and 0.5–6.6 μg/kg (Gumus, Arici, & Demirci, 2004). In 1996 transmission of OTA and other mycotoxins into beer during brewing was studied (Scott, 1996). Korgh et al. observed substantial OTA losses (40–89%) (Krogh, Hald, Gjertsen, & Myken, 1974) in the grist during mashing, most probably due to proteolytic degradation. Another 16% was eliminated with the spent grains. During fermentation OTA losses ranged from 2–69% (Scott & Kanhere, 1995). The remaining OTA is transmitted to beer. For the first time OTA content in beer was described in 1983 (Payen, Girard, Gaillardin, & Lafont, 1983). A number of studies performed worldwide since 1998 reported OTA levels in beer ranging from 0 to 0.5 ng/ml (Mateo, Medina, Mateo, Mateo, & Jiménez, 2007 and Refs. cited herein), with the exception of the study on South African beers performed in 2002 (Odhav & Naicker, 2002), where the value of 2340 ng/ml was the highest level ever reported for OTA content in beer. We can conclude that beer is not a relevant contributor to the OTA exposure for population. The European Commission did not set the maximum allowable limit for OTA content for beer.

Barley, malt and beer represent a very complicated matrix, sample preparation and clean-up procedure prior to the analysis are necessary. The methods most frequently used are liquid–liquid and solid–liquid extraction or immunoaffinity columns (IAC) (Şenyuva & Gilbert, 2010 and Refs. cited herein,). The possibilities of extraction, separation and detection of OTA and other mycotoxins including HPLC, TLC, GC, mass spectrometry, capillary electrophoresis and ELISA tests were summarised in the latest review in January 2009 (Turner, Subrahmanyam, & Piletsky, 2009).

The aim of this study was to determine the ochratoxin A content in raw brewing materials, during the brewing process and in the beer samples by a new UPLC separation technique coupled to fluorescence detection.

2. Experimental

2.1. Materials and reagents

OTA standard (solution, 10 μg/ml in acetonitrile), analytical and HPLC reagents were obtained from Sigma–Aldrich (Steinheim, Germany). The immunoaffinity columns Ochraprep were purchased from the company R-Biopharm (Germany).

Phosphate buffer (PBS) was prepared by mixing 800 ml of deionised water containing 19.1 ± 0.1 g of disodium hydrogen phosphate and 200 ml of deionised water containing 1.8 ± 0.1 g of potassium dihydrogen phosphate. The pH of the resulted phosphate buffer was adjusted to 7.4 with the solution of 2 M sodium hydroxide.

2.2. Samples

OTA content was determined in three sets of samples: (1) OTA content in brewing materials – barley, hop, malt, (2) OTA content during beer production, (3) OTA content in beers purchased in local shops.

2.2.1. OTA content in brewing materials – barley, hop, malt

Samples of malting barley, hop and malt were obtained from different localities and suppliers in the Czech Republic. Various matrixes of hop were delivered (cones, extract, paste). This set included 39 barley samples, 58 malt samples and 5 hop samples, i.e. totally 102 samples. All samples came from harvests 2008–2009.

2.2.2. OTA content during beer production

Ochratoxin A was determined in samples of two malting barley varieties Bojos and Sebastian during malting and brewing. The samples from the harvest year 2008 were collected from the growing station Hrubčice in the Czech Republic. The malting barley samples included both the fungicide treated and nontreated variants. Malts from these barleys were prepared in the micromalting plant of the Malting Institute of the Research Institute of Brewing and Malting in Brno using the procedure with short steeping and CO_2 extraction. Sweet wort, hopped wort and beer were prepared in the Brewing Institute of the Research Institute of Brewing and Malting in Prague. Beer was prepared by a classical decoction method. This set included 20 samples.

2.2.3. OTA content in beer

Beer samples were purchased in local Czech stores in 2009. Beer samples included pale beer, lager, blended beers, special beers, dark and nonalcoholic beers both of inland and outland production. This set included 115 samples.

2.3. Sample preparation and extraction

OTA from a sample was concentrated on the immunoaffinity column. The antibodies contained in the immunoaffinity column were reversibly bound to the mycotoxin in the extract and thus trapped. Subsequently, the mycotoxin was eluted from the column using the elution mixture, as described below.

The barley, malt samples (50 ± 0.1 g) and hop samples (25 ± 0.1 g) were blended for 2 min or ultrasonicated for 10 min with 200 ml acetonitrile–water (60:40 v/v). The homogenised sample was centrifuged at 4000 rev/min for 10 min. The supernatant was filtered through a glass filter. 22 ml of PBS was added to 2 ml filtered extract. After complete homogenisation the sample was applied to the Ochraprep immunoaffinity column with flow rate equal to 2–3 ml/min. The column was washed with 20 ml of PBS.

Elution was repeated three times with 1.5 ml of methanol/acetic acid (98:2, v/v) at a flow rate less or equal to 2–3 ml/min. The eluate was rotary evaporated to dryness under vacuum and redissolved in 1 ml of methanol–water (50:50, v/v). The sample was filtered through a 0.22 μm nylon microfilter.

In the liquid matrixes, the extraction step was omitted. 50 ml of beer sample was degassed using the ultrasound cleaning device, pH was adjusted to 7.2 with 2 M NaOH. The whole volume of the sample was passed through the immunoaffinity column and elution was performed as described above.

2.4. Preparation of standard calibration curve

Concentration of OTA standard (Sigma–Aldrich) was 10 μg/ml in acetonitrile (ACN). Concentration of stock solution prepared from this standard was 100 ng/ml in methanol–water 50:50. A seven-point calibration curve of methanol–water 50:50 (at concentrations of 0.5, 1, 2, 4, 6, 8, and 10 ng/ml) was constructed. Calibration solutions were freshly prepared each day before the measurement. Each solution was injected twice, mean was

calculated from two measurements. Calibration curve was constructed as the dependence of the peak area on concentration of the standard. Ochratoxin A was identified by the comparison of retention time of a corresponding peak with peak of the standard. The external standard method was used for the quantitative evaluation.

2.5. Instrumentation and OTA analyses

OTA standards and samples were analysed on the Acquity UPLC chromatographic system (Waters, USA). The system is equipped with a binary high-pressure gradient pump, vacuum degasser, autosampler with the Rheodyne injector, thermostat of columns and programmable PDA and fluorescence detector. Data were collected and processed by the Empower software.

Analyses were performed on a Waters ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm particle size) using binary gradient acetonitrile (ACN)-water adjusted with H_3PO_4 to pH 2 (0 min 40% ACN, 2 min: 60% ACN, 2–2.2 min: 60% ACN, 2.5 min: 40% ACN). The flow rate of the mobile phase was 0.500 ml/min, the column temperature was 40 °C, injection volume was 10 μl. The total analysis time was 5 min. The system was equipped with a fluorescence detector set to an excitation wavelength of 335 nm and emission wavelength of 440 nm.

For the identification and confirmation of OTA in some selected samples, the HPLC/ITP/MS LCQ Advantage (Thermo-Fisher, USA) with atmospheric pressure ionisation controlled by the Excalibur software was used. Conditions were as follows: The Kinetex PFP column (3.0 × 100 mm, 2.6 μm particle size) was employed using isocratic elution with a methanol – 10 mM ammonium acetate in water (70:30, v/v) mixture as mobile phase. The flow rate was 0.300 ml/min and injection volume was 25 μl. The retention time of OTA was 2.5 min. The following MS parameters were employed: For electrospray ionisation with positive ion polarity the capillary voltage was set to 3.0 kV, the capillary temperature to 250 °C, the sheath gas flow (nitrogen) to 40 l/min. The collision energy was 40% and the fragmentation time was 30 ms. To determine the product ion of OTA, the protonated molecule ($[M + H]^+$) at m/z 404 was isolated, and the fragment ion was detected in a scan range of m/z 300–450. The most intensive product ion was m/z 358 ($[M + H–HCOOH]^+$). OTA was detected in full scan mode of MS^2 404 → 358.

3. Results and discussion

3.1. Method parameters

Matrixes of barley, malt, hop, and beer being very complicated, the use of selective immunoaffinity columns was the most suitable method for obtaining high purity OTA extracts without the content of matrix components that could cause interferences in the assay. The method for the extraction of ochratoxin A from different matrixes was optimised. Both the methods used for sample homogenisation, i.e. blending and ultrasonic homogenisation, gave similar results. For the extraction on immunoaffinity column, different volumes of PBS buffer and beer samples were tested to achieve optimal recovery. Conditions described in Section 2.3 proved to be the most suitable.

For the chromatographic analyses, gradient elution was optimised. Elution mentioned above in Section 2.5 was assessed as the best one. Fig. 1 shows the chromatogram of OTA standard at the concentration of 0.5 ng/ml and chromatogram of beer sample with 0.795 ng/ml of OTA, extract was pre-concentrated fifty times.

Calibration curve was linear in the given range with the regression coefficient $R^2 = 0.9992$. The equation of the calibration curve was $y = 7430 \pm 94\ x + 715 \pm 527$. EffiValidation program was used for calculation of the confidence intervals (a < 7189.40–76971.50 >; b < −640.47–2069.80 >).

The limit of detection (LOD) was defined as the concentration at which the signal to noise ratio equals 3. The limit of quantification (LOQ) was defined as the concentration where the signal to noise ratio equals 10. LOD and LOQ values were 0.0003 and 0.001 ng/ml for beer, 0.05 and 0.2 μg/kg for barley and malt, 0.16 and 0.5 μg/kg for hop, respectively. RSD for repeatability was 5.3% in barley and malt, 4.2% in beer.

Recovery for barley and malt was calculated using the certified reference material (CRM), cereal flour enriched with the known OTA concentration. The sample with zero OTA concentration was selected for the determination of recovery in beer. The sample was spiked with the OTA standard of the given concentration and then it was extracted and analysed pursuant to the method described above. Relative extended uncertainty of the determination for barley and malt was 14.90%. Relative extended uncertainty of the determination for beer was 8.94%. Recovery and relative extended uncertainty of determination for hop was not calculated due to a small number of samples and differences in hop matrixes (cones, extract, paste). Results are given in Table 1.

3.2. Analyses of brewing materials

Within the period of two years (2008 and 2009) the analyses of 39 malting barley samples, 58 malt samples and 5 hop samples were performed. These brewing material samples came from different localities of the Czech Republic. OTA was detected in one barley sample (0.3 μg/kg) and one malt sample (0.7 μg/kg). One hop sample was contaminated with 0.6 μg/kg of ochratoxin A. All three contaminated samples were from harvest 2009. Results (without recalculation for recovery) are given in Table 2.

In comparison to other studies (Gumus, Arici, & Demirci, 2004; Thelman & Weber, 1997; Wolf, 2000), very low OTA contamination of studied brewing materials was found.

The level of OTA depends on number of key factors. Cereals can be contaminated during harvest, the occurrence and persistence of *P. verrucosum* in soil has been proved (Elmholt & Hestbjerg, 1999). Good storage practice must be also kept (Magan & Aldreda, 2007). In addition, admixtures of impurities, namely dust, weed seeds, and grain fractures (Tangni & Pussemier, 2006) can affect OTA contamination. Dust can either contaminate cereals directly or it can act as an inoculum. Seed and weed admixtures can temporarily increase moisture in a cereal and thus create suitable conditions for mycotoxin production. Early grain clearance and moisture decrease under critical limit can prevent cereal contamination with OTA.

It can be concluded, that the very low contamination of studied brewing materials with OTA was probably due to the fact that impurities were removed as the samples were determined for further processing and beer production. Another reason can be, that all producers and suppliers kept an excellent drying and storage conditions, which helped avoid mould growth and subsequent OTA contamination.

3.3. Transmission of OTA during the technological process of beer

Transmission of OTA during the technological process of beer production was analysed in 2 samples of barley, malt, sweet wort and hopped wort and beer produced from them. The barley varieties Bojos and Sebastian were from one locality, the variants were both chemically (fungicide) treated and nontreated. OTA content was not detected in any barley and malt samples. The trace amounts of OTA were detected in sweet wort made from the variety Sebastian. Sweet wort was stored in the refrigerator for 72 h during analyses. During this time an eye-visible mould was

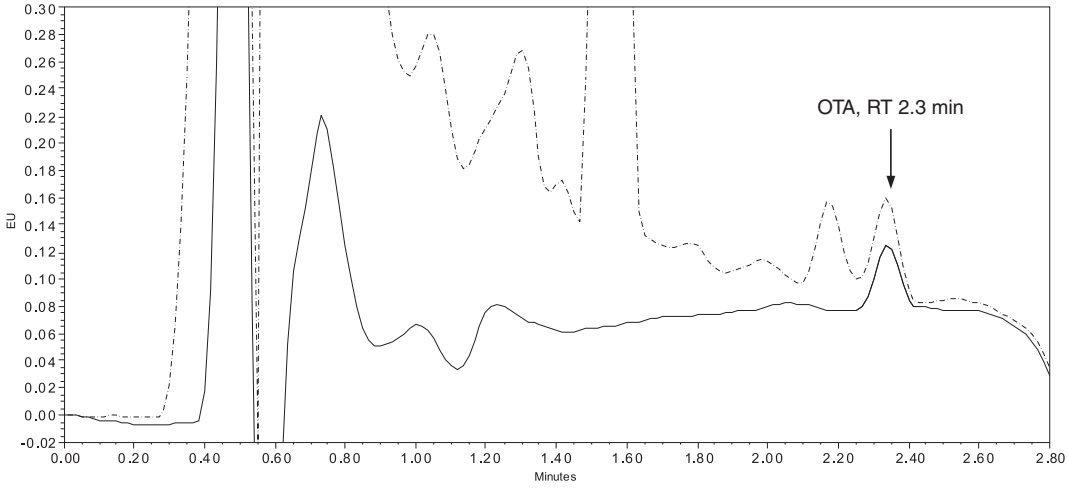

Fig. 1. OTA standard (full line) and beer contaminated with OTA (interrupted line).

Table 1
Limits of detection and quantification for different matrixes.

Matrix	LOD	LOQ	Recovery%
Beer	0.015 ng/ml	0.05 ng/ml	95
Barley, malt	0.05 μg/kg	0.2 μg/kg	82 (CRM)
Hop	0.16 μg/kg	0.5 μg/kg	–

Table 2
OTA concentrations in brewing materials.

		Total	Positive	Min	Max
Barley	μg/kg	39	1	0	0.3
Malt	μg/kg	58	1	0	0.7
Hop	μg/kg	5	1	0	0.6

Table 3
OTA concentrations during technological process.

