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Analýza senzoricky a toxikologicky významných látek v pivu
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Abstrakt

Jednou z nejvýznamnějších skupin toxikologicky významných látek v pivu jsou N-nitrosaminy. V této práci byla pozornost zaměřena zejména na netěkavé nitroso sloučeniny, jejichž koncentrace v pivu, chemická struktura a biologický účinek nejsou dosud známy. Z důvodu nedostatku znalostí o této skupině látek byla vyvinuta metoda pro jejich citlivou detekci prostřednictvím chemiluminiscenčního detektoru po plynově chromatografické separaci. Tato metoda zároveň umožňuje klasifikovat detekované nitroso sloučeniny do různých skupin (N-nitroso, C-nitroso a kombinace C-nitroso a nitro) a odlišit je od interferencí. Metoda využívá záznamu pyrolyzních profilů jednotlivých chromatografických píků, které jsou zpracovány pomocí diskriminační analýzy. Metoda byla vyvíjena za účelem nalezení a strukturní identifikace těchto dosud neznámých látek. Její aplikace na uměle nitrosovaný vzorek piva spolu s plynově chromatografickou analýzou s tandemovou hmotnostní detekcí vedl k strukturní identifikaci několika zástupců nitroso sloučenin.

Senzoricky aktivní látky v této práci zastupují karbonylové sloučeniny a mastné kyseliny. Karbonylové sloučeniny – furfural a hydroxymethylfurfural – byly využity při vývoji mobilní, jednoduché, rychlé a objektivní metody pro *in situ* stanovení intenzity staré chuti piva, která je odrazem jeho nevhodného skladování. Metoda využívá barevné reakce na testovacím proužku, reflektometrické detekce a vytvořeného kalibračního modelu z reálných dat ze senzorické a chemické analýzy piv. Výsledná metoda stanovuje intenzitu staré chuti piva na čtyřbodové škále, s nejistotou ± 0,5 bodu a mezí stanovitelnosti 1,0 bodu.

Poslední experimentální část této práce je zaměřena na studium distribuce mastných kyselin v průběhu pivovarského procesu za pomocí analyticko-chemometrického nástroje zvaný senzomické profilování. Tímto přístupem byly odhaleny některé nově popsané zákonitosti vzniku mastných kyselin v průběhu pivovarského procesu.

Klíčová slova

analytická chemie, separační metody, chemiluminiscenční detekce, hmotnostní spektrometrie, analýza potravin, kontaminanty, senzoricky aktivní látky, pivo

Abstract

One of the most relevant groups of toxicologically significant compounds in beer are N-nitrosamines. In this thesis, the attention was paid especially to non-volatile nitroso compounds whose concentrations in beer, a chemical structure, and a biological effect, have not been known yet. For the reason of lack of knowledge regarding this compound group, a method for their sensitive detection by the chemiluminescence detection after the gas chromatographic separation was developed. This method allows a classification of detected nitroso compounds to different groups (N-nitroso, C-nitroso, and a combination of C-nitroso and nitro) and distinguishing them from interferences. The method is based on recording a pyrolytic profile of each chromatographic peak, the profiles are consequently processed by discriminant analysis. The method has been developed for the detection and structural identification purposes of these unknown compounds. Its application on an artificially nitrosated beer sample, together with gas chromatographic tandem mass spectrometric analysis, led to a structural identification of several representatives of nitroso compounds.

Sensory active compounds in this thesis are represented by carbonyl compounds and fatty acids. Carbonyl compounds – furfural and hydroxymethylfurfural – were used during the development of a mobile, easy-to-use, rapid, and objective *in situ* method for stale flavor intensity of beer determination – stale flavor is a reflection of inappropriate storage conditions of beer. The method is based on a color reaction on a test strip, the reflectometric detection, and a calibration model constructed by data from sensory and chemical analysis of a real samples. Resulted method permits determination of stale flavor of beer on a four-point scale, the with uncertainty ± 0.5 and a limit of determination 1.0.

The last part of this thesis is focused on a study of distribution of fatty acids during brewing process by an analytical-chemometric tool named sensomic profiling. Some of the new patterns of a fatty acid formation during the brewing process were uncovered.

Key words

analytical chemistry, separation methods, chemiluminescence detection, mass spectrometry, food analysis, food contaminants, sensory active compounds, beer

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1 Úvod

Pivo je jedním z celosvětově nejoblíbenějších nápojů, a právě díky tomu je nedílnou součástí kultury, tradicí, stravování a společenského života v mnoha zemích, např. v Německu, Irsku a České republice. Z těchto důvodů tyto země pravidelně obsazují první příčky v roční spotřebě piva připadající na jednoho obyvatele [1]. Kromě jeho oblíbenosti je také významným zdrojem jak některých nutrientů, tak i zdravotně prospěšných látek, jako jsou vitamíny B a řada polyfenolických sloučenin s antioxidačními, antikarcinogenními a antimikrobiálními účinky [2, 3]. Oproti tomu prokázaných zdravotně nežádoucích látek obsahuje pivo v porovnání s ostatními potravinami jen minimální množství. Mezi tyto látky patří zejména N-nitrosaminy, biogenní aminy a mykotoxiny. Přestože jsou jejich koncentrace v pivu za běžných podmínek velice nízké, jejich analytická kontrola je vysoce doporučována (zejména díky velké spotřebě piva v některých zemích) [4]. Bez ohledu na zastoupení výše zmíněných skupin látek v pivu jsou z pohledu konzumenta jedním z nejdůležitějších parametrů při výběru stylu a značky piva jeho senzorické vlastnosti, které jsou dány celkovým složením piva a zejména koncentrací různorodé skupiny senzoricky aktivních látek.