Variety	Barley (μg/kg)	Malt (μg/kg)	Sweet wort (ng/ml)	Hopped wort (ng/ml)	Beer (ng/ml)
Bojos (nontreated)	<LOD	<LOD	<LOD	<LOD	0.0076
Bojos (treated)	<LOD	<LOD	<LOD	<LOD	0.013
Sebastian (nontreated)	<LOD	<LOD	0.0126	<LOD	<LOD
Sebastian (treated)	<LOD	<LOD	0.0126	<LOD	<LOD

created, probably causing production of OTA. This suggests a possible contamination of a sample during storage at lowered temperature. OTA was not found in the control sample of wort. OTA was not detected in the beer made from the variety Sebastian. We can assume that secondary fungal contamination can be eliminated by maintaining the proper technological procedure. Trace amounts of OTA were only detected in beer produced from the variety Bojos; OTA concentration in other samples was below LOD. Results are given in Table 3.

3.4. Occurrence of OTA in beer

In 2009 analyses of 115 beers (i.e. pale, dark, special and nonalcoholic beers) from local stores were performed. OTA was present in 39% of samples. With the exception of one sample, Ochratoxin concentration ranged from 0.001–0.0544 ng/ml, which corresponds to the data given in the literature. The highest ochratoxin A level (0.2438 ng/ml) was found only in one sample of pale beer. Results are given in Table 4.

Table 4
OTA concentrations in beer samples.

		Total	Positive	Min	Max
Pale beer	ng/ml	72	30	0.001	0.2438
Dark beer	ng/ml	18	8	0.0022	0.0478
Special beer	ng/ml	10	4	0.0018	0.045
Nonalcoholic beer	ng/ml	15	5	0.0014	0.0508

4. Conclusion

The method for the extraction of ochratoxin A from different matrixes was optimised and the analytical UPLC method with fluorometric detection was developed and validated. This method enables to obtain results in a substantially shorter period compared to the classical HPLC method. LOD and LOQ were significantly lower compared to the data given in the literature for the FLD or MS detection. It is also much cheaper than the frequently used MS detection. The method is simple and fast and it is suitable for routine checking of the brewing materials, intermediates and a final product in the whole technological process of the beer production. It is also suitable for monitoring the OTA occurrence in beer in common trade network.

During the two-year period (2008–2009) this method was used for the analysis of samples of malting barley, malt, hop and beer. Only one barley, one malt and one hop sample from 2009 were contaminated with ochratoxin A. 115 beer samples of local and foreign provenience purchased in local stores were analysed, OTA content was determined in 39% of samples in the range of 0.001 to 0.2438 ng/ml.

Acknowledgement

Results were achieved in the framework of the Research Plan of the MEYS (id.no. 6019369701).

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Food Control 25 (2012) 626–630

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Monitoring of selected aflatoxins in brewing materials and beer by liquid chromatography/mass spectrometry

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ARTICLE INFO

Article history:
Received 22 September 2011
Received in revised form
21 November 2011
Accepted 26 November 2011

Keywords:
Aflatoxins
Mass spectrometry
Brewing materials
Beer
Immunoaffinity column

ABSTRACT

In 2008–2011 a total set of 333 samples of brewing raw materials and beer were analyzed for the presence of aflatoxins B_1, B_2, G_1 and G_2. The standard analytical method using high-performance liquid chromatography coupled to mass spectrometric detection with immunoaffinity column clean-up was applied. The method was validated. Limits of detection varied from 0.04 to 0.12 μg/kg in barley and malt, 0.08–0.58 μg/kg in different hop samples, 0.04–0.12 μg/kg in brewers' yeast and spent grains and 1.5–4.7 ng/l in beer. Limits of quantification varied from 0.13 to 0.39 μg/kg in barley and malt, 0.25–1.94 μg/kg in different hop samples, 0.13–0.39 μg/kg in brewers' yeast and spent grains and 5.1–15.2 ng/l in beer. In 7 of 216 samples of brewing raw materials (3.2%), aflatoxins were found at trace concentrations to 1.2 μg/kg. In 6 of 117 (5.1%) beer samples, aflatoxins were detected at concentrations to 31.0 ng/l. Values in barley and malt did not exceed the maximum allowable limit set by the European Union.

1. Introduction

Mycotoxins are low molecular secondary metabolites of filamentous microscopic fungi that cause various toxic syndromes, collectively called mycotoxicoses. Bennett (1987) defined mycotoxins as natural products of fungi that evoke a toxic response of the organism even at small concentrations. Therefore, any contamination with toxigenic fungi may be considerably dangerous (Richard, 2007; Speijers & Speijers, 2004). Microscopic fungi occur in soil and in a form of spore also frequently in the air. Mycotoxins are found throughout all levels of the food chain, contamination may already occur in the phase of growing in a field as well as during storage (Krska, Baumgartner, & Josephs, 2001). The most serious effects include hepatotoxicity and neurotoxicity (Eaton, Beima, Bammler, Riley, & Voss, 2010). Acute toxic mycotoxin effects are observed only exceptionally, late toxic effects, such as carcinogenity, immunotoxicity, mutagenity and developmental toxicity caused by the uptake of single low doses or repeated low doses from food, being more significant. For this reason, their presence in food materials is continuously monitored. In 2006, the European Commission set the new maximum allowable limits (MAL) for some food commodities (Commision of the European Communities, 2006).

The best known mycotoxin producers are fungi of *Aspergillus*, *Penicillium* and *Fusarium* species. Aflatoxins (*Aspergillus flavus* toxins) are products of toxigenic strains *A. flavus*, *A. parasiticus*, *A. argeninicus*, and *A. nomius*. They are typical toxins of tropical and subtropical regions. Under favorable conditions (relatively high temperature and humidity) *A. flavus* and *A. parasiticus* are able to grow and produce aflatoxins on nearly each organic substrate including all agricultural commodities. They mostly occur in various unprocessed products, such as cereals, spices, all kinds of nuts, oil crops, figs and dried fruits (Imperato, Campone, Piccinelli, Veneziano, & Rastrelli, 2011). Food of the animal origin is a much less suitable substrate for aflatoxin production. Of more than 20 aflatoxins known, only four (i.e. B_1, B_2, G_1, and G_2) contaminate food. Chemically, they belong to difuranocoumarins. Aflatoxin B_1 occurs most frequently, it is considered as the most toxic and powerful carcinogens (IARC, 1993). Hydroxylated derivates of aflatoxin B_1, aflatoxins M_1 and M_2, are found in milk and dairy products (e.g Bilandžić, Varenina, & Solomun, 2010; López, Ramos, Ramadán, & Bulacio, 2003). These metabolites are produced mainly during the digestion process of ruminants fed with contaminated feed.

Beer is a traditional beverage in many countries. Mycotoxins get to beer either from contaminated input materials or adjuncts (barley, malt, hop, brewery yeasts) added in the course of brewing. The first experiments to trace the mycotoxin fate in the brewing process were carried out in the 1970s when the authors Chu, Chang, Ashoor, and Prentice (1975) added before the micromalting process

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0956-7135/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodcont.2011.11.033

purified standards of toxins AF B_1 and ochratoxin A at concentrations of 1 and 10 μg/g of malt. A great portion of mycotoxins was removed during the production process, nevertheless 18% (at concentration of 1 μg/g) and 27% (at concentration of 10 μg/g) of aflatoxin B_1 passed into beer. The aflatoxins are only moderately heat-stable when boiled in water alone, but if present in foods and bound to a substrate, they are not nearly degraded (Tabata et al., 1992). In 2010, the authors Pietri, Bertuzzi, Agosti, and Donaldini (2010) studied the transfer of aflatoxin B_1 and fumonisin B_1 from naturally contaminated raw materials to beer. Content of AF B_1 ranged from 0.31 to 14.85 μg/kg of the raw material, 0.6–2.2 % of AF B_1 passed into beer. Before 1991 aflatoxins were not detected in European beers (e.g. Cerutti, Vecchio, Finoli, & Trezzi, 1987; Fukal, Prošek, & Rákosová, 1990; Payen, Girard, Gaillardin, & Lafont, 1983; Woller & Majerus, 1982), later, in the 1990s trace amounts were scarcely found in several samples of beers imported from south Europe. They occurred in beers coming from South America or equatorial Asian countries (Mably et al., 2005; Scott, 1996; Nakajima, Tsubouchi, & Miyabe, 1999). The presence of aflatoxins in raw materials and beers from tropical countries is common (e.g. Nikander et al., 1991; Odhav & Naicker, 2002), increased aflatoxin concentration is frequently reported in sorghum, a traditional African cereal, and beers brewed from it (Matumba, Monjerezi, Khongha, & Lakudzala, 2011).

A number of analytical methods have been used for the mycotoxin detection in food matrices (Turner, Subrahmanyam, & Piletsky, 2009). The most commonly used test for screening of aflatoxins in food is the commercially available set ELISA (e. g. Ghali, Hmaissia-khlifa, Ghorbel, Maaroufi, & Hedili, 2008), high-performance liquid chromatography with fluorescent (Jaimez et al., 2000) or mass - spectrometric detection is employed for more accurate determination. Currently, multiresidual methods for the analysis of more mycotoxins from one food matrix have been developed (e. g. Berthiller, Sulyok, Krska, & Schuhmacher, 2007; Ibáñez-Vea et al., 2011; Ventura et al., 2006; Zachariasova et al., 2010). The advantage is a lower price of the analysis and detection of simultaneous occurrence of more toxins.

The aim of this study was to summarize results of a four-year (2008–2011) research project monitoring the occurrence of aflatoxins B_1, B_2, G_1 and G_2 in brewing materials – malting barley, malt, hop, brewers' yeast, spent grains and beer. All samples were obtained from malt plants and breweries in the EU countries. The method of high-performance liquid chromatography with mass spectrometry (HPLC–MS/MS) was used for the analysis.

2. Experimental

2.1. Materials and reagents

Aflatoxin standards (B_1 and G_1 2.0 μg/ml, B_2 and G_2 0.5 μg/ml, solution in acetonitrile), analytical and HPLC reagents were obtained from Sigma–Aldrich (Steinheim, Germany). The immunoaffinity columns AFLAPREP were purchased from the company R-Biopharm (Germany). Multifunctional SPE clean-up columns MycoSep® 226 Aflazon+ were obtained from the company Romer Labs Diagnostic GmbH (Austria).

Phosphate buffer (PBS) was prepared by mixing 800 ml of deionized water containing 19.1 ± 0.1 g of disodium hydrogen phosphate (Na_2HPO_4) and 200 ml of deionized water containing 1.8 ± 0.1 g of potassium dihydrogen phosphate (KH_2PO_4). The pH of the resulted phosphate buffer was adjusted to 7.4 with the solution of 2 M sodium hydroxide (NaOH).

Mobile phase for chromatographic analyses was prepared by dissolving 0.38 ± 0.001 g of ammonium acetate (CH_3COONH_4) in 400 ml of deionized water and the volume was adjusted to 500 ml. The solution was filtered before use through the 0.45 μm nylon filter.

2.2. Samples

Aflatoxin content was analyzed in different brewing materials – malting barley, malt, hop, brewers' yeast and spent grains. Barley samples were collected during harvests 2008–2010. All samples were obtained from EU malting plants or breweries. The set included 61 barley samples, 77 malt samples, 54 hop samples, 12 brewers' yeast samples, 12 spent grains samples and 117 beer samples, i.e. totally 333 samples.

2.3. Preparation of samples

All samples were analyzed shortly after delivery. Barley and malt samples were ground and homogenized in the malt mill (Super Jolly SJ 500, Mezos, Czech Republic). Various matrices of hop were obtained (cones, extract, paste). Dried hop cones or pellets were ground in a mortar. 50 ± 0.1 g of a homogenized sample (barley, malt) or 25 ± 0.1 g (hop pellets) or 10 ± 0.1 g (hop cones) were weighed into a lyofilization bottle and 2.0 g of sodium chloride was added. 125 ml of MeOH:H_2O (60:40 v/v) was poured into the sample. After 10 min of ultrasonic treatment, deionized H_2O water (125 ml) was added and the homogenized sample was centrifuged at 4000 rev/min for 10 min. The supernatant was filtered through a glass filter.

10 ml of filtrated extract of barley, malt or hop were applied to the AFLAPREP immunoaffinity column at a flow rate equaling to 2–3 ml/min or slower. The column was washed with 2 × 10 ml of PBS. Elution was repeated three times with 1.0 ml of methanol at a flow rate lower than or equaling to 2–3 ml/min. The eluate was rotary evaporated to dryness under vacuum and redissolved in 1 ml of methanol–water (50:50, v/v). The sample was filtered through a 0.22 μm nylon microfilter.

In the beer matrix, the extraction step was omitted. 50 ml of beer sample was degassed using the ultrasound cleaning device; pH was adjusted to 7.2 with 2 M NaOH. The whole volume of the sample was passed through the immunoaffinity column and elution was performed as described above.

Brewers' yeast and spent grains were thoroughly homogenized (shaken) and 25 g aliquot was immediately taken. 100 ml of ACN:H_2O (84:16 v/v) was added to the sample and the sample was placed in ultrasonic bath for 10 min. The sample was then centrifuged at 4000 rev/min for 15 min and the supernatant filtered through a glass filter. 10 ml of the filtered extract was transferred into the glass tube and the whole volume was pushed through the MycoSep® 226 Aflazon + clean-up column. Pure extract (4 ml) was removed, rotary evaporated to dryness under vacuum, redissolved in 1 ml of methanol–water (50:50, v/v) and analyzed.

2.4. Preparation of standard calibration curve

Aflatoxin standards B_1, B_2, G_1 and G_2 were purchased from Sigma–Aldrich. Concentrations of B_1 and G_1 were 2.0 μg/ml and concentrations of B_2 and G_2 were 0.5 μg/ml in acetonitrile. Concentration of stock solution prepared from this standards was 100 ng/ml of B_1 and G_1 and 50 ng/ml of B_2 and G_2 in methanol:-water 50:50, v/v. A six-point calibration curve of methanol:water 50:50 was constructed from this stock solution by gradual dilution. Concentrations were as follows: B_1 and G_1 0.5, 1, 2, 5, 8, and 10 ng/ml, B_2 and G_2 0.25, 0.5, 1, 2.5, 4, and 5 ng/ml. Fresh calibration solutions were prepared before the measurement every day. Each solution was injected twice; mean was calculated from two measurements. The calibration curve was constructed as the dependence of the peak area on concentration of each standard.

Each aflatoxin was identified by comparing the retention time and MS/MS spectrum of a corresponding peak with the peak of the standard. The external standard method was used for the quantitative evaluation.