Pivo obsahuje velké množství sloučenin s různou chemickou povahou [5], a proto je z pohledu analytické chemie velice složitou matricí. Jak již z předchozího odstavce vyplývá, základní konstituenty piva lze rozdělit na senzoricky aktivní, zdravotně prospěšné, nutričně významné a toxikologicky významné. Tato práce je zaměřena pouze na dvě z těchto skupin – senzoricky aktivní a toxikologicky významné látky. Vzhledem k velké různorodosti a obsáhlosti obou skupin je tato práce zacílena pouze na vybrané podskupiny. Za skupinu senzoricky aktivních látek to jsou karbonylové sloučeniny a mastné kyseliny, a nitroso sloučeniny za skupinu toxikologicky významných látek.

Cíle experimentální části této práce jsou tedy rozděleny mezi tato základní témata a to následovně: a) vývoj metody pro *in situ* stanovení míry senzorického poškození piva jeho

nevhodným skladováním, b) vývoj metody pro detekci nitroso sloučenin v pivu a jejich klasifikaci do skupin N-nitroso a C-nitroso sloučenin, schopnou odlišit je od interferencí, prostřednictvím plynové chromatografie s pyrolyzně profilovací chemiluminiscenční detekcí (GC-PPNCD), c) studium distribuce mastných kyselin v průběhu pivovarského procesu a v závislosti na způsobu rmutování prostřednictvím senzomického profilování.

2 Teoretická část

2.1 Toxikologicky významné látky v pivu

Přestože je pivo obecně považováno za nápoj s vysokým obsahem zdravotně prospěšných látek, může za určitých podmínek obsahovat i relativně vysoké koncentrace toxikologicky významných sloučenin. Mezi nejčastější toxické kontaminanty piva patří nitrosaminy, biogenní aminy a mykotoxiny.

2.1.1 Nitrosaminy

Jednou z nejvýznamnějších skupin toxikologicky významných látek v pivu jsou N-nitrosaminy. Vznik těchto látek je spojen s vysokou koncentrací dusitanů, či vystavení vzorku oxidům dusíku. Dusitany nejčastěji pocházejí z redukce dusičnanů prostřednictvím bakteriální kontaminace a oxidy dusíku hrají hlavní roli při tvorbě nitrosaminů při závěrečné fázi výroby sladu – hvozdění [6]. Bakteriemi nejčastěji zodpovědnými za zvýšený obsah ATNC v pivu jsou dle literatury považovány *Obesumbacterium proteus* a *Escherichia coli* [7].

Nitrosaminy lze na základě fyzikálně-chemické povahy rozdělit na těkavé a netěkavé. Těkavým nitrosaminům jsou prokázány silně karcinogenní a mutagenní účinky [8]. Dohromady lze obě tyto skupiny stanovit jako zdánlivé celkové N-nitroso sloučeniny (ATNC, z angl. Apparent Total N-nitroso Compounds, vyjádřené jako koncentrace N-nitroso skupiny v μg(N-NO)/l). Metoda stanovení ATNC je založena na chemickém štěpení N-NO vazby denitrosační směsí bromovodíku a kyseliny octové. Uvolněný oxid dusnatý je následně

detekován a kvantifikován chemiluminiscenčním detektorem (NCD) [9]. Metoda však poskytuje pouze informaci o zdánlivé koncentraci N-nitroso skupiny, nikoliv o koncentraci jednotlivých zástupců.

Výskyt a vznik těkavých nitrosaminů byl v pivu, potažmo sladu, již dobře popsán a byly tak zavedeny kroky pro jejich eliminaci. Typickým zástupcem těkavých nitrosaminů je N-nitrosodimethylamin (NDMA), který se do piva dostává zejména extrakcí ze sladu, kde vzniká reakcí oxidů dusíku v sušícím vzduchu s alkaloidy (hordenin a gramin) v naklíčeném zrnu ječmene [10]. Příprava vzorků pro analýzu těkavých nitrosaminů v pivovarských matricích zahrnuje vakuovou destilaci původního vzorku, následnou extrakci nitrosaminů z destilátu dichlormethanem a zakoncentrování pod proudem dusíku. Samotná analýza je pak prováděna pomocí plynové chromatografie s chemiluminiscenční nebo hmotnostní detekcí [11]. Vyvinuté byly také metody využívající kapalinovou chromatografii s hmotnostní, spektrofotometrickou či fluorescenční detekcí [12]. Tyto metody však v porovnání s GC metodami nejsou v pivovarství příliš rozšířené.

Koncentrace těkavých nitrosaminů v pivu jsou za běžných podmínek velice nízké (<0,2 µg/kg), stejně tak koncentrace ATNC (<20 µg(N-NO)/l). Oproti tomu jejich koncentrace může v případě použití sladu s vysokým obsahem NDMA nebo v případě bakteriální kontaminace vzrůst až k 2 µg/kg u NDMA a u ATNC až na 560 µg(N-NO)/l. Zvýšené koncentrace ATNC byly např. popsány v pivech některých minipivovarů, kde systém důkladné sanitace prostředí a provozního zařízení není natolik propracován jako ve velkých pivovarech [3]. Nejvyšší přípustné množství NDMA v pivu je 0,5 µg/kg a sumy těkavých nitrosaminů 1,5 µg/kg (dle doporučení Českého svazu pivovarů a sladoven vycházející z požadavků kompendií JECFA – Joint FAO/WHO Expert Committee on Food Additives – které byly součástí zrušené vyhlášky č. 305/2004). Pro ATNC není uplatňována

žádná koncentrační hranice, nicméně hodnota pod 20 µg(N-NO)/l (odpovídající mezi stanovitelnosti metody) se obecně považuje za uspokojivou.

Na opačném pólu, z pohledu znalostí o výskytu, mechanismů vzniku, analýze a biologických účincích, stojí netěkavé nitrosaminy. To je dáno především dosavadní neznalostí chemických struktur zástupců této skupiny látek, která tak vede k nemožnosti jejich cílené analýzy či toxikologického testování. Přítomnost netěkavých nitrosaminů (respektive obecně nitroso sloučenin) v pivu je evidentní z porovnání koncentrace ATNC a koncentrace těkavých nitrosaminů. Při zvýšené hladině ATNC tvoří těkavé nitrosaminy pouze kolem 1 % z ATNC. Koncentrace dosud jediného potvrzeného zástupce netěkavých nitrosaminů v pivu, N-nitrosoprolinu, dosahuje okolo 10 % z ATNC [13]. Z výše uvedeného je zřejmé, že velká většina koncentrace ATNC (cca 89 %) není přisouzena přítomnosti konkrétních látek. Proto nelze ze zvýšeného obsahu ATNC v pivu vyvozovat závěry s ohledem na vliv na lidské zdraví – přesto se takové zvýšené množství považuje za zdraví škodlivé, což vychází z předpokladu, že všechny nitroso sloučeniny mají stejné účinky jako těkavé nitrosaminy. Z těchto faktů vyplývá, že detailní studium netěkavých nitrosaminů je velice důležité pro budoucí možnost posuzování míry zdravotní závadnosti piv se zvýšeným obsahem ATNC.

Přestože se v minulosti mnohé výzkumy snažily o rozklíčování látek přispívajících k ATNC v pivu a sladu, významnějšího pokroku nebylo dosud dosaženo. Tento cíl si klade současný výzkumný projekt Výzkumného ústavu pivovarského a sladařského, jehož základní součástí je metoda pyrolyzně profilovací chemiluminiscenční detekce vypracovaná v rámci této disertační práce.

2.1.2 Biogenní aminy

Biogenní aminy (histamin, tyramin, tryptamin, kadaverin, putrescin, spermidin a spermin) jsou látky vznikající enzymatickou dekarboxylací aminokyselin prostřednictvím

mikrobiální kontaminace piva či jeho meziproduktu. Zvýšené koncentrace těchto látek v pivu mohou mít u konzumenta za následek zdravotní potíže zahrnující boleti hlavy, zažívací problémy nebo vysoký či nízký krevní tlak [14, 15]. Přítomnost biogenních aminů v pivu je spojena s přítomností nitrosaminů [3], díky stejnému původu těchto látek (bakteriální kontaminace). Koncentrace biogenních aminů v pivu se pohybuje řádově v desetinách až desítkách miligramů na litr, avšak za běžných podmínek je velice nízká či nedetekovatelná. Analytické metody stanovení zahrnují širokou škálu instrumentálních technik jako je kapilární elektroforéza, plynová, kapalinová či iontová chromatografie [15].

2.1.3 Mykotoxiny

Mykotoxiny jsou toxické sekundární metabolity vláknitých hub a do piva se dostávají zejména prostřednictvím kontaminovaného ječmene/sladu, případně chmelu či kvasinek. Nejsledovanější zástupci jsou ochratoxin A, patulin, deoxynivalenol a zearalenon [16]. Účinky těchto látek na člověka závisí na jednotlivých zástupcích a popisují se jako karcinogenní, cytotoxické a imunotoxické [17]. Koncentrace těchto látek v pivu se vyskytují v jednotkách až desítkách mikrogramů na litr. Nedávným screeningem mykotoxinů v pivech z českého, polského a slovenského trhu byl zjištěn deoxynivalenol (<1,0 až 12,6 µg/l) a ochratoxin A (<1,0 až 82,5 ng/l) v koncentracích nepředstavující významné zdravotní riziko [4]. Metody jejich stanovení jsou založeny na kapalinové chromatografii s hmotnostní detekcí či na komerčně dostupných ELISA testech [4, 18]

2.1.4 Ostatní toxikologicky významné látky

Další toxikologicky významné látky v pivu mohou být těžké kovy, alifatické halogenované uhlovodíky či polycyklické aromatické uhlovodíky. Kontaminace piva těmito látkami není tolik častá oproti látkám diskutovaných v předchozích podkapitolách [19].

2.2 Senzoricky aktivní látky piva

Senzoricky aktivní látky jsou nejvýznamnější skupinou látek, které se v pivu vyskytují, protože jejich koncentrace a vzájemný poměr udává celkovou senzorickou kvalitu piva. Senzoricky aktivní látky lze obecně rozdělit na látky s pozitivním a negativním vjemem. Jelikož je v mnohých případech toto rozdělení určeno jejich koncentrací, analytická kontrola či výzkum vlivu jejich koncentrace na senzorickou kvalitu piva je velice důležitá pro pochopení funkce těchto látek při senzorickém posuzování finálního výrobku. Pro stanovení senzoricky aktivních látek v pivu je využíváno zejména metod plynové a kapalinové chromatografie.