2.5. Analysis of aflatoxins by HPLC/ITP/MS

For the identification and quantification of aflatoxins, the HPLC (Finnigan Surveyor) coupled to ion trap LCQ Advantage (ThermoFisher, USA) with atmospheric pressure ionization was used. Conditions were as follows: The Synergi Hydro RP 80A HPLC column (3.0 × 150 mm, 4.0 μm particle size) was employed using isocratic elution with a methanol:10 mM ammonium acetate in water (50:50, v/v) mixture as the mobile phase. The column temperature was 40 °C. The flow rate was 0.3 ml/min and injection volume was 25 μl. The following MS parameters were employed: For electrospray ionization with positive ion polarity, the capillary voltage was set to 3.0 kV, the capillary temperature to 250 °C, the sheath gas flow (nitrogen) to 40 l/min. The collision energy was 40% and the fragmentation time was 30 ms. To determine the product ions, the protonated molecules $[M + H]^+$ (at m/z 313 for AFB_1, 315 for AFB_2, 329 for AFG_1 and 331 for AFG_2) were isolated, and the fragment ions were detected in a scan range of m/z 200–400. The selected fragment ions were 285 and 241 m/z for AFB_1, 287 and 259 m/z for AFB_2, 311 and 243 m/z for AFG_1 and 313 and 245 m/z for AFG_2. Data were collected and processed with Excalibur software.

3. Results and discussion

3.1. Analytical methods

Brewing materials are very complicated matrices, therefore, the selective immunoaffinity column (IAC) was chosen as the most suitable method for obtaining high purity extracts of aflatoxins with the low content of matrix components that could cause interferences in the assay. Purification of a sample also helps prevent contamination of the ion source. Multifunctional SPE clean-up columns are another alternative for complex matrices. Currently, the commercially available IAC and SPE columns can be used for simultaneous extraction of several mycotoxins, which makes sample preparation cheaper and less laborious.

Calibration curves were linear in the given range. EffiValidation program was used for calculation of the confidence intervals a and b. Equations of the calibration curves were as follows: For B_1 $y = 4232 \pm 1466x + 49405 \pm 8427$ with the regression coefficient $R^2 = 0.9969$. ($a < -17383 - 29402 >$; $b < 44606 - 52747 >$), for G_1 $y = 5331 \pm 1512x + 37423 \pm 8645$ with the regression coefficient $R^2 = 0.9943$. ($a < 32737 - 41130 >$; $b < -16432 - 31562>$), for B_2 $y = 9534 \pm 1115x + 32271 \pm 3171$ with the regression coefficient $R^2 = 0.9909$. ($a < 4727 - 22335 >$; $b < 28085 - 34275 >$) and for G_2 $y = 2398 \pm 1625x + 29858 \pm 5052$ with the regression coefficient $R^2 = 0.9929$. ($a < -12211 - 19940 >$; $b < 24300 - 34643 >$).

The limit of detection (LOD) was defined as the concentration at which the signal to noise ratio equals 3. The limit of quantification (LOQ) was defined as the concentration where the signal to noise ratio equals 10. LOD and LOQ values for barley and malt are comparable to the values given in the literature for cereals (Beltrán, Ibáñez, Sancho, & Hernández, 2009; Frenich, Martínez, Romero-González, & Aguilera-Luiz, 2009; Lattanzio, Solfrizzo, Powers, & Visconti, 2007) and beer (Nakajima et al., 1999; Ventura et al., 2006; Zachariasova et al., 2010). Results are summarized in Table 1.

Recovery was calculated using the spiked sample since the certified reference material is not commercially available for the raw materials studied. The sample with zero concentration of aflatoxins was selected and spiked with the aflatoxin standards; hop, brewers' yeast and spent grains to two, and barley, malt and beer to three given concentration levels. Then the samples were extracted and analyzed using the method described above. Recovery values for barley and malt were comparable to those given in the literature for barley (Ibáñez-Vea at al., 2011) and other cereals, such as maize (Lattanzio et al., 2007; Rahmani, Jinap, Soleimany, Khatib, & Tan, 2011). Recovery for beer was also in compliance with the published data (Nakajima et al., 1999; Ventura et al., 2006; Zachariasova et al., 2010). No comparable data for other brewing matrices exist in the literature. Hop is one of the most complicated matrices. It contains many complex substances (polyphenols, phenolic acids, flavonoids, bitter acids, hop oils and resins) which can complicate sample purification and following analytical determination. Each hop sample has a slightly different character (hop cones, hop pellets). Nowadays hop pellets are most frequently used for beer production. Recovery values attained by purification of hop through the immunoaffinity column were very good. Yeast and spent grain samples contained different percentage of water. The yeast and spent grain samples were treated in the same way as solid cereal matrix and results were not calculated to dry matter content in a sample. Extraction of yeasts and spent grains through the IAC columns was tested but extract yields were quite low, particularly in aflatoxins G_1 and G_2. In yeasts, the values were 61%, 63%, 41% and 28% for AFB_1, AFB_2, AFG_1 and AFG_2; in spent grains, the values were 60%, 87% 41% and 27% for AFB_1, AFB_2, AFG_1 and AFG_2. The immunoaffinity column may not be suitable for this kind of matrices. The sample clean-up procedures employing the multifunctional SPE column provided very good recovery values. Considering the complexity of matrices and relatively high "noise" on the MS detector, these three matrices were spiked only at two concentration levels, corresponding to the EU limits. The results are summarized in Table 2. The average recovery value for the sum of all aflatoxins (total aflatoxins) was 91.97% for barley and malt, 98.50% for hop, 86.64% for yeasts, 92.70% for spent grains and 93.98% for beer.

Table 1
Limits of detection (LOD) and limits of quantificiation (LOQ) for different matrices.

Matrix	Aflatoxin	LOD	LOQ
Barley, malt μg/kg	B_1	0.04	0.13
	B_2	0.08	0.26
	G_1	0.08	0.25
	G_2	0.12	0.39
Hop (granular formulation) μg/kg	B_1	0.08	0.25
	B_2	0.16	0.52
	G_1	0.15	0.51
	G_2	0.23	0.78
Hop (cones) μg/kg	B_1	0.19	0.64
	B_2	0.39	1.29
	G_1	0.38	1.27
	G_2	0.58	1.94
Brewer's yeast μg/kg	B_1	0.04	0.13
	B_2	0.08	0.26
	G_1	0.08	0.25
	G_2	0.12	0.39
Spent grains μg/kg	B_1	0.04	0.13
	B_2	0.08	0.26
	G_1	0.08	0.25
	G_2	0.12	0.39
Beer ng/l	B_1	1.5	5.1
	B_2	3.1	10.3
	G_1	3.1	10.2
	G_2	4.7	15.2

3.2. Occurrence of aflatoxins in brewing materials

The aflatoxin contents in real samples are given without recalculation for recovery. Values above the limit of quantification are

Table 2
Recovery values of aflatoxin-spiked brewing matrices and beer at three (barley, malt, beer) or two (hop, yeast, spent grains) different levels.

Matrix	Preparation of sample	Aflatoxin	Recovery (%) ± RSD		
			Low level (1.0 B_1/G_1, 0.5 B_2/G_2)	Medium level (2.0 B_1/G_1, 1.0 B_2/G_2)	High level (4.0 B_1/G_1, 2.0 B_2/G_2)
Barley, malt μg/kg	IAC	B_1	97.7 ± 18.63	83.1 ± 8.83	75.5 ± 16.74
		B_2	98.1 ± 16.18	103.2 ± 19.94	88.3 ± 12.68
		G_1	91.8 ± 11.58	81.4 ± 11.92	88.9 ± 10.97
		G_2	117.0 ± 25.03	87.8 ± 29.31	90.8 ± 15.76
Hop (granular formulation) μg/kg	IAC	B_1		86.8 ± 12.99	71.8 ± 7.38
		B_2		79.8 ± 17.15	87.5 ± 17.34
		G_1		98.7 ± 12.48	92.1 ± 9.92
		G_2		65.7 ± 16.18	109.6 ± 12.63
Brewer's yeast μg/kg	Clean-up column	B_1		82.6 ± 17.19	88.2 ± 11.44
		B_2		73.9 ± 17.15	82.1 ± 15.47
		G_1		101.3 ± 16.54	90.5 ± 9.09
		G_2		98.7 ± 16.87	75.8 ± 13.83
Spent grains μg/kg	Clean-up column	B_1		96.6 ± 11.62	90.3 ± 10.86
		B_2		93.5 ± 14.27	93.0 ± 12.83
		G_1		96.1 ± 9.17	95.5 ± 12.27
		G_2		101.8 ± 16.77	74.8 ± 12.27
			Low level (0.04 B_1/G_1, 0.02 B_2/G_2)	Medium level (0.08 B_1/G_1, 0.04 B_2/G_2)	High level (0.16 B_1/G_1, 0.08 B_2/G_2)
Beer μg/l	IAC	B_1	98.2 ± 17.96	88.4 ± 16.30	87.0 ± 8.39
		B_2	96.8 ± 14.94	84.3 ± 15.83	105.6 ± 14.79
		G_1	96.8 ± 18.67	90.1 ± 10.34	80.6 ± 10.78
		G_2	106.0 ± 17.82	103.6 ± 16.68	90.4 ± 18.84

taken as positive. Table 3 summarizes the results expressed according to currently valid European legislation (Commision of the European Communities, 2010) setting the maximum allowable limit for aflatoxin B_1 2.0 μg/kg and for total content of all aflatoxins (Σ B_1, B_2, G_1, G_2) 4.0 μg/kg. Only three positive samples of barley were found (4.9%). One sample contained 0.4 μg/kg AFB_1 and at the same time 0.4 μg/kg of the sum of the other three aflatoxins (AFB_2, AFG_1 and AFG_2), the second sample only 0.3 μg/kg AFB_1 and the third one only 1.1 μg/kg of the sum of the other three aflatoxins. The values measured in this study are slightly higher than those given by Ibáñez-Vea et al. (2011), who analyzed barley samples for food and feed products. The authors gave five positive (above LOQ) barley samples with maximal AFB_1 content 0.185 μg/kg. AFB_2 was detected in several samples but only one of them exceeded LOQ (0.042 μg/kg) and no samples with detected AFG_1 and AFG_2 exceeded limit of quantification.

In this study, one positive sample of malt (1.3%), one sample of brewers' yeast (8.3%) and one sample of spent grains (8.3%) were detected. These samples contained only aflatoxin B_1 at maximal concentration of 0.4 μg/kg. Most afltoxins are removed during brewing, the exact mechanism is not known. Significant losses during malt production may be caused by non-specific interactions or adsorption of mycotoxins by the solid particles removed by the filtration process (Chu et al., 1975) as spent grains. Brewing spent grains are a major by-product – up to 85% of all waste produced at brewing is used as feed for livestock, power industry and biotechnological processes. Due to its high protein and fiber contents it can serve as a dietary supplement (Mussatto, Dragone, & Roberto, 2006).

Table 3
Content of aflatoxins in brewing materials and beer.

Matrix	Total	Positive		Range	Range
		B_1	$\sum B_1, B_2, G_1, G_2$	B_1	$\sum B_1, B_2, G_1, G_2$
Barley μg/kg	61	2	3	0.3–0.4	0.3–1.1
Malt μg/kg	77	1	0	0.2	
Hop μg/kg	54	0	1		1.2
Brewers yeast μg/kg	12	1	0	0.2	
Spent grain μg/kg	12	1	0	0.4	
Beer ng/l	117	5	2	5.0–10.6	15.4–31.0

One positive hop sample (1.9%) contained only aflatoxins B_2, G_1 and G_2 above LOQ. Aflatoxins in malt, hop, yeast or spent grains aflatoxins have not been detected yet, nevertheless, monitoring of contamination by these mycotoxins in these matrices might be desirable also in the future.

3.3. Occurrence of aflatoxins in beer

Six positive beer samples (5.1%) contained aflatoxins in the values exceeding LOQ. In five of them, only AFB_1 at maximum concentration 10.6 ng/l was detected. This sample also contained 4.8 ng/l of other aflatoxins. The sixth positive beer sample contained 31.0 ng/l of the other three aflatoxins (AFB_2, AFG_1 and AFG_2). The given concentrations are comparable with the results given by Nakajima et al. (1999) and Mably et al. (2005) for European beers.

Presumably, malting barley and malt are not main sources of aflatoxin exposure for consumers if properly dried and secondary contamination during storage is avoided. We can conclude that beers brewed from quality, purified and well stored raw materials do not represent any health risk of aflatoxin exposure for consumers.

4. Conclusion

Aflatoxins may pass from the naturally contaminated raw materials or adjuncts added during the brewing process even to the final product – beer. In 2008–2011, a total set of 216 samples of brewing materials coming from the European Union were analyzed. Aflatoxins in trace concentrations to 1.2 μg/kg were found in 7 samples, i.e. 3.2%. In addition, trace amounts of aflatoxins were detected in six (5.1%) of 117 analyzed beer samples coming from the EU countries (AFB_1 to 10.5 ng/l and sum of all aflatoxins to 31.0 ng/l). Values in barley and malt did not exceed the maximum allowable limit given by the European Union.

Acknowledgment

Results were achieved in the framework of the Research Plan of the MEYS 6019369701.

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Food Control 47 (2015) 108–113

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Determination of seventeen mycotoxins in barley and malt in the Czech Republic

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ARTICLE INFO

Article history:
Received 5 March 2014
Received in revised form
13 June 2014
Accepted 21 June 2014
Available online 30 June 2014

Keywords:
Mycotoxins
QuEChERS
Mass spectrometry
Malting barley
ELISA

ABSTRACT

In this study, an analytical method for the determination of 17 mycotoxins was developed and validated: common (aflatoxins B_1, B_2, G_1, and G_2, fumonisins B_1 and B_2, ochratoxin A, deoxynivalenol, nivalenol, zearalenone, T-2 and HT-2 toxin) and "emerging" toxins (enniatins A, A_1, B, and B_1, and beauvericin) were detected using ultra-performance liquid chromatography coupled to mass spectrometry. A modified QuEChERS method was used for extraction. The method was applied to a total set of 52 barley and malt samples. All samples were contaminated with at least one of mycotoxins. None of the investigated samples contained any of four aflatoxins nor ochratoxin A. Fumonisin B_1 occurred only in one sample, Fumonisin B_2 and zearalenone were found in two barley samples. Enniatins were detected in all samples. The values did not exceed the maximum allowable limit for the selected mycotoxins in unprocessed or processed cereals set by the European Union.

1. Introduction

Barley (*Hordeum vulgare* L.) belongs to the oldest and economically most important crops. Currently, most of barley production is used as feed; the best quality barley is utilized for production of malt, beer and whisky. *Fusarium*, *Aspergillus*, and *Penicillium* species are the most significant fungal pathogens attacking cereals. Mycotoxins, secondary metabolites of these microscopic filamentous fungi (molds) are dangerous natural contaminants of crops. The target organs for mycotoxins are the immune and hematological system, the liver, kidneys, neurological and respiratory system. They can also induce dermatological reactions; in addition, they have mutagenic, teratogenic, carcinogenic, and estrogenic effects. The common occurrence of several mycotoxins accumulates their effects (Speijers & Speijers, 2004). Products of fungi of *Fusarium* (trichothecenes, zearalenone, fumonisins), *Aspergillus* and. *Penicillium* sp. (aflatoxins, ochratoxin A) are the most significant mycotoxins found in cereals. These mycotoxins have been studied for decades and numerous studies on their acting and occurrence are available (Creppy, 2002; Groopman, Kensler, & Wu, 2013; Marin, Ramos, Cano-Sancho, & Sanchis, 2013; Zain, 2011). On the other hand, "emerging" mycotoxins are neither routinely determined nor legislatively regulated. This group belongs to fusarium mycotoxins, including namely enniatins (Enns), beauvericin (Bea), moniliformin, and fusaproliferin. Important producers of these toxins are *F. subglutinans, F. proliferatum, F. avenaceum, F. tricinctum, F. acuminatum, F. oxysporum, F. sporotrichioides*, and *F. sambucinum* (Jestoi, 2008). Enniatins and beauvericin are usually cyclic hexadepsipeptides consisting of alternating units of D-α-hydroxy-isovaleryl- (2-hydroxy-3-methyl butan acid) and amino acids (Oueslati, Meca, Mliki, Ghorbel, & Mañes, 2011). Enniatins exhibit both antibiotic and insecticidal activity (Grove & Pople, 1980). Beauvericin exhibits cytotoxic activity (Fornelli, Minervini, & Logrieco, 2004) and can induce apoptosis (programmed cell death) and DNA fragmentation (Ojcius, Zychlinsky, Zheng, & Young, 1991). Enniatins and beauvericin act as ionophores; they disturb the pH and physiological ionic balance (Ivanov et al., 1973; Kamyar, Rawnduzi, Studenik, Kouri, & Lemmens-Gruber, 2004; Kouri, Lemmens, & Lemmens-Gruber, 2003).

Mycotoxins are thermostable and can pass from contaminated cereals to bakery and cereal products. Therefore, their occurrence is monitored, some of them have been regulated by European

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http://dx.doi.org/10.1016/j.foodcont.2014.06.045
0956-7135/

legislation (EC, 2006). In 2009, the European Food Safety Authority (EFSA) launched a call for monitoring the "emerging" mycotoxins and providing data on their occurrence (Verstraete, 2009).

Common mycotoxins in cereals are usually detected with screening ELISA tests, their advantage being cost effectiveness. More accurate and sensitive analytical methods based on high-performance liquid chromatography (HPLC) with fluorescence (FL) or ultraviolet (UV) detection or mass spectrometry (MS) are applied for simultaneous determination of several mycotoxins in one sample (Sulyok, Krska, & Shuhmacher, 2007, 2010, Malachova et al., 2011). Due to modern, sensitive mass spectrometers, the current trend is minimal or none sample pre-treatment. Besides cost savings, another advantage is a limited use of organic environmentally-unfriendly solvents (Malachova et al., 2011; Rubert et al., 2012; Zachariasova et al., 2010).

The objective of this study was to develop and validate a method based on ultra-performance liquid chromatography with tandem mass spectrometry for the determination of multiple mycotoxins, namely aflatoxins B_1, B_2, G_1 and G_2, fumonisins B_1 and B_2, ochratoxin A, deoxynivalenol, nivalenol, zearalenone, T-2 and HT-2 toxin, and "emerging" mycotoxins beauvericin and enniatins A, A_1, B, and B_1 in samples of spring barley and barley malt. Our method can be a useful tool for obtaining data on the occurrence of mycotoxins needed for their further regulation by legislation.

2. Experimental

2.1. Materials and reagents

Mycotoxin standards (solid and solution in acetonitrile) were purchased from different suppliers. Deoxynivalenol (DON), ochratoxin A (OTA), HT-2, zearalenone (ZON), aflatoxins B_1, B_2, G_1 and G_2 as well as isotope-labeled standards AFB_1-$^{13}C_{17}$, FB_1-$^{13}C_{34}$, DON-$^{13}C_{34}$, OTA-$^{13}C_{20}$ and T-2 toxin-$^{13}C_{24}$ were obtained from Sigma–Aldrich (Steinheim, Germany). Nivalenol (NIV), T-2 toxin and fumonisins FB_1 and FB_2 were purchased from Biopure (Wien, Austria). Enniantins EnnA, $EnnA_1$, EnnB, $EnnB_1$ and beauvericin were obtained from Enzo Life Science (New York, USA). Before use, the standard stock solutions were prepared in acetonitrile or mixture of toluene: acetonitrile (20–200 μg/ml) and stored at 4 °C in the dark.

Analytical, HPLC and MS reagents (acetonitrile, methanol, formic acid, ammonium formate, $MgSO_4$, and NaCl) were obtained from Sigma–Aldrich, Fluka (Steinheim, Germany) and Lach-Ner (Czech Republic). Deionized water was prepared using the Milli-Q system (Millipore Corporation, USA). The reference material (maize matrix contaminated with the assigned values of selected mycotoxins) was obtained during the participation in the inter-laboratory Proficiency Test for multi-mycotoxin methods based on LC-MS (MS) IRM MoniQA FOOD-CT-2006-036337.

2.2. Samples

Three sets of samples (52 in total) from harvest 2012 were analyzed. The first set included 22 samples ($n = 22$) of the following malting barley varieties: Bojos, Xanadu, Blaník, Radegast, Malz, and Wintmalt from different localities in the Czech Republic. DON detected in these varieties using the screening ELISA method exceeded 250 μg/kg. The second set consisted of six barley samples with DON content lower than 250 μg/kg. This set was then used as the control ($n = 6$). The third set included 24 samples, i.e. 12 barley samples of four malting varieties, Malz, Bojos, Sebastian, and Xanadu, from three localities in the Czech Republic (Čáslav, Uherský Ostroh, and Jaroměřice nad Rokytnou) and 12 malt samples made from these barleys ($n = 24$). In these samples, the ELISA test was not performed. The malts were prepared in a micromalting plant in the Malting Institute of the Research Institute of Brewing and Malting in Brno using the procedure with short steeping and CO_2 extraction according to the MEBAK method (Anger, 2006). The matrix-matched calibration was used for the quantification of mycotoxin content.

2.3. Detection of DON by the ELISA method

The competitive direct enzyme-linked immunosorbent assay (ELISA) is based on an antigen – antibody reaction. The number of bound substances is measured by adding an enzyme-labeled antigen or antibody. A commercially available AgraQuant® DON Assay 0.25/5.0 Test Kit (Romer Labs, Tulln, Austria) was used for the routine measurement of DON in barley samples. The method was validated for barley and malt. Extraction and determination were performed according to the manufacturer's instructions. Values of LOD and LOQ were 200 μg/kg and 250 μg/kg, respectively.

2.4. Preparation of samples for the UPLC-MS/MS analysis

Samples were prepared using the modified QuEChERS method. 10 ml of 0.1% HCOOH + 10 ml acetonitrile were added to the homogenized sample (5 g) and the mixture was shaken for 20 min at 250 rpm. Then 4 g $MgSO_4$ + 1 g NaCl were added, shaken and centrifuged at 5000 rpm for 5 min 0.5 ml of the mixture was taken from the supernatant and diluted in 0.5 ml of H_2O. Before the analysis, the sample was filtered through a 0.2 μm nylon microfilter.

2.5. Analysis of mycotoxins by UPLC/MS/MS

For the identification and quantification of the 17 targeted mycotoxins, the UPLC Acquity instrument (Waters) coupled to Xevo TQ MS (Waters) triple quadrupole mass spectrometer with atmospheric pressure ionization was used. Chromatographic separation was performed on the Acquity UPLC BEH C18 (50 mm × 2.1 mm × 1.7 μm) column equipped with the Acquity UPLC BEH C18 guard column (5 mm × 2.1 mm × 1.7 μm) at 40 °C using gradient elution. Mobile phase A consisted of 0.1% (v/v) formic acid in water, mobile phase B consisted of 0.1% (v/v) formic acid and 1 mM ammonium formate in methanol. Gradient elution started at 0 min with 10% B and was linearly increased to 20% B in 0.3 min, followed by another linear increase to 99.5% B over next 4.5 min, these conditions were maintained for 7 min (99.5% B) and then at 7.1 min switched back to 10% B. Prior to the next injection, the column was re-equilibrated to initial conditions for 10 min. The flow rate was 0.4 ml/min and injection volume 2.5 μl. Mass spectrometric conditions were as follows: The ESI interface was used in positive ion mode, source temperature – 150 °C, desolvation temperature – 450 °C, cone nitrogen gas flow – 15 L/h, desolvation gas flow – 700 L/h, capillary voltage – 3 kV. Mycotoxins were analyzed in MRM mode. The precursor and fragment ions for each analyte are given in Table 1. Data were collected and processed with MassLynx software.

3. Results and discussion

3.1. Analytical method

Accuracy, precision, selectivity, recovery and extended uncertainty of the determination of multiple mycotoxins were verified within the validation process. Spiking was performed in six replicates at three levels for cereal (barley) matrix. The validation criteria are given in Table 2. The uncertainty was determined based on the multiple measurement of the matrix spiked at three

Table 1
MS/MS parameters for each analyte, Xevo TQ MS (Waters) triple quadrupole mass spectrometer.

Mycotoxin	RT (min)	Precursor ion	Cone voltage (V)	Product ion 1	Collision energy (eV)	Product ion 2	Collision energy (eV)
Nivalenol	0.84	313.0	26	125.0	12	177	13
Deoxynivalenol	1.10	297.1	20	203	20	231.1	20
Aflatoxin G_2	2.07	331.1	45	245.1	40	313.1	33
Aflatoxin G_1	2.21	329.1	45	243.1	37	311.1	30
Aflatoxin B_2	2.33	315.1	45	259.1	40	287.1	35
Aflatoxin B_1	2.46	313.1	45	213.0	40	241	40
HT-2 Toxin	3.02	442.0	16	215.0	13	263	13
Fumonisin B_1	3.15	722.4	45	334.2	40	352.2	40
T-2 Toxin	3.29	484.1	20	185.2	12	215.3	12
Zearalenone	3.48	319.0	20	185.0	20	187	20
Ochratoxin A	3.54	404.2	25	221.1	25	239.1	25
Fumonisin B_2	3.62	706.4	50	318.2	55	336.2	50
Enniatin B	4.35	640.4	50	196.1	25	214.1	25
Beauvericin	4.44	784.4	50	244	25	262.4	25
Enniatin B_1	4.45	654.4	50	196.1	30	214.2	30
Enniatin A_1	4.54	668.5	50	210.2	27	228.2	27
Enniatin A	4.62	682.5	50	210.3	27	228.3	27

different concentrations. The extended uncertainty corresponded to the standard deviation of the data multiplied by a coverage factor of 2. With the exception of fumonisin B_2 and zearalenone, all validated mycotoxins fulfilled the requirements for the extended maximum uncertainty. Fumonisin B_2 and zearalenone met the requirements for the maximum extended uncertainty at the nearest validated level. The recovery in nivalenol was lower than 80%, nivalenol content was recalculated to the given recovery.

The accuracy of the method was checked with the reference material containing the assigned amount of selected mycotoxins. Further, for comparison, mycotoxins were quantified by the isotope dilution method. This method is appropriate only for the analytes with available isotope-labeled analogs, thus only some of the analytes were quantified by this method. In all cases, mycotoxin contents determined using this reference material were in compliance with the declared values both of matrix quantification and isotope-labeled standards.

The method is suitable for the determination of mycotoxins in food barley as the given limits of quantification (LOQ) are lower than, or in case of ochratoxin A equal to, maximum allowable limit (MAL) set by the EU regulation (EC, 2006) for the content of certain mycotoxins in selected foods.

3.2. Occurrence of mycotoxins in malting barley and malt

None of four aflatoxins and no ochratoxin A were detected in any of 52 investigated barley and malt samples. Fumonisin B_1 occurred only in one barley sample from the first set where contamination with other mycotoxins was presumed at the level of LOQ (<20 μg/kg). The samples with the mycotoxin content lower than the limit of detection (LOD) were considered negative, positive samples for the calculation of average values were those with the mycotoxin content exceeding the LOQ.

3.2.1. Comparison of DON contents detected by ELISA and UPLC-MS/MS

Although the ELISA assays are widely used for their easy sample preparation and low costs, on the other hand, their main limitations are cross-reactivity and false positive or over-estimated results. Kits have been developed for the detection of DON and DON-related compounds, e.g. 3-acetyl-DON, 15-acetyl-DON, de-epoxy-DON (DOM-1), and deoxynivalenol-3-glucoside (Ran et al., 2013). Besides cross-reactivity with conjugated deoxynivalenol-3-glucoside (D3G), the result of the ELISA assay may also be affected by cross-reactivity between the kit antigen and compounds of the matrix. In this study, we did not determine separately D3G or any other conjugated forms of DON. Therefore, the results of DON assessed by the ELISA method in the first set are overvalued. DON concentrations determined by this method varied from 255 to 917 μg/kg in the first set, while concentrations determined by UPLC-MS/MS moved from 69.9 to 602.3 μg/kg (Table 3). In the control set, DON content determined by ELISA in all six samples was below the LOQ, i.e.250 μg/kg. The UPLC-MS/MS detected DON only in one of six samples in the amount below LOQ (Table 3).

3.2.2. The first set and control set of barley samples

Samples for the first set were selected with respect to the presence of trichothecene DON as this frequently occurring toxin is considered a marker of mycotoxin contamination indicating the presence of other mycotoxins. This fact was confirmed in this study as well. The maximum content of DON determined by UPLC-MS/MS was 602.2 μg/kg; this corresponds to approximately half of the legal limit of 1250 μg/kg (EC, 2006) and is comparable with the data given for barley in the literature (Běláková, Benešová, Čáslavský, Svoboda, & Mikulíková, 2014). Contents of other present mycotoxins were also in compliance with the data reported in the literature. Contamination with ZON and FB_2 was negligible; these two mycotoxins were detected only in two samples. Contents of nivalenol, zearalenone, T-2 and HT-2 toxins are comparable with results of studies on barley and cereals carried out in the Czech Republic and neighboring countries in recent years (Běláková et al., 2014; Ibáñez-Vea, Lizzaraga, González-Peñaz, & López de Cerain, 2012; Malachová et al., 2010; Mankevičiene, Butkutė, Gaurilčikienė, Dabkevičius, & Supronienė, 2011; Pleadin at al., 2013, Škrbič, Malachová, Živančev, Vepříková, & Hajšlová, 2011).