2.2.1 Karbonylové sloučeniny

Karbonylové sloučeniny v pivu jsou spojeny především s oxidací a/nebo stárnutím piva [20] a proto jsou jejich vyšší koncentrace v pivu nežádoucí. Tyto látky způsobují v pivu oxidační, papírovou či starou chuť. Nejvýznamnějšími zástupci jsou furan-2-karbaldehyd, 5-(hydroxymethyl)furan-2-karbaldehyd (HMF), trans-2-nonenal, benzaldehyd a fenylacetaldehyd [21]. Stanovení těchto látek v pivu je nejčastěji prováděno pomocí plynové chromatografie s hmotnostně spektrometrickou detekcí po jejich derivatizaci pomocí o-(2,3,4,5,6-pentafluorobenzyl)hydroxylaminu (PFBHA), kdy derivatizace je prováděna na vláknu pro mikroextrakci na pevné fázi, nebo přímo ve vzorku piva, kdy výsledné oximy jsou následně extrahovány hexanem [22, 23]. Koncentrace karbonylových látek v pivu se pohybují v jednotkách až stovkách mikrogramů na litr. Výjimkou je HMF, jehož koncentrace je řádově v jednotkách miligramů na litr, čímž je koncentračně nejzastoupenější karbonylovou sloučeninou v pivu. Díky polární hydroxy skupině je analýza HMF výše popsanými metodami problematická, a proto se volí buď jiná příprava vzorku, jiný typ derivatizace (silylace) nebo analýza kapalinovou chromatografií [24, 25]. Kapalinová chromatografie v tomto případě

umožňuje přímý nástřik, kdy příprava vzorku zahrnuje pouze odplynění vzorku piva a filtraci, což výrazně zkracuje celkovou dobu přípravy a analýzy vzorku.

Sledování koncentrací karbonylových látek v pivu je využíváno při studiích zaměřených na stárnutí piva, což je jev samovolného zhoršení senzorické kvality piva při jeho skladování [23, 26]. Míra stárnutí piva a jeho výsledné senzorické poškození je závislé na podmínkách a době skladování, kdy s vyššími teplotami a delšími dobami skladování toto poškození vzrůstá – typické je senzorické stárnutí piva během skladování v tržní síti. Není překvapivé, že vyšší stupeň poškození piva jeho stárnutím může mít negativní vliv na vnímání dané značky piva spotřebitelem. Spolehlivá analytická/senzorická kontrola takového piva přímo v terénu je momentálně téměř neproveditelná. Analytická kontrola vyžaduje odběr vzorku a transport do laboratoře, což může v mnohých případech vést k falešně pozitivním výsledkům, naopak pro spolehlivou senzorickou kontrolu je zapotřebí určité množství nezávislých hodnotitelů, což v praxi při kontrole v terénu není možné zabezpečit. Z těchto důvodů byla v rámci této disertační práce vyvíjena metoda pro *in situ* stanovení staré chuti piva, která je odrazem senzorického poškození piva jeho nevhodným skladováním.

2.2.2 Vyšší alkoholy a estery

Vyšší alkoholy a estery jsou sekundárními metabolity kvašení a významně přispívají k celkovému senzorickému dojmu piva. Vyváženost mezi koncentracemi těchto a ostatních senzoricky aktivních látek v pivu je jedním z rozhodujících faktorů, zda pivo bude pozitivně či negativně přijímáno z pohledu spotřebitele. Nadměrné množství esterů vede k příliš intenzivnímu ovocnému aromatu, a naopak nadměrné množství vyšších alkoholů zapříčiňuje alkoholový až rozpouštědlový charakter piva, který ač je v mnohých pivních stylech žádoucí, tak naopak v nejčastěji konzumovaném pivním stylu v České republice, ležáku, je nežádoucí. Koncentrace těchto látek se v pivu pohybují řádově v jednotkách až desítkách mikrogramů

na litr. Pro jejich stanovení v pivu se nejčastěji používá plynová chromatografie s plamenově ionizačním detektorem s headspace dávkováním [27]. Tato metoda je obecně preferována díky její jednoduchosti, finanční dostupnosti a také na základě faktu, že tato metoda je doporučována komisí EBC (z angl. European Brewery Convention) jako standardní metoda stanovení vyšších alkoholů a esterů v pivu [28].

2.2.3 Chmelové silice

Další významnou skupinou senzoricky aktivních látek piva jsou chmelové silice, které zahrnují terpeny, terpenové alkoholy, některé karbonylové sloučeniny a některé estery mastných kyselin. Mezi zástupce těchto látek patří např. myrcen, karyofylen, humulen, farnesen, limonen, linalool, terpineol, geraniol, hexanal, 3-methyl-2-pentanon, methylheptanoát a isoamylpropionát [29]. Tyto látky jsou z pohledu senzorického vnímání obecně považovány v pivu za pozitivní – tvoří zejména příjemné chmelové, květinové a ovocné aroma. Pro kvalitu a intenzitu těchto aromat je rozhodující koncentrace chmelových silic v pivu, které jsou dány množstvím a typem chmele/ů použitého/ých při výrobě piva. Stejně tak i fáze výroby piva, ve které byl chmel přidáván (např. počátek či konec chmelovaru, studené chmelení apod.), rozhoduje o výsledné koncentraci silic v pivu. Koncentrace těchto látek se v pivu řádově pohybuje v jednotkách až desítkách mikrogramů na litr a metody jejich stanovení zahrnují destilaci [30] či mikroextrakci na pevné fázi [31] před plynově chromatografickou analýzou s hmotnostně spektrometrickou detekcí.

2.2.4 Vicinální diketony

Mezi vicinální diketony v pivu patří 2,3-butandion a 2,3-pentandion. Tyto látky jsou tvořeny jako meziprodukty anabolismu aminokyselin valinu a isoleucinu během kvašení, kdy jejich koncentrace nejprve stoupá a posléze jsou kvasinkami zpět metabolizovány, a jejich

koncentrace klesá [32]. Zvýšené koncentrace mohou být však způsobeny i bakteriální kontaminací pediokoky či laktobacily [33]. Tyto látky způsobují v pivu chuť i vůni po másle. Vyšší koncentrace jsou ze senzorického hlediska považovány za nežádoucí. Avšak mírné máselné aroma způsobené vicinálními diketony je jedním ze znaků českého piva a pozitivně přispívá k jeho typickému senzorickému charakteru v porovnání s ležáky produkovanými v zahraničí [34]. Analytické metody pro stanovení vicinálních diketonů mohou zahrnovat metody spektrofotometrické, plynově nebo kapalinově chromatografické s přípravou vzorku využívající extrakci na pevné fázi, mikroextrakci na pevné fázi, mikroextrakci na míchací tyčince i derivatizaci. Nejrozšířenější metoda v praxi je založena na plynové chromatografii s headspace technikou a detekcí elektronového záchytu [35].

2.2.5 Mastné kyseliny

Mastné kyseliny v pivu mohou pocházet ze všech fází výroby piva, tzn. ze sladu při rmutování, z chmele při chmelovaru a z produkce kvasinkami. Během fáze kvašení se produkují především nižší mastné kyseliny (zejména kyselinu máselnou, valerovou a isovalerovou), které mohou při překročení prahových koncentrací způsobovat nepříjemná aromata – např. po zrajícím sýru, po zpocených nohou či žluklou [36]. Vyšší mastné kyseliny jsou výrazně eliminovány po chmelovaru, kdy jsou odstraňovány spolu s chmelovým mlátem, a do piva se tak dostávají v minimálním množství. Vzhledem k jejich vyšším senzorickým prahům ovlivňují senzorický charakter piva jen minimálně [37]. Mastné kyseliny negativně ovlivňují také stabilitu pivní pěny [38]. Koncentrace mastných kyselin v pivu se pohybují řádově v desítkách až stovkách mikrogramů na litr. Analýza mastných kyselin je obecně velice dobře zvládnutá napříč různými matricemi. Stejně tak je tomu i v pivu, kdy se využívá extrakce na pevné fázi, derivatizace na příslušné methyl estery a následné plynově chromatografické analýzy s plamenově ionizačním detektorem [39, 40].

2.2.6 Organické kyseliny

Senzorickou kyselost piva ovlivňují zejména organické kyseliny (zahrnující především kyselinu citronovou, mléčnou, jablečnou, octovou, pyrohroznovou, jantarovou, fumarovou a α-hydroxyglutarovou). Různé zastoupení těchto látek může významně určovat celkový charakter výsledného nápoje díky ovlivnění jeho pH, které kromě vlivu na vnímání ostatních senzoricky aktivních látek ovlivňuje i citlivost piva k růstu kontaminujících bakterií [41, 42]. Organické kyseliny jsou v určité míře produkovány kvasinkami při kvašení nápoje, významným zdrojem těchto látek je také slad. Reálné koncentrace organických kyselin v pivu se pohybují od jednotek po desítky miligramů na litr [43]. V případě kontaminace bakteriemi mléčného kvašení se mimo jiné výrazně zvyšuje koncentrace kyseliny octové a mléčné, čímž se významně snižuje senzorická kvalita piva [33]. Naopak, pro výraznou kyselou chuť některých specifických stylů piva (např. Lambic) jsou vysoké koncentrace organických kyselin nezbytné (např. koncentrace kyseliny mléčné může být až několik gramů na litr) [44].

Analytické metody stanovení organických kyselin zahrnují kapalinovou chromatografii se spektrofotometrickou detekcí [41], iontovou chromatografii s vodivostní detekcí [43], kapilární elektroforézu s nepřímou spektrofotometrickou detekcí [45] nebo isotachoforézu s vodivostní detekcí [46]. Bez ohledu na tyto instrumentální metody se v praxi nejčastěji používá stanovení tzv. titrační kyselosti, která je určitou mírou celkové koncentrace kyselin v pivu [47].

2.2.7 Hořké kyseliny

Jedněmi z nejdůležitějších senzoricky aktivních látek piva jsou chmelové hořké kyseliny, které se dále rozdělují na α-kyseliny (humulony) a β-kyseliny (lupulony). Z těchto skupin látek jsou důležité zejména jejich izomerizační a transformační produkty

vznikající při varu mladiny. Nejdůležitější z těchto produktů jsou iso-α-kyseliny (trans/cis-isokohumulon, trans/cis-isohumulon a trans/cis-isoadhumulon), které jsou hlavními složkami hořké chuti piva. Transformační produkty β-kyselin, přispívající k hořkosti piva, jsou hulupony. Kromě těchto látek lze v pivu také nalézt velkou škálu dalších degradačních či oxidačních produktů hořkých kyselin, jako jsou humulinony, hydroxytricyklolupony, dehydrotricyklolupony, hydroperoxytricyclolupony a hulupinové kyseliny [48, 49]. Poměrné zastoupení hořkých látek v pivu má za následek nejen intenzitu výsledné hořké chuti, ale také její charakter a doznívání. Koncentrace iso-α-kyselin v pivu se pohybují od jednotek až několik desítek miligramů na litr. Hořké chmelové látky jsou v pivu stanovovány kapalinovou chromatografií se spektrofotometrickou či hmotnostně spektrometrickou detekcí po extrakci z původního vzorku piva [49, 50].