Beauvericin and enniatin A occurred sporadically in our samples and only at low concentrations (max. 47.9 and 11.7 μg/kg, respectively). The samples were most contaminated with Enn A_1, Enn B_1, and Enn B, which occurred in all samples. The highest concentration (ca 1000 μg/kg) was detected in Enn B, which is in compliance with the data reported for barley (Uhlig, Torp, & Heier, 2006; Václavíková et al., 2013). Higher enniatin concentrations have been reported from the Mediterranean countries with warmer weather. In 2011, Oueslati et al., 2011 found in barley average content of Enn A 33.6, Enn A_1 116.4, Enn B 27.5 and Enn B_1 31.0 mg/kg.

Trace amounts of beauvericin were detected in six samples from the control set, one sample contained a low quantity of Enn A (6.72 μg/kg) and 3–4 × lower amount of Enns A_1, B and B_1 compared to the first set. The samples from this set also contained

Table 2
Validation parameters of UPLC-MS/MS method.

Mycotoxin	Spike level (μg/kg)	Recovery (%) ± RSD	LOD (μg/kg)	LOQ (μg/kg)
Nivalenol	80.0	50.0 ± 2.0	24.0	80.0
	160.0	51.0 ± 8.0		
	800.0	50.0 ± 8.0		
Deoxynivalenol	50.0	75.0 ± 5.0	15.0	50.0
	100.0	78.0 ± 8.0		
	500.0	85.0 ± 10.3		
Aflatoxin G_2	2.5	103.0 ± 7.0	0.75	2.5
	5.0	103.0 ± 8.0		
	25.0	98.0 ± 5.0		
Aflatoxin G_1	2.5	95.0 ± 14.0	0.75	2.5
	5.0	92.0 ± 12.0		
	25.0	98.0 ± 4.0		
Aflatoxin B_2	1.0	86.0 ± 13.0	0.3	1.0
	2.0	97.0 ± 12.0		
	10.0	99.0 ± 4.0		
Aflatoxin B_1	1.0	79.0 ± 12.0	0.3	1.0
	2.0	93.0 ± 4.0		
	10.0	101.0 ± 2.0		
HT-2	5.0	105.0 ± 28.0	1.5	5.0
	10.0	99.0 ± 13.0		
	50.0	99.0 ± 12.0		
Fumonisin B_1	10.0	94.0 ± 17.0	3.0	10.0
	20.0	106.0 ± 13.0		
	50.0	75.0 ± 14.0		
T-2	5.0	90.0 ± 14.0	1.5	5.0
	10.0	94.0 ± 9.0		
	50.0	92.0 ± 5.0		
Zearalenone	5.0	82.0 ± 44.0	3.0	10.0
	10.0	86.0 ± 15.0		
	50.0	102.0 ± 7.0		
Ochratoxin A	5.0	110.0 ± 1.0	1.5	5.0
	10.0	110.0 ± 3.0		
	50.0	95.0 ± 2.0		
Fumonisin B_2	5.0	124.0 ± 38.0	6.0	20.0
	20.0	96.0 ± 10.0		
	50.0	119.0 ± 7.0		
Enniatin B	5.0	96.0 ± 12.0	1.5	5.0
	10.0	101.0 ± 14.0		
	50.0	114.0 ± 7.0		
Beauvericin	5.0	100.0 ± 2.0	1.5	5.0
	10.0	97.0 ± 3.0		
	50.0	98.0 ± 2.0		
Enniatin B_1	5.0	107.0 ± 10.0	1.5	5.0
	10.0	99.0 ± 6.0		
	50.0	105.0 ± 5.0		
Enniatin A_1	5.0	96.0 ± 7.0	1.5	5.0
	10.0	95.0 ± 4.0		
	50.0	99.0 ± 3.0		
Enniatin A	5.0	94.0 ± 4.0	1.5	5.0
	10.0	96.0 ± 4.0		
	50.0	99.0 ± 2.0		

very low or trace amounts of other monitored mycotoxins. Results from both series are summarized in Table 3.

3.2.3. Transfer of mycotoxins from barley to malt

The fate of some mycotoxins during the malting process and during brewing has been monitored in the past (e.g. Scott, 1996), however, there are only two studies on the transfer of enniatins and beauvericin from barley to malt and beer in a laboratory (Hu, Gastl, Linkmeyer, Hess, & Rychlik, 2014; Václavíková et al., 2013). In 2007, Václavíková et al. malted the spring barley variety Radegast and collected samples during the malting process. Barley was naturally contaminated and grown in a field in two variants: treated and untreated with fungicide. The authors observed, that green malt contained ca 13–40% of enniatins compared to the initial barley, the enniatin content in dry kilned malt decreased to 10–30% of the amount detected in the original raw material. The effect of chemical treatment was not significant.

Table 3
Results of mycotoxins in the first and second (control) sets of barley samples.

Sample group	Mycotoxins	No. of positive samples above LOD (% positive)	Mean (samples positive above LOQ)	Range (samples positive above LOQ)	Median
First set ($n = 22$)	NIV	8 (36.4)[a]	93.4	52.0–123.3	0
	DON	19 (86.4)[b]	284.14	69.9–602.3	127.8
	ZON	2 (9.1)	192,8	181.2–204.4	0
	FB_1	1 (4.5)	Trace (<20)		0
	FB_2	2 (9.1)	13.36	11.08–15.64	0
	Enn B	22 (100)	498.6	15.90–1416	427.17
	Bea	22 (100)[c]	18.2	5.13–47.91	0
	Enn B_1	22 (100)	142.8	5.56–476.2	126.35
	Enn A_1	22 (100)[d]	45.6	5.85–174.3	25.56
	Enn A	22 (100)[e]	9.7	5.96–12.36	0
	HT-2	10 (45.5)	14.6	5.9–57.15	0
	T-2	8 (36.4)[f]	24.0	14.85–30.5	0
Second (control) set ($n = 6$)	NIV	1 (16,7)	Trace (<80)		0
	DON	1 (16,7)	Trace (<50)		0
	FB_2	1 (16,7)	Trace (<20)		0
	Enn B	6 (100)	143.4	14.5–338.4	133.72
	Bea	6 (100)	Trace (<5)		0
	Enn B_1	6 (100)	52.5	6.5–171.6	35.99
	Enn A_1	6 (100)[g]	28.8	3.6–84.7	7.57
	Enn A	6 (100)[h]	6.72	6.72	0
	HT-2	4 (66.7)	9.1	6.9–11.54	3.45
	T-2	2 (33.3)	Trace (<5)		0

[a] 5 samples were positive above LOQ, 3 below LOQ (<80 μg/kg).
[b] 16 samples were positive above LOQ, 3 below LOQ (<50 μg/kg).
[c] 6 samples were positive above LOQ, 16 below LOQ (<5 μg/kg).
[d] 19 samples were positive above LOQ, 3 below LOQ (<5 μg/kg).
[e] 3 samples were positive above LOQ, 19 below LOQ (<5 μg/kg).
[f] 5 samples were positive above LOQ, 3 below LOQ (<5 μg/kg).
[g] 4 samples were positive above LOQ, 2 below LOQ (<5 μg/kg).
[h] 1 sample was positive above LOQ, 5 below LOQ (<5 μg/kg).

Hu et al., 2013 malted the spring barley variety Quench in three replicates, one of which was infected during flowering in a field with spores of *F. culmorum*, the second with *F. avenaceum* and the third was left to natural infection. Their results were partially contrary to the findings of Václavíková et al., 2013. Content of enniatins and beauvericin in naturally contaminated (non-infected) variants increased in green malt versus barley on average by ca 50%. Compared to the initial barley, the resulting dry kilned malt contained 76% of Enn A, 83% of Enn A_1, 100% of Enn B, 103% of Enn B_1, and 84% of Bea. The mycotoxin contents of the initial barley and final kilned malt did not change substantially. In these studies only one or two samples of one naturally contaminated variety were malted, each had a different initial content of mycotoxins.

In the scope of this research, only barley and malt prepared from it were analyzed. Malt prior to the analysis was stored at the laboratory temperature in an air-conditioned room.

Based on the experiments published previously, it could be presumed that enniatin and beauvericin levels in malt would keep the same values as the initial barley or would rather decline. This assumption was confirmed only in some cases. We can summarize that barley used for malting belonged to those less contaminated (Rubert et al., 2012).

Fig. 1 shows the average contents (from four varieties) of enniatins and beauvericin in the barley and malt samples.

The detected Enn A levels were below the LOQ with the exception of the barley variety Bojos from Jaroměřice nad Rokytnou (9.4 μg/kg) and two malts from Uherský Ostroh (9.6 and 25.3 μg/kg) (not included in the figure).

It has been proven that the range of the mycotoxins present depends on the locality, weather, variety and first of all year of growing. In this study, significant differences among the individual

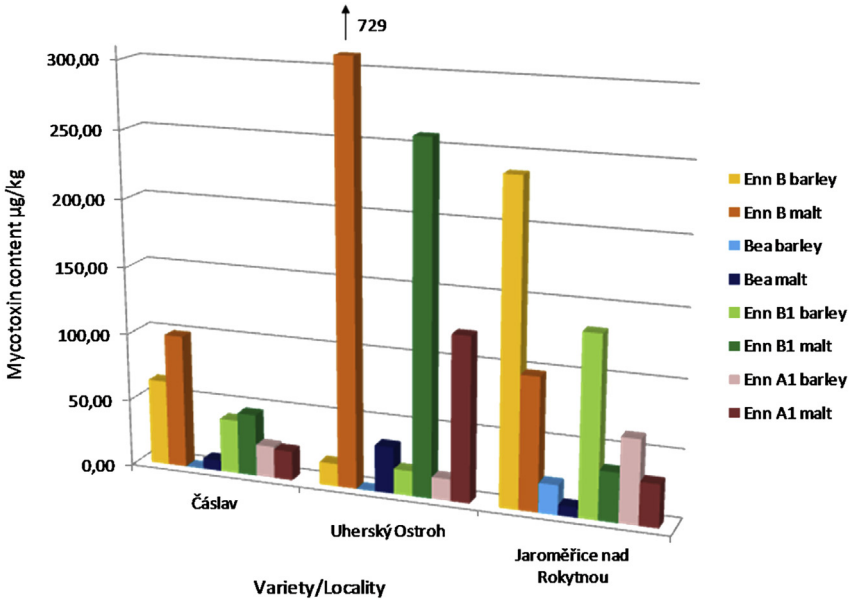

Fig. 1. Enniatin and beauvericin contents in barley and malt from different localities.

localities were found. The relatively highest concentration of these mycotoxins was detected in malts from the locality Uherský Ostroh.

The least contaminated barley and malts were from the locality Čáslav. Enn A_1 occurred at concentrations to 34.1 µg/kg, Enn B to 111.9 µg/kg, Enn B_1 to 68.4 µg/kg and their levels both in barley and malt were comparable. Beauvericin was detected in two malts at low concentration (7.8 µg/kg), and it was always under the LOQ in barley. Of the other mycotoxins, only DON occurred in the barley variety Sebastian at concentration of 62.12 µg/kg.

The mycotoxin levels in the samples from Jaroměřice nad Rokytnou were also quite low. The highest concentrations were detected in the variety Bojos: Enn B 420.7 µg/kg, Enn B_1 307.6 µg/kg, and Enn A_1 154.4 µg/kg; concentrations of Enn B_1 and A_1 were the highest of all the examined samples. Compared to barley, Enn B, B_1 and A_1 contents decreased in all malts with the exception of the variety Xanadu. Beauvericin was detected at low concentrations (to 30.1 µg/kg) in all barley samples with even lower concentration in malt. All barley samples also contained nivalenol and two samples contained DON, these values, however, were below the LOQ. Two barley samples and one malt sample contained low amounts of T-2 and HT-2 toxins.

Barleys from the locality Uherský Ostroh were the least mycotoxin-contaminated. In two barley samples, nivalenol (<80 µg/kg), Bea, T-2 and HT-2 toxin were below the LOQ (5 µg/kg), Enns did not exceed 33.5 µg/kg (Enn B_1 in the barley variety Bojos). After malting, concentration of some mycotoxins increased in orders of hundreds to thousands of percents in all four samples. In all malts, Bea occurred at concentrations to 41.7 µg/kg, Enn A_1 to 281 µg/kg, Enn B to 1110 µg/kg, and Enn B_1 to 504 µg/kg. Nivalenol (220 µg/kg), DON (65 µg/kg) were found in the variety Sebastian; HT-2 toxin in the varieties Bojos and Xanadu (20 and 12 µg/kg, respectively).

Although the micromycetes of the *Fusarium* species are "field molds", under favorable conditions they can also grow during storage (Fakhrunnisa, Hasmhi, & Ghaffar, 2006; Vaughan, Sullivan, & Sinderen, 2005). Microspores of fungi can be present everywhere, for example, contact of harvested crops with soil during harvest can be risky. The previous crop is also an important factor. During malting, mycotoxin levels decline, however, their production may increase during germination as the warm, humid environment of a malthouse is a suitable environment for mold growth. Further mycotoxin production can occur during kilning. High temperatures reduce fungal growth, but in some fungal species the increase in temperature in the initial stages of kilning can stimulate an increased mycotoxin production (Wolf-Hall, 2007). In compliance with previous results, Oliveira, Mauch, Jacob, Waters, and Arend (2012) refered that the increased temperature during kilning created unfavorable conditions for the growth of molds, on the other hand, the stress response led to the increased DON production. Concentration of DON can also increase as a result of the activity of amylolytic enzymes involved in the release of DON and D3G from their bound forms (Malachová et al., 2010).

4. Conclusion

The ultra-performance liquid chromatography method with mass spectrometry for the detection of 17 mycotoxins in barley and malt was developed and validated. This method was applied in a set of 52 samples of spring barley and barley malt from crop 2012. The detected mycotoxin concentrations did not differ from the values given in the literature in previous years. It was confirmed that the range of the mycotoxins present depended on the locality, weather, variety, and namely on the growing year. The mycotoxin values detected in barley in this study were lower compared to the data given in the literature. Twelve barley samples from three localities were malted. Mycotoxin contents in produced malts were lower or did not change significantly compared to the initial raw material. Only in one locality, concentration of some mycotoxins (enniatins) significantly increased after malting barley from all four studied varieties. This may be due to contamination of barley with mold microspores already in a field and enhanced mycotoxin production during malting. However, no general conclusions can be made and further research is necessary.

Acknowledgment

Results were achieved within the Project FCH-S-13-2087 of the Ministry of Education, Youth and Sports of the Czech Republic and institutional support of the Ministry of Agriculture of the Czech

Republic for a long-term conceptual development of the Research Institute of Brewing and Malting.

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Assessment of the Authenticity of Fruit Spirits by Gas Chromatography and Stable Isotope Ratio Analyses

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Abstract

Winterová R., Mikulíková R., Mazáč J., Havelec P. (2008): **Assessment of the authenticity of fruit spirits by gas chromatography and stable isotope ratio analyses**. Czech J. Food Sci., **26**: 368–375.