Velice rozšířená je i spektrofotometrická metoda stanovení mezinárodních jednotek hořkosti, která je založena na extrakci iso-α-kyselin z piva iso-oktanem a jejich spektrofotometrické detekci v UV oblasti (275 nm) [51]. Tato rutinní metoda je používána laboratořemi po celém světě, zejména díky své jednoduchosti, dostupnosti a faktu, že ji světové pivovarsko-analytické komise doporučují jako metodu standardní. Z principu metody je patrné, že se nejedná o metodu selektivní a ostatní látky přecházející z piva do extraktu mohou ovlivňovat úroveň výsledného analytického signálu. To se projevuje zejména v posledních letech, kdy spolu s rozvojem nových pivovarských technologií a stále větší popularitou široké škály pivních stylů se objevují slabiny této metody. Diskutován je zejména vliv studeného chmelení (technologie chmelení piva, kdy chmel je přidáván ve studených fázích výroby – kvašení a ležení piva – čímž se dosahuje bohatšího a intenzivnějšího chmelového aromatu) na výsledek této metody, kdy z chmele do piva přecházejí α-kyseliny a humulinony, které prakticky vůbec nepřispívají k hořkosti piva nebo výrazně méně než iso-α-kyseliny [52].

2.2.8 Sacharidy

Sacharidy v pivu zahrnují jednoduché cukry, jako je glukósa a fruktósa, ale i disacharidy, jako je maltósa, a složitější oligosacharidy se třemi až deseti monosacharidickými jednotkami. Sacharidy pocházejí z extrakce ze sladu a enzymatického štěpení škrobů při rmutování. Do finálního piva se dostává jen relativně malá část zkvasitelných cukrů, které jsou spotřebovány kvasinkami během kvašení. Jednoduché sacharidy přispívají k intenzitě sladké chuti piva a delší oligosacharidy pravděpodobně pozitivně přispívají k dalšímu důležitému senzorickému parametru piva – plnosti [53]. Koncentrace sacharidů v pivu se pohybují v řádech jednotek miligramů na litr až jednotek gramů na litr. Metody stanovení sacharidů v pivu jsou založeny na kapalinové chromatografii, nejčastěji s refraktometrickým detektorem, ale vyvinuty byly také metody s hmotnostně spektrometrickou detekcí [54]. Sacharidy lze stanovit také sumárně ve formě glukosy, kdy oligomery jsou enzymaticky štěpeny pomocí amylasy či amyloglukosidasy [55].

2.2.9 Sirné látky

Sirné látky v pivu pocházejí z různých zdrojů, avšak téměř všechny jsou charakteristické negativním dopadem na senzorickou kvalitu piva. Často se jedná o nízkomolekulární látky pocházející ze starého chmele, kvašení, autolýzy kvasnic či vystavení piva světelnému záření. Mezi zástupce těchto látek patří například sirovodík, dimethylsulfid, methanthiol, 3-methyl-2-buten-1-thiol, p-menthan-8-thiol-3-on [56]. Tyto látky mají zároveň velice nízké prahy senzorického vnímání, a proto je sebemenší koncentrace těchto látek v pivu senzoricky detekována – v mnohých případech je často senzorická analýza jediný nástroj jak některé z těchto látek v pivu detekovat, protože analytické metody stále nedosahují u těchto látek dostatečných mezí detekce. To platí hlavně pro 3-methyl-2-buten-1-thiol (práh senzorického vnímání 4,4 - 35 ng/l [67]) vznikající v pivu fotolýzou isohumulonů a následnou

reakcí produktu se sirnými sloučeninami a způsobující tzv. světelnou vůni piva, popisovanou také jako letinková, skunk či pavilon opic [58]. Nicméně i pro tento problematický analyt byly vyvinuty metody stanovení jako např. metoda využívající purge-and-trap systém a plynové chromatografie s hmotnostní detekcí [57] či plynovou chromatografii s headspace technikou nebo mikroextrakcí na pevné fázi [58]. Nicméně vzhledem k problematickým fyzikálně-chemickým vlastnostem 3-methyl-2-buten-1-thiolu (vysoká těkavost a reaktivita) nejsou často tyto metody v praxi využívány a na místo nich je častěji pro prokázání přítomnosti využívána senzorická analýza (díky nízkému prahu senzorického vnímání a nezaměnitelnou vůní). Oproti tomu jsou ostatní sirné látky v pivu častěji stanovovány instrumentálními analytickými metodami – díky jejich těkavosti pomocí plynové chromatografie s chemiluminiscenčním detektorem citlivým na sirné sloučeniny nebo plamenově fotometrickým detektorem [56].

Vzhledem k tomu, že pivo je velice složitou směsí různorodých látek, jeho senzorické vnímání člověkem je také velice komplexní. Z tohoto důvodu nelze v mnohých případech pro popis senzorického charakteru piva jednoduše použít analyticky stanovenou koncentraci zodpovědných látek, ale velice důležitý je celkový pohled na senzoricky aktivní látky a také látky nepřímo ovlivňující senzorický charakter piva (prostřednictvím senzorických interakcí). Tento úkol si klade nový obor odvozený ze zavedených omických disciplín (jako je metabolomika, lipidomika a proteomika) nazvaný senzomika [49, 59]. Jelikož jde o relativně nový přístup, není v literatuře dosud příliš rozšířený. Metodika založená na senzomickém profilování byla využita v jedné z experimentálních částí této práce – studia distribuce mastných kyselin v průběhu pivovarského procesu a v závislosti na způsobu rmutování.

Souhrn senzoricky a toxikologicky aktivních látek piva diskutovaných v této práci, spolu s jejich nejčastějšími metodami stanovení, je v Tabulce 1.

Tabulka 1: Souhrn nejvýznamnějších skupin toxikologicky a senzoricky aktivních látek v pivu, jejich nejvýznamnějších zástupců, vlivu na kvalitu piva, řádový rozsah koncentrací a metody stanovení

skupina látek	nejvýznamnější zástupci v pivu	vliv na kvalitu piva	rozsah koncentrací	metody stanovení	zdroj
Toxikologicky významné látky					
nitrosaminy	N-nitrosodimethylamin	negativní – karcinogenní a mutagenní účinky	desetiny až jednotky µg/kg	GC-NCD, GC-MS	3, 11
biogenní aminy	tyramin, kadaverin	negativní – bolest hlavy, zažívací potíže, vysoký či nízký krevní tlak	setiny až desítky mg/l	HPLC-UV, GC-MS	3, 15
mykotoxiny	ochratoxin A, deoxynivalenol	negativní – karcinogenní, cytotoxické, imunotoxické účinky	jednotky až desítky mg/l	HPLC-MS, ELISA	4, 18
Senzoricky významné látky					
karbonylové sloučeniny	furfural, hydroxymethylfurfural, trans-2-nonenal	negativní – oxidační, papírová, stará	jednotky až stovky mg/l	GC-MS, HPLC-UV	20–25
vyšší alkoholy	propanol, 2- a 3-methylbutanol	v nižších koncentracích pozitivně přispívá k celkovému vjemu, ve vyšších koncentracích negativní – rozpouštědla	jednotky až desítky µg/l	HS-GC-FID	27

estery	ethylacetát, isoamylacetát	v nižších koncentracích pozitivní ovocné aroma, ve vyšších koncentracích negativní	jednotky až desítky µg/l	HS-GC-FID	27
chmelové silice	myrcen, farnesen, linalool, geraniol, humulen	pozitivní – chmelová, květinová, ovocná, pryskyřičná	jednotky až desítky µg/l	GC-MS	29-31
vicinální diketony	2,3-butandion, 2,3-pentandion	negativní – po másle	stovky µg/l	HS-GC-ECD	34, 35
mastné kyseliny	kyselina máselná, valerová, isovalerová	negativní – žluklá, zrající sýr, zvratky	desítky až stovky mg/l	GC-FID	36-40
organické kyseliny	kyselina citronová, mléčná, jablečná, octová	do určitých koncentrací působí pozitivně (mírná kyselost, ovlivnění senzorického vnímání ostatních látek), ve zvýšených koncentracích nepříjemná kyselost	jednotky až desítky mg/l	HPLC-UV, CZE-UV, ITP-UV, ITP-CD, titrace	43–47
hořké kyseliny	iso-humulon, iso-adhumulon, iso-kohumulon	pozitivní – hořkost	jednotky až desítky mg/l	UV, HPLC-UV, HPLC-MS	48–51
sacharidy	maltósa, glukósa, fruktósa	pozitivní – sladkost, plnost piva	jednotky až tisíce mg/l	HPLC-RI	53, 54

Seznam zkratek: GC – plynová chromatografie, HPLC – vysokoúčinná kapalinová chromatografie, CZE – kapilární zónová elektroforéza, ITP – izotachoforéza, HS – headspace, NCD – chemiluminiscenční detektor, MS – hmotnostní spektrometrie, UV – spektrofotometrie v ultrafialové oblasti, FID – plamenově ionizační detektor, ECD – detektor elektronového záchytu, CD -vodivostní detektor, RI – refraktometrický detektor, ELISA – enzyme-linked immuno sorbent assay

3 Experimentální část

3.1 Vývoj metody pro detekci nitroso sloučenin v pivu a jejich klasifikaci pomocí GC-PPNCD

Detailní popis experimentální práce, zahrnující použité chemikálie, vzorky a jejich úpravu, podmínky analýz a zpracování dat, jsou uvedeny v přiloženém publikovaném článku Pyrolytic Profiling Nitrosamine Specific Chemiluminescence Detection Combined with Multivariate Chemometric Discrimination for Non-targeted Detection and Classification of Nitroso Compounds in Complex Samples.

3.2 Vývoj metody pro *in situ* stanovení míry senzorického poškození piva jeho nevhodným skladováním

Detailní popis experimentální práce, zahrnující použité chemikálie, vzorky a jejich úpravu, podmínky analýz a zpracování dat, jsou uvedeny v přiloženém publikovaném článku The usage of a reflectometric method for 5-(hydroxymethyl)furan-2-carbaldehyde determination as a stale flavor sensor for beer.

3.3 Studium distribuce mastných kyselin v průběhu pivovarského procesu a v závislosti na způsobu rmutování prostřednictvím senzomického profilování

Detailní popis experimentální práce, zahrnující použité chemikálie, vzorky a jejich úpravu, podmínky analýz a zpracování dat, jsou uvedeny v přiloženém publikovaném článku The chemical profiling of fatty acids during the brewing process.

4 Publikované články

Z provedených experimentální prací byly publikovány (nebo přijaté k publikaci) tři články v impaktovaných zahraničních časopisech. Tyto články jsou níže vloženy jako součást této práce.

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Pyrolytic profiling nitrosamine specific chemiluminescence detection combined with multivariate chemometric discrimination for non-targeted detection and classification of nitroso compounds in complex samples

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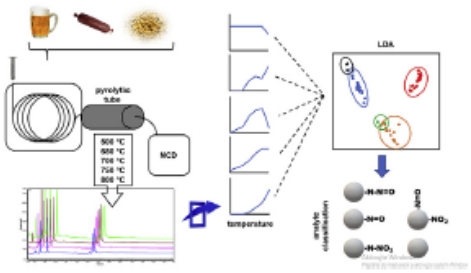
HIGHLIGHTS

- The NCD method for selective discrimination of N- and C-nitroso compounds from other interfering compounds was developed.
- Gas chromatography with chemiluminescence detection and chemometric discriminant analysis was used for this method.
- Resulted classification model shows good performance with total accuracy of 96.12%.
- The method is usable in non-targeted detection of nitroso compounds in complex samples.
- In connection with MS/MS instrument, the method provides a highly valuable tool in nitroso compounds identification tasks.