The gas chromatographic (GC) determination of volatile constituents and the determination of $^{13}C/^{12}C$ isotope ratios by isotope ratio mass spectrometry – IRMS analysis as well as SNIF–NMR analysis of (D/H)I and (D/H)II ratios in ethanol are prospective analytical methods which can be used for checking the authenticity of fruit spirits and for detecting their adulteration. Different concentrations of volatile compounds such as acetaldehyde, ethyl acetate, diethyl acetal, methanol, 1-butanol, 2-butanol, 1-propanol, 2-methyl-1-propanol, 2- and 3-methyl-1-butanol, volatile fatty acids and isotopic data were demonstrated using discriminant analysis. The results show that the determination of isotope ratios can be used especially for distinguishing between fruit spirits and others spirits, i.e. those made from beet sugar, maize, cane sugar, grain, potato, or synthetic alcohol. Gas chromatography also makes it possible to discriminate between respective spirits derived from one kind of fruit such as sweet cherry brandy, sour cherry brandy, pear brandy, apple brandy, apricot brandy, or plum brandy.

Keywords: authenticity; fruit spirits; gas chromatography; stable isotope ratio analysis; IRMS; SNIF-NMR; linear discriminant analysis

The requirements for quality food products have been increasing in recent years and the interest in the quality and purity of fruit spirits has grown in this connection as well. The everyday practice of market supervision reveals that high-quality distillates are often blended with cheaper raw materials of lower quality. Sugar is sometimes added during fermentation of fruits to obtain a higher yield of spirit, at other times ethanol made from cheaper raw materials (beet sugar, maize, cane sugar, grain, potato) or synthetic alcohol is added.

One of the possibilities of preventing the adulteration of fruit spirits is an advanced analytical control. A study of proving the authenticity and identification of respective kinds of fruit spirits was therefore started. This study includes the creation of a statistical file of analytical data.

Methods based on the determination of fruit spirit components were developed for these purposes. These methods include gas chromatography – GC (Bauer-Christoph *et al.* 1997; Kelly *et al.* 1999; Council Regulation EEC No. 2870/2000), the determination of stable isotope ratio using nuclear magnetic resonance – ^{2}H-NMR, and $^{13}C/^{12}C$ isotope ratio using mass spectrometry – IRMS (Council Regulation EEC No. 2676/90; Bauer-Christoph *et al.* 1997, 2003).

The assessment of the $^{13}C/^{12}C$ carbon isotope ratio reliably reveals the adulteration of fruit spirits

with sugar. The determination of deuterium/hydrogen (D/H)I and (D/H)II ratios in the ethanol molecule by ^{2}H- NMR serves to detect the source of non-fruit ethanol.

Gas chromatography is a suitable method for the identification and specification of respective kinds of fruit spirits. The use of gas chromatography can determine major as well as minor components of fruit spirits (Kelly *et al.* 1999; Bauer-Christoph *et al.* 1997). The contents of volatile compounds, especially aroma components, in the finalised spirits can verify the use of single fruit materials for their production.

The requirements for determining the authenticity of fruit spirits are described in the Council Regulation EEC No. 2870/2000. The authentic fruit spirits cannot contain ethanol other than that of fruit origin.

MATERIALS AND METHODS

Materials. A total of 153 samples of fruit spirits (from years 2003–2006) made from different kinds of fruit (plum brandy, pear brandy, apple brandy, apricot brandy, sweet-cherry brandy, sour-cherry brandy) were analysed and the results were processed statistically.

The samples were provided by three producers located in the Czech Republic, who had guaranteed the authenticity of spirits.

Gas chromatography

Methods. Volatile components such as acetaldehyde, ethyl acetate, methanol, and higher alcohols (1-propanol, 1-butanol, 2-butanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol) were analysed by gas chromatography (Council Regulation EEC No. 2870/2000), using a FID detector and a Hewlett Packard gas chromatograph with split injection (20:1). The injector temperature was 150°C; the detector temperature was 250°C. The capillary column CP-WAX 57CB (length 50 m, I.D. 0.32 mm, film thickness 0.2 μm) was used.

The oven temperatures were programmed as follows: starting at 40°C with 17 min isothermal period, then increasing to 70°C at the rate of 12°C/min, with the final 5 min thermal persistence. The carrier gas was helium at the flow of 2.7 ml/minute. The standards from Fluka and Aldrich companies were used for qualitative and quantitative calibrations. All determinations were executed by the internal standard method. Pentane-3-ol was used as the internal standard substance. The components contents were expressed in mg/l of pure ethanol (p.e.).

Volatile components such as ethyl esters of fatty acids, benzaldehyde, and flavour compounds were analysed by gas chromatography using mass selective detection (MSD) (Wardencki 2003; Pino 2002; Ng 2002; Gomez 2005; Pawliszyn 2000; Soufleros 2004). The apparatus used was a Finnigan gas chromatograph. The SPME (Solid Phase Micro Extraction) method was chosen for extracting these substances. This technique is suitable for the organic components concerned. It is based on the adsorption of substances from the sample to the surface of a siliceous fibre covered with the appropriate stationary phase. The substances adsorbed to the fibre were desorbed in the injector of the gas chromatograph. A 75 μm CAR^{TM}/PDMS fibre was used for SPME extraction.

SPME extraction conditions: sample temperature –25°C; time of extraction 15 min; desorption in injector 3 min; injector temperature 280°C (splitless).

A GC/MSD capillary column DB-WAX (length 30 m, I.D. 0.25 mm, film thickness 0.25 μm) was used for the analysis by gas chromatography. The oven temperatures were programmed in two steps. In the first step, the temperature increased from 55°C (isothermal 3 min) to 150°C (isothermal 5 min) at 10°C/min, in the second step it ramped from 150°C to 200°C (isothermal 1 min) at 10°C per minute. The carrier gas used was helium at the flow of 2.0 ml per minute. The internal standard method (heptanoic acid) was used for the determinations.

^{2}H-NMR analysis

The D/H ratios of ethanol in the samples were determined according to the official analytical method for wine analysis by quantitative deuterium NMR spectroscopy, as described in the Council Regulation EEC 2676/90, method No. 8. A 70 ml subsample was distilled using the Cadiot spinning band column. To prevent the isotopic fractionation, the minimal distillation yield of 95% was acquired. To determine the water content in the distillate, a Mettler DL18 Karl Fischer titrator was used.

The Bruker AVANCE DPX 400 spectrometer equipped with a 10 mm dual deuterium probehead (fluorine lock) and a BACS-60 automatic sample changer was used for ^{2}H-NMR measurement. The

NMR tubes were prepared as follows: 2.3 ml of distillate were placed into a pre-weighed bottle and weighed nearest to 0.1 mg; 1.3 ml of the internal standard (tetramethylurea with known value of D/H) was then added and weighed nearest to 0.1 mg; finally, 150 µl of the lock substance (10:1 mixture of C_6F_6 and trifluoroacetic acid) was added and weighed nearest to 0.1 mg; the blend obtained was then filtered into the NMR tube. For each tube, 10 NMR spectra were recorded at a frequency of 61.4 MHz with the acquisition time 6.2 s, 90° pulse, and 200 scans at 30°C. The processing of the FIDs and the calculation of D/H of ethanol were performed using the EUROSPEC software.

Carbon isotope analysis by IRMS

The determination of $\delta^{13}C$ ratio of ethanol contained in the samples was carried out according to the official analytical method for wine analysis by EA-IRMS spectroscopy, as described in the Council Regulation EEC 2676/90, method No. 45. Approximately 1 µl of the distillate was injected into the EA 1110 CHN (Fisons Instruments) using a liquid autosampler CTC-AS200S. The CO_2 obtained by the combustion of the distillate was introduced into the Thermo Finnigan DELTA Plus Advantage IRMS spectrometer using the ConFlo interface. CO_2 calibrated by certified reference materials was used as the reference gas.

RESULTS AND DISCUSSION

Volatile components, esters and aroma components suitable for the specification of fruit spirits, analysed by GC/FID and GC/MSD

Tables 1 and 2 summarise the minima and maxima of the volatile compound contents in the authentic samples of individual sorts of fruit spirits. These volatile compounds are important for characterising alcoholic distillates and fruit spirits.

The amounts of methanol in the samples varied from 932 to 12 053 mg/l p.e. Methanol is a constituent arising from the enzymatic degradation of pectin contained in fruits. Generally, its quantity is related to the amount of pectin present in fruits used for fermentation. The methanol concentration is suitable for proving the authenticity of fruit spirits.

Limits are posed by the Council Regulation EEC No. 1576/89 on the methanol content in many spirits. Its determination is part of the quality control of spirit drinks.

Following this regulation, the authentic fruit spirits should meet the maximum limit approved for the methanol concentration, i.e. 10 000 mg/l p.e. This limit was exceeded in 9 samples (4 apricot brandies, 1 pear brandy, 2 sweet cherry brandies, 2 plum brandies).

Fruit spirits typically had high contenst of methanol and 1-propanol, whereas spirits made from grain contained significantly less of these. This compares well with the data by Bauer-Christoph *et al.* (1997), who found that the grain spirits mostly contained only 100 mg/l p.e. of methanol and 1-propanol. In the fruit spirits, the concentrations of higher alcohols were significantly lower than those of methanol. The contents of higher alcohols fluctuated over a wide range of values.

Higher alcohols are characteristic components which are metabolised from amino acids by yeasts during alcoholic fermentation of fruits and other raw materials. The amounts of these compounds depend on the quantity of amino acids in fruits.

The higher alcohols most frequently found in low concentrations were 1-butanol and 2-butanol. The lowest values measured (5–31 mg/l p.e.) were those of 1-butanol in sweet cherry and sour cherry brandies. Wencker *et al.* (1981) showed that 1-butanol is a strongly discriminating parameter for the fruit spirits.

Table 2 show the values of the aroma components that were present in concentrations significantly lower than those of higher alcohols (Table 1).

The concentrations of esters and aroma components were mostly lower than 1 mg/l p.e., in some cases 1–50 mg/l p.e., and only sporadically higher than 50 mg/l p.e.

The lowest concentrations were found of β-citronellol (below 0.3 mg/l p.e.) in all sorts of fruit spirits. Low contents of β-linalool, α-terpineol, and eugenol (0–15.3 mg/l p.e.) were also observed. Although the aroma compounds were only found in smaller amounts, they should also contribute to the verification of fruit spirit authenticity.

In all fruit spirits, the contents of volatile components were probably correlated with technological parameters, such as the activity of yeasts during fermentation or the conditions of fermentation, and with the distillation process, i. e. the separation of particular fractions. The contents of the respective components may also depend on fruit ripeness and storage.

Table 1. Concentrations (minimum–maximum) of volatile components of fruit spirits, determined by GC/FID (mg/l of pure ethanol; *n* = number of samples)

	Pear brandy (*n* = 44)	Apple brandy (*n* = 12)	Sweet cherry brandy (*n* = 31)	Plum brandy (*n* = 29)	Sour cherry brandy (*n* = 21)	Apricot brandy (*n* = 16)
Acetaldehyde	13–562	30–260	16–355	26–385	13–597	25–320
Ethyl acetate	76–2937	125–2334	270–6921	563–2359	199–6565	279–3394
Diethyl acetal	20–375	63–778	17–254	18–321	19–361	42–203
Methanol	932–10 809	1794–9168	4520–10 695	2877–11 414	4376–8784	6723–12 053
2-Butanol	6–733	8–323	15–1531	13–195	5–176	7–1715
1-Propanol	141–7068	121–2290	244–3758	356–3084	129–1562	292–2869
2-Methyl-1-propanol	341–1116	392–968	178–1366	222–1361	113–1955	511–1776
1-Butanol	16–228	80–205	5–31	21–126	7–31	27–516
2-Methyl-1-butanol	201–753	333–705	110–618	149–735	128–726	254–818
3-Methyl-1-butanol	900–3998	1705–4225	589–3017	591–2649	674–3120	799–2878

Analysis of stable isotope ratios for determining the authenticity of fruit spirits

The amount of stable isotopes in raw materials is influenced by the growing location and the growth conditions of the plant from which ethanol has been made.

The ^{2}H-NMR analysis is based on the measurement of the deuterium to hydrogen (D/H) ratio of the methyl (D/H)I and methylene (D/H)II groups in the ethanol molecule.

Isotopic parameters (D/H)I, (D/H)II, and $\delta^{13}C$ of ethanol from the fruit spirits are summarised in Table 5. It should be noted that the parameters

Table 2. Concentrations (minimum-maximum) of esters, aroma components of fruit spirits, determined by GC/MSD (mg/l of pure ethanol; *n* = number of samples)

	Pear brandy (*n* = 44)	Apple brandy (*n* = 12)	Sweet cherry brandy (*n* = 31)	Sour cherry brandy (*n* = 21)	Plum brandy (*n* = 29)	Apricot brandy (*n* = 16)
Ethyl caprylate	0.3–39.1	14.2–106.7	1.1–46.6	1.4–52.7	2.9–107.6	0.6–49.1
Benzaldehyde	< 0.1–5.9	0.8–73.8	1.3–47.0	0.7–195.0	0.3–31.2	0.3–46.7
β-Linalool	< 0.9	< 0.9	< 0.9–2.8	< 0.9–1.7	< 0.9–1.8	< 0.9–85.5
Methyl caprinate	< 0.3–1.6	< 0.3–5.1	< 0.3–2.4	< 0.3–1.8	< 0.3–3.3	< 0.3–1.3
Ethyl caprinate	0.8–167.1	13.4–360.0	1.3–136.2	3.1–176.8	6.0–306.0	2.1–144.2
α-Terpineol	< 0.5–0.8	< 0.5–2.3	< 0.5–1.4	< 0.5–3.1	< 0.5–1.2	0.7–63.2
β-Citronellol	< 0.3	< 0.3	< 0.3	< 0.3–3.2	< 0.3	< 0.3–3.5
Ethyl laurinate	< 0.1–134.9	< 0.1–254.2	1.4–157.5	1.1–186.6	3.6–226.1	2.4–206.0
Ethyl myristate	0.9–43.1	4.2–72.7	0.4–55.8	< 0.4–69.5	< 0.4–47.1	2.2–54.6
Eugenol	< 0.7–2.7	< 0.7–1.9	< 0.7–2.3	< 0.7–15.3	< 0.7–9.4	< 0.7–13.2
Methyl palmitate	< 0.2–8.0	< 0.2–4.1	< 0.2–4.0	< 0.2–10.8	< 0.2–9.8	0.4–13.2
Ethyl palmitate	1.0–192.8	22.1–156.4	1.0–179.3	0.4–229.1	< 0.2–490.7	13.2–289.7
Phenylethyl oktanoate	< 0.4–93.3	< 0.4–147.0	< 0.4–5.1	0.6–269.2	< 0.4–11.8	< 0.4–10.4

Table 3. Stable isotope concentrations (minimum and maximum) in ethanol from fruit spirits (*n* = number of samples)

Fruit spirit	(D/H)I (ppm)	(D/H)II (ppm)	$\delta^{13}C$ (‰)
Pear brandy (*n* = 44)	94.46 to 98.95	118.92 to 127.41	–28.17 to –25.27
Apple brandy (*n* = 12)	94.40 to 96.09	119.88 to 124.73	–28.80 to –26.52
Sweet cherry brandy (*n* = 31)	94.17 to 100.46	120.68 to 140.13	–28.44 to –25.42
Sour cherry brandy (*n* = 21)	95.50 to 98.68	121.06 to 131.40	–27.21 to –25.74
Plum brandy (*n* = 29)	95.67 to 99.51	120.60 to 126.27	–27.50 to –24.30
Apricot brandy (*n* = 16)	95.08 to 99.80	121.78 to 127.20	–27.27 to –23.30

(D/H)I, (D/H)II, and $\delta^{13}C$ of all kinds of the fruit spirits tested had similar values.

The typical values for ethanol of non-fruit origin are shown in Table 6 (Bauer-Christoph *et al.* 1997). Tables 6 and 5 present the values of isotope parameters found in the samples of commercial spirits originating from various raw materials.

The values displayed show that isotopic parameters (D/H)I of the spirits from cane sugar or maize and especially of those made from synthetic alcohol are significantly higher than isotopic parameters (D/H)I of the fruit spirits. On the other hand, the spirits from beet sugar have isotopic parameters (D/H)I lower than the fruit spirits. The spirits from cane sugar and maize have the isotopic parameters $\delta^{13}C$ markedly lower than the fruit spirits.

It is not possible to distinguish between the fruit spirits of different origins using solely their isotopic parameters because the variation ranges of these parameters overlap too much. On the other hand, the isotopic parameters enable the recognition of the fruit spirits containing ethanol of non-fruit (such as beet sugar, cane sugar or maize) origin. The only exception is ethanol from grain, which fits to the isotopic parameters otherwise typical for the fruit spirits. There is also slight overlap of the ranges of isotopic parameters of ethanol from the fruit spirits and ethanol from potatoes (Figure 1). The graph demonstrates the differences in the position of stable parameters of the fruit spirits and of other materials (alcohol from beet sugar, cane sugar, maize, potato, and synthetic alcohol).

Statistical evaluation

Misselhorn and Grafahrend (1990) were the first to use linear discriminant analysis (LDA) in conjunction with the isotope parameters of ethanol in order to differentiate between highly rectified ethyl alcohols made from diverse raw materials.

If the separation potential of LDA is efficient, the resulting discriminant variables can be used as a means of assigning an unknown sample to one of the groups considered.

A total of 153 samples of fruit spirits were statistically evaluated using discriminant analysis (Meloun & Militský 2002). The purpose of the discriminant analysis was to find new variables. These variables should sufficiently distinguish between the samples of particular spirit types made from fruits such as plumes, sweet cherries,

Table 4. Stable isotope concentrations (minimum and maximum) in ethanol derived from various raw materials (Bauer-Christoph *et al.* 1997)

Raw material	(D/H)I (ppm)	(D/H)II (ppm)	$\delta^{13}C$ (‰)
Beet sugar	91 to 93	116 to 120	–28 to –26
Cane sugar, maize	108 to 110	127 to 130	–13 to –11
Grain	96 to 99	121 to 124	–26 to –24
Potato	93 to 95	124 to 126	–28 to –25
Synthetic alcohol	123 to 124	138 to 139	–32 to –25

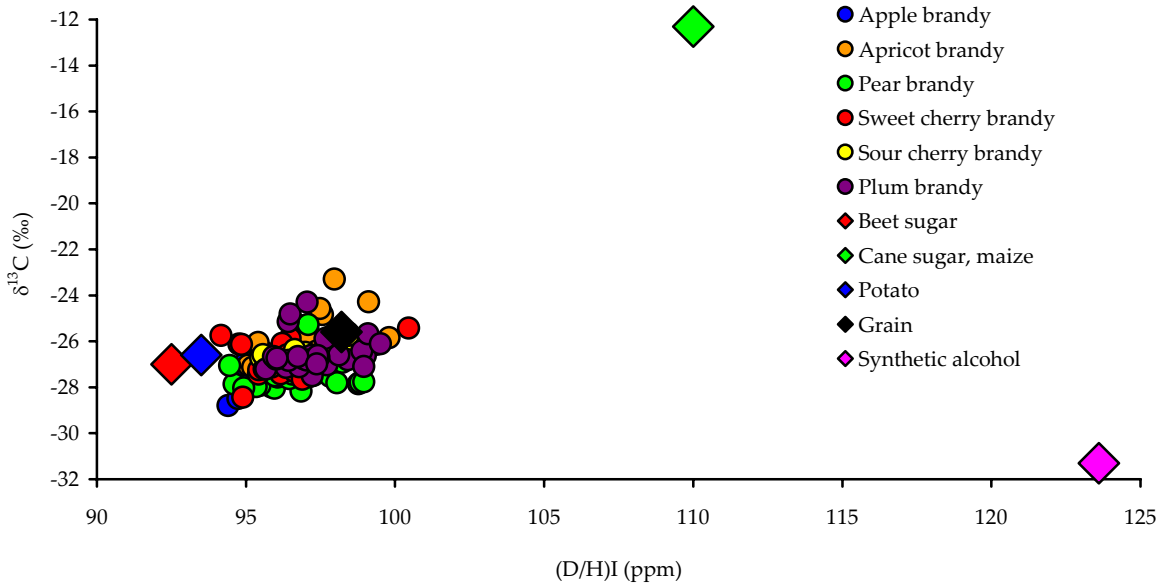

Figure 1. Correlation between the means of (D/H)I and $\delta^{13}C$ isotope ratios in ethanol from fruit spirits, beet sugar, cane sugar, potato, maize, grain, and synthetic alcohol

sour cherries, pears, apples and apricots. In addition, the discriminant analysis with different ranges of parameters was carried out in order to recognise the sole influence of particular groups of parameters including:

– all 26 parameters (data from GC-FID and GC-MSD with isotopic parameters used);
– 10 parameters (only data from GC-FID used);
– 3 parameters (only data from isotopic analysis used);

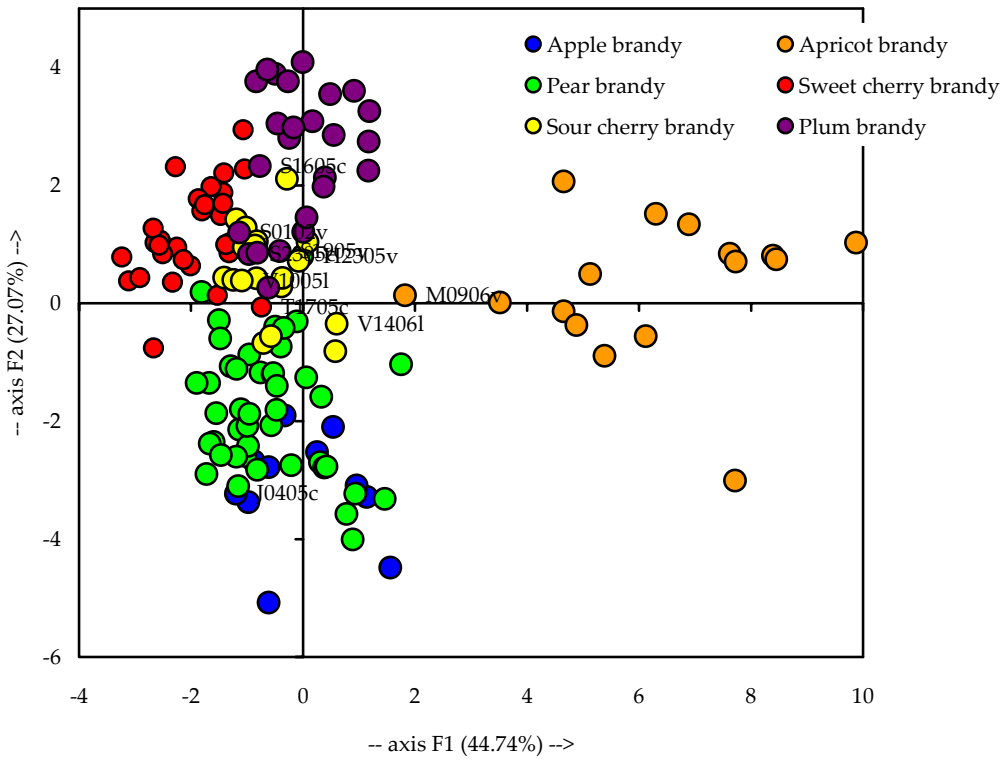

Figure 2. Plot of data from all samples set along first and second new canonical axes

– 13 parameters (only data from GC-MSD used);
– 23 parameters (data from GC- FID and GC-MSD without isotopic parameters used).

The results of the discriminant analysis of the respective data sets with different data sizes showed that the discriminant success rate between the individual types of spirits reached 93% and was influenced by the production date. Figure 2 displays the distribution of the data for all samples, set along the first and second new canonical axes. The similarity of spirit pairs such as apple–pear brandy, sour cherry–sweet cherry brandy is shown. Contrariwise, the conspicuous dissimilarity between the apricot spirits and other spirits is obvious. By employing a test against an independent data set, the discrimination success rate was found to be from 73 to 93% (if covered by the discrimination model) or 45% (not covered by the discrimination model).

Furthermore, it was possible to identify the important parameters for discrimination, also with respect to the sufficient distinction between the ranges of the values of individual analytical parameters. However, the evaluation of the data sets as well as the practical point of view revealed the suitability of using all parameters from GC-FID and GC-MS analyses. The results of the isotopic analyses showed to be very appropriate for the identification of outliers (suspected of containing components of different botanical origin), but not for distinguishing between the individual types of spirits.

CONCLUSIONS

The results of this work and the statistical processing of the data showed the possibilities of authenticity detection of fruit spirits based on stable isotope determination by using nuclear magnetic resonance – ^{2}H-NMR and mass spectrometry – IRMS (Figure 1). This paper also describes the way of identification of the individual kinds of fruit spirits based on the determination of higher alcohols and aroma components using gas chromatography.

Figure 2 shows the similarity of spirit pairs such as apple – pear brandy, sour cherry – sweet cherry, and the dissimilarity of apricot brandy to other spirits.

The data obtained show that the combined use of the volatile compounds and isotope parameters in LDA provide an efficient tool for detecting the adulteration of fruit spirits.

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Received for publication November 20, 2007
Accepted after corrections July 14, 2008

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Chromatographia (2011) 73 (Suppl 1):S157–S161
DOI 10.1007/s10337-011-1958-x

FULL SHORT COMMUNICATION

Optimization of Modern Analytical SPME and SPDE Methods for Determination of *Trans*-2-nonenal in Barley, Malt and Beer

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Received: 30 September 2010 / Revised: 21 January 2011 / Accepted: 4 February 2011 / Published online: 22 February 2011

Abstract *Trans*-2-nonenal is an aldehyde contributing to an unpleasant off-flavor and odor of rancid butter in stored beer. The automated solid-phase microextraction technique (SPME) coupled with gas chromatography (GC) and solid-phase dynamic extraction (SPDE) coupled with gas chromatography were optimized and introduced to determine *trans*-2-nonenal in barley, malt and beer. Five types of SPME fibers coated with different stationary phases (100 μm PDMS, 65 μm PDMS/DVB, 85 μm CAR/PDMS, 50/30 μm DVB/CAR/PDMS, 85 μm PA) and two needles (PDMS, PDMS/AC) were compared and tested for their efficiencies in the headspace (HS) SPME and SPDE determination of *trans*-2-nonenal in barley, malt and beer. The highest extraction efficiency of HS-SPME was achieved with the PDMS/DVB fiber, and addition of 1.5 g of NaCl, extraction time was 20 min at 60 °C. The highest extraction efficiency of HS-SPDE was obtained with the PDMS needle, 15 extraction strokes at 60 °C and addition of 1.5 g of NaCl. *Trans*-2-nonenal was identified with the method of HS-SPME coupled gas chromatography-mass spectrometry (GC–MS); the samples were analyzed using the HS-SPME-GC-coupled gas chromatography-flame ionization detector (GC-FID) technique.

Keywords Gas chromatography · Mass spectrometry · Solid-phase microextraction · Solid-phase dynamic extraction · Barley · Malt and beer · *Trans*-2-nonenal

Introduction

Spring barley has been a main raw material for production of malt and subsequently beer in the territory of the Czech Republic since the late 19th century. Requirements for the quality of malting barley have gradually been increased and specified.

The current state of knowledge on enzymatic processes in barley caryopses including changes during technological processing is not sufficient. Lipoxygenase enzyme catalyzes the oxidation of unsaturated fatty acids with more double bonds containing *cis*-1,4-pentadien group, with a molecular oxygen. Thus in a chain reaction transient peroxides of unsaturated fatty acids are formed, these are further degraded to carbonyl compounds (aldehydes, ketones) or short-chain fatty acids. In this way compounds with characteristic flavors and odors are formed. Nutritiously important essential fatty acids, linoleic, linolenic, and arachidonic, belong to lipoxygenase substrates. Acyl glycerols and other esters of the fatty acids mentioned above can also be oxidized [1, 2].

An aldehyde *trans*-2-nonenal is a basic component contributing to the rancid taste in stored beers [3]. The mechanism of formation of *trans*-2-nonenal in beer is enzymatic or nonenzymatic oxidation of fats and oxidation

Presented at: 16th International Symposium on Separation Science, Recent Advancements in Chromatography and Capillary Electromigration Techniques. Rome, Italy, September 6–10, 2010.

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of free fatty acids, enzyme lipoxygenase plays a role here [4].

Due to low *trans*-2-nonenal contents in barley, malt and beer, an extraction technique suitable both for isolation and increase in concentration of this substance must be used. Distillation with water vapor, headspace methods (gas extraction) and solid-phase microextraction (SPME) are usually used for concentration of volatile substances [5, 6].

The SPME is a solvent-free sample preparation technique. This method does not require a complicated instrumentation and is based on sorption of the analytes on the surface of a silica fiber. The amount of the extracted analyte depends on the value of the analyte/fiber partition coefficient, polarity and fiber thickness, extraction time and analyte concentration in a sample. The extraction yield can be increased by stirring, addition of salt into the sample, or change of pH or temperature [7–11].

Solid-phase dynamic extraction (SPDE) is a newer and more modern setting of SPME for dynamic extraction of liquid or gaseous samples. Unlike the SPME method, sorbent in the SPDE technique is coated on the internal layer of a needle connected to a gas-tight syringe. Repeated sampling of the gaseous (HS-SPME) or liquid phase (DI-SPME) of the sample results in concentrating the determined analytes in the needle. This extraction technique is more robust than SPME and it allows assessing even very low concentrations. The disadvantage of this system is that it must be connected to a robotic system. This extraction technique cannot be conducted manually as required repeatability would not be reached.

For the determination of *trans*-2-nonenal in barley, malt and beer the automated SPME-GC and SPDE-GC methods were optimized and compared.

Experimental

Chemicals and Standards

Trans-2-nonenal, ethanol and NaCl were purchased from Sigma-Aldrich (Sigma-Aldrich, Praha, Czech Republic). The standard working solution was prepared in ethanol with concentration of 50.0 μg L^{-1}. Calibration solutions with concentrations in the range from 0.03 to 3.4 μg L^{-1} were prepared daily with deionized water obtained from the purification system Millipore (Millipore, Billerica, MA, USA).

Instrumentation

The gas chromatograph Trace Ultra with a flame ionization detector (FID) (Thermo Electron Corporation, Milano, Italy) was used for the assessment of *trans*-2-nonenal in samples. SPME and SPDE extractions were carried out in the automatic injection autosampler CombiPal (CTC Analytics, Zwingen, Switzerland). The column Supelcowax (30 m × 0.25 mm I.D, 0.25 μm) (Supelco, Bellefonte, PA, USA) was held at 50 °C for 2 min, then programmed at 8 °C min^{-1} to 200 °C (5 min). The temperature of the split/splitless PTV injector (metal liner 2.75 mm × 1 mm × 120 mm) was 250 °C, 2 min splitless time. The flow rate of helium carrier gas (99.999%) was 1.5 mL min^{-1}.

The gas chromatograph Trace GC Ultra Finnigen (Thermo Electron Corporation, Milano, Italy) with a mass detector Trace DSQ Thermo Finnigen (Thermo Electron Corporation, Austin, TX, USA) was used for the identification of *trans*-2-nonenal in samples. The column, thermal regime and flow rate of helium carrier gas were the same as those used for GC/FID method. The transfer line temperature was 200 °C. The mass selective detector operated in full scan mode with positive electron impact (EI) ionization. The manual HS-SPME method was used for the identification of *trans*-2-nonenal.

Sample Preparation

Barley, Malt

A ground sample of barley or malt (±5 g) was weighed into an Erlenmeyer flask, 50 mL of deionized water was added and the flask was closed. The sample was then extracted in a laboratory shaker for 15 min. After the extraction, the sample was transferred into a centrifuge tube and centrifuged at high-speed rotation at low temperature for 15 min. 5 mL of supernatant was used for the SPME procedure described below.

Beer

1.5 g of NaCl was placed into a 20 mL head space vial and a magnetic stirrer was inserted. 5 mL of cooled beer was then added with a pipette into the vial and the sample was analyzed using the automated HS-SPME-GC-FID method.

SPME Procedure

Five types of fibers were compared for the HS-SPME (100 μm PDMS, 65 μm PDMS/DVB, 85 μm CAR/PDMS, 50/30 μm DVB/CAR/PDMS, 85 μm PA).

Preparation of Individual SPME Fibers

The PDMS-SPME fibers were conditioned in the GC injector before use as follows: PDMS 250 °C, 0.5 h;

PDMS/DVB 250 °C, 0.5 h; CAR/PDMS 300 °C, 2 h; DVB/CAR/PDMS 270 °C, 1 h; PA 300 °C, 2 h.

Manual SPME

To perform the SPME extraction, 1.5 g of NaCl was placed in a 20 mL head space vial. 5 mL of supernatant was then pipetted into the vial. The vial was closed with a metal cap with septum and the sample was analyzed using a manual HS-SPME-GC–MS.

A SPME holder for manual sampling was used for fixing the fiber. The depth of needle insertion into a container was always 0.5 cm. Selected sorption conditions were 60 °C for 20 min. Desorption of the analyte from the fiber was performed in the PTV injector at 250 °C for 2 min. The depth of fiber insertion into the PTV injector was 4.5 cm.

Automated SPME

To perform the SPME extraction, 1.5 g of NaCl was placed in a 20 mL head space vial and a magnetic stirrer was inserted. 5 mL of supernatant was then pipetted into the vial. The vial was closed with a metal cap with septum and the sample was analyzed using the automated HS-SPME-GC-FID.

For *trans*-2-nonenal extraction from the analyzed samples, the PDMS/DVB fiber was used. Sample incubation time was 10 min at 60 °C. Time of sorption (extraction) of the analyte to the SPME fiber was 20 min at 60 °C. Desorption of the analyte from the fiber was conducted in the PTV injector at 250 °C for 2 min. The depth of fiber insertion into the PTV injector was 4.5 cm.

SPDE Procedure

Two needles (50 μm PDMS, 50 μm PDMS/AC) were tested for their efficiencies in the HS-SPDE extraction.

Preparation of the Individual SPDE Needles

The SPDE needles were conditioned in the automatic injection autosampler CombiPal before use as follows: PDMS 250 °C, 0.5 h; PDMS/AC 300 °C, 2 h.

Automated SPDE

To perform the SPDE extraction, 1.5 g of NaCl was placed in a 20 mL head space vial and a magnetic stirrer was inserted. 5 mL of supernatant was then pipetted into the vial. The vial was closed with a metal cap with septum and the sample was analyzed using the automated HS-SPDE-GC-FID.

For *trans*-2-nonenal extraction from the analyzed samples, the PDMS needle was used. Sample incubation time was 10 min at 60 °C. Needle temperature was 35 °C. For extraction, 15 extraction cycles were used. Desorption of the analyte from the needle was performed in the PTV injector at 250 °C for 2 min. Volume of He (99.999%) in the syringe after desorption was 500 μL. Desorption gas rate was 50 μL s^{-1}. The depth of needle insertion into the PTV injector was 4.5 cm.

Result and Discussion

Optimization of HS-SPME Extraction

For HS-SPME extraction of *trans*-2-nonenal, the type of a SPME fiber, extraction temperature, extraction time and the amount of added NaCl on extraction yield were tested and optimized.

Selection of the SPME Fiber

Following five types of SPME fibers were tested: 100 μm PDMS, 65 μm PDMS/DVB, 85 μm CAR/PDMS, 50/30 μm DVB/CAR/PDMS, 85 μm PA. The PDMS/DVB fiber had the highest affinity for *trans*-2-nonenal extraction.

Optimization of Extraction Temperature and Time

For optimization of the extraction, the dependence of efficiency of *trans*-2-nonenal extraction on the sample temperature and extraction time was studied. The highest extraction efficiency was achieved at the sample temperature of 60 °C and at the extraction for 20 min.

Salt Addition

The effect of NaCl concentration in the analyzed sample on the extraction efficiency was studied at the optimized extraction temperature and time. The highest extraction efficiency of *trans*-2-nonenal was achieved with the addition of 1.5 g of NaCl.

Optimization of SPDE Extraction

For the SPDE extraction of *trans*-2-nonenal the type of the SPDE needle and number of extraction cycles were optimized. Two SPDE needles (50 μm PDMS, 50 μm PDMS/AC) were tested. For SPDE a needle with the PDMS phase was more suitable.

Figure 1 shows the dependence of the extraction efficiency on the number of extraction strokes. It is evident that the highest extraction efficiency was achieved with 15

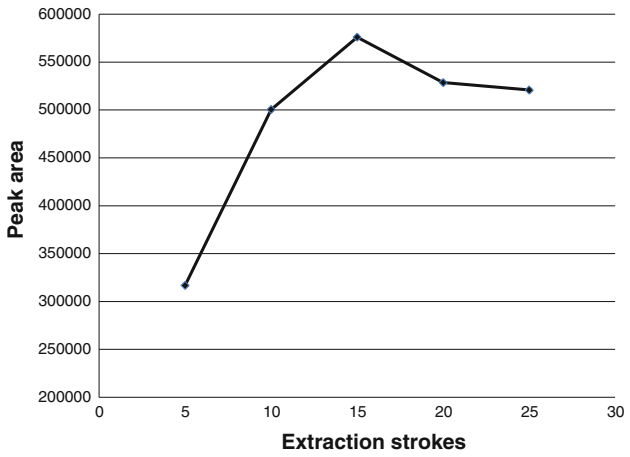

Fig. 1 Optimization of the number of extraction strokes for the SPDE needle with the PDMS phase

Table 1 Validation parameters for HS-SPME determination of *tans*-2-nonenal in barley, malt and beer

Sample	LOQ	LOD	RSD (%)
Barley and malt	15×10^{-2} μg kg^{-1}	5×10^{-2} μg kg^{-1}	8.3
Beer	15×10^{-3} μg L^{-1}	5×10^{-3} μg L^{-1}	9.5

extraction cycles. With a higher number of extraction strokes reversed desorption from a needle to the sample occurred.

The comparison of relative extraction efficiency of the optimized automated HS-SPME and HS-SPDE techniques suggests that SPME extraction with the PDMS/DVB fiber is more suitable for the extraction of *trans*-2-nonenal than the extraction with the tested SPDE needles.

Validation Parameters

Quantification of *trans*-2-nonenal in samples was performed using a calibration curve. The calibration curve was linear in the range from 0.03 to 3.4 μg L^{-1} with the correlation coefficient of 0.9998.

Validation parameters of the HS-SPME-GC-FID method for the determination of *trans*-2-nonenal content in the analyzed samples are given in Table 1.

Analysis of the Samples with the Automated HS-SPME-GC-FID Method

A type of the SPME fiber, extraction temperature and time and the effect of NaCl addition on extraction yield were optimized for SPME extraction of *trans*-2-nonenal. The PDMS/DVB fiber had the highest affinity for *trans*-2-nonenal extraction.

The comparison of relative extraction yields of the optimized automated HS-SPME and HS-SPDE techniques suggested that SPME extraction with the PDMS/DVB fiber was more suitable for the extraction of *trans*-2-nonenal than the extraction with the SPDE needles tested.

Based on the experimental results, the automated HS-SPME-GC-FID method with the PDMS/DVB fiber, time of extraction 20 min at 60 °C and with the addition of 1.5 g NaCl was chosen for the determination of *trans*-2-nonenal content in barley caryopses, malts and beers. The HS-SPME-GC-FID method with the PDMS/DVB fiber for the determination of *trans*-2-nonenal content in a barley caryopsis, malt and beer was validated.

A total set of 54 samples (21 barley varieties and 21 malts produced from them and 12 beers) was analyzed and contents of *trans*-2-nonenal in barley caryopses, malts and beers were determined with the optimized HS-SPME-GC-FID

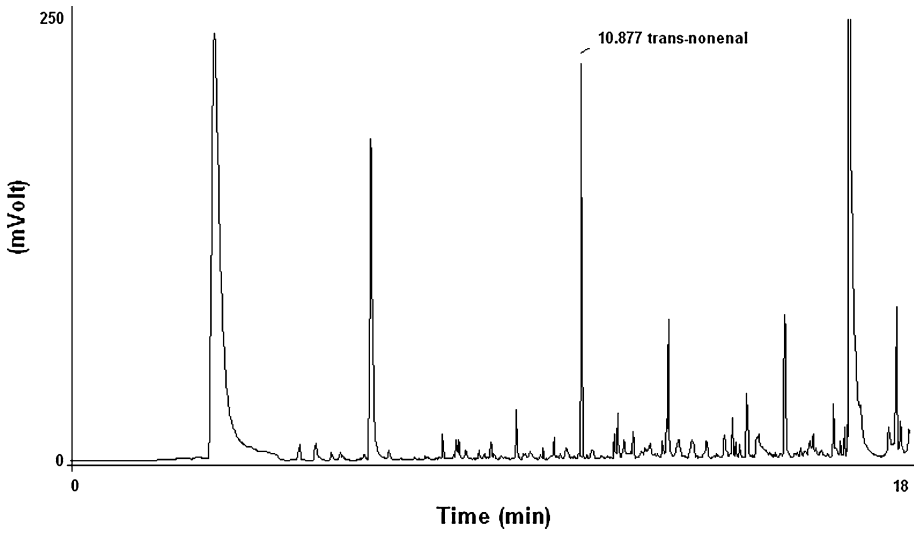

Fig. 2 A chromatogram of *trans*-2-nonenal determination in beer using the automated HS-SPME-GC-FID method with the PDMS/DVB fiber

Table 2 *Trans*-2-nonenal content in barley and malt samples

Barley variety	*Trans*-2-nonenal (μg kg^{-1})	
	Barley	Malt
Wikingett	1.14	17.26
Troon	0.98	23.93
Cruiser	1.24	10.29
Bellevue	0.97	29.43
Biatlon	1.39	18.16
Mauritia	0.81	27.59
Ebson	0.83	38.54
NFC Tipple	0.89	10.31
Westminster	1.42	15.66
Publican	0.30	8.90
Marthe	1.74	26.86
Maltasia	3.06	12.07
Lissane	0.96	10.96
Musikant	0.28	15.82
Xanadu	1.18	2.77
Jersey	0.59	22.38
Malvaz	0.16	23.96
Binder	0.24	61.70
Tepelský 421	0.65	13.96
Ratbořský	0.38	9.07
Timori	0.96	1.89

method with the PDMS/DVB fiber. Figure 2 shows a chromatogram of the determination of *trans*-2-nonenal in a beer sample with the automated HS-SPME-GC-FID method with the PDMS/DVB fiber.

Table 2 summarizes the results of *trans*-2-nonenal in barley and malt samples. *Trans*-2-nonenal content in barleys ranged from 0.28 to 3.06 μg kg^{-1}, in malts it varied from 8.90 to 38.54 μg kg^{-1}. Multiple higher *trans*-2-nonenal content in malt versus the barley grain was caused by an increased enzymatic activity at malting.

Trans-2-nonenal was determined in the set of 12 beer samples (4 pale dispensed beers, 4 lagers and 4 non-alcoholic beers). *Trans*-2-nonenal in pale dispensed beers varied from 1.01 to 3.44 μg L^{-1}, in lager beers from 1.06 to 4.02 μg L^{-1} and non-alcoholic beers it moved from 3.39 to 20.28 μg L^{-1}.

Conclusion

Trans-2-nonenal content was determined with the optimized automated HS-SPME-GC-FID method with the PDMS/DVB fiber. *Trans*-2-nonenal contents in barleys ranged from 0.28 to 3.06 μg kg^{-1}, in malt it moved from 8.90 to 38.54 μg kg^{-1}. The highest *trans*-2-nonenal content was determined in non-alcoholic beers and the lowest in dispensed beers.

Acknowledgments The presented results were obtained within the solution of the research projects of NAZV MA CR (identification code QH 81056) and MYES CR (identification code2B08057).

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