GRAPHICAL ABSTRACT



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ABSTRACT

The problems of contamination of many products by nitroso compounds have been discussed since 1970's and have been partially solved, namely, the contamination by carcinogenic volatile N-nitrosamines. However, there is still a gap in knowing non-volatile nitroso compounds in terms of both the determination of these compounds and the description of their toxicity. Therefore, a procedure for their detailed non-targeted study is necessary to be developed. Based on these facts, a new method permitting the detection and the classification of nitroso compound groups, such as N-nitroso, C-nitroso, and interfering substances in the nitrosamine specific chemiluminescence detection after previous gas chromatographic separation, was developed. The method is based on signal profiling of chromatographic peaks recorded by a chemiluminescence detector at different pyrolytic temperatures and subsequent multivariate chemometric classification. The resulting classification function by linear discriminant analysis shows good performance with total accuracy of 96.12% after the method validation. The method was successfully applied and demonstrated on a non-targeted beer sample analysis. Nitroso compounds detected by the method were selected for detailed structural analysis by GC-MS/MS. The combination of

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the presented method with the MS/MS instrumentation provides a really powerful analytical tool for the identification of unknown nitroso compounds in complex samples. This study represents a valuable contribution to the protocols of identification of organic compounds with the nitrogen functional groups – toxicologically and analytically important nitroso compounds.

1. Introduction

Nitroso compounds, specifically carcinogenic N-nitrosamines, are well-known contaminating compounds in many beverages and foodstuffs or industrial products. The authors usually divided these compounds into volatile and non-volatile ones and it is possible to determine these two groups together as their apparent total concentration - Apparent Total N-nitroso Compounds (ATNC) in μg(N-NO)/kg [1]. Volatile N-nitrosamines are routinely determined by gas chromatography with the nitrosamine specific chemiluminescence detection (GC-NCD, sometimes referred as GC-TEA) [2,3]. Gas chromatography with mass spectrometry is also frequently used in nitrosamine determination, using either positive chemical ionization or electron ionization. Tandem mass spectrometric detection is greatly appropriate technique for nitrosamine detection especially in matrices where very low detection and quantification limits are required. Liquid chromatography in nitrosamine testing is not very common (in comparison with GC methods), however, methods based on spectrophotometric, mass spectrometric and fluorescence detection were developed [3]. However, currently there is not a routine and reliable analytical method for determination of non-volatile N-nitrosamines as the structure of these compounds has not been elucidated yet (with some exceptions, e.g., N-nitrosoproline). Nitroso compounds can be further classified into C-, N-, S-, and O-nitroso compounds but the greatest attention is paid to N-nitroso compounds (due to their toxic effect). The presence of non-volatile nitroso compounds in some types of food and beverages, especially in beer and/or malt, is evident from the comparison of volatile N-nitrosamine concentrations and ATNC concentrations determined by a denitrosation reaction by hydrogen bromide in acetic acid solution and consequent quantification of released nitric oxide by NCD [4].

Sources of N-nitrosamines in beer are malt and nitrite contamination by bacterial reduction of nitrates in brewing intermediate (wort) and/or the final product. N-nitrosamines in malt are formed during the malting process, especially during kilning, where the nitrogen oxides from the environment or from direct heating react with precursors in sprouted grain [5]. Whereas the problem of volatile N-nitrosamines originating from the malting process was nearly eliminated due to the technological changes, the risk of a high amount of ATNC in beer via microbial contamination still persists. Volatile N-nitrosamine concentrations in beer are very low, even after microbial contamination, e.g., N-nitrosodimethylamine concentration is usually lower than 0.2 μg/kg (<0.12 μg(N-NO)/kg expressed as ATNC) [1]. Based on our previous study [6], common concentrations of ATNC in beer is lower than 20 μg(N-NO)/kg and only approximately 1% of ATNC in beer is formed by volatile N-nitrosamines, therefore, non-volatile ones represent the vast majority of total ATNC in beer (non-volatile N-nitrosoproline represents approximately 5%). This would not be problematic in cases where ATNC concentrations is below 20 μg(N-NO)/kg, but in some cases, especially in some beers from microbreweries or brewpubs, ATNC concentrations occurs at much higher values (up to 560 μg(N-NO)/kg) [7]. In spite of these facts, nobody has described the spectrum of ATNC compounds in beer in more detail yet to evaluate the toxicological risk from the consumption of beer with a higher ATNC amount.

Besides N-nitrosamines, this study is also focused on C-nitroso compounds due to the likelihood of formation of these types of substances during nitrosation in beer and/or malt (in more detail below). The remaining S- and O-nitroso compounds are not within our focus because of their destruction during the sample preparation prior ATNC determination.

From the nature of compounds presented in beer (relatively high concentration of polyphenolic compounds) it is likely that a part of ATNC is formed by C-nitroso compounds arising from an interaction of phenolic compounds with nitrosation agents [8,9] produced after bacterial contamination. Based on the molar ratio between polyphenols and nitrosation agents, polyphenolic compounds inhibit or catalyze N-nitrosamine formation [10,11]. Inhibition occurs by its C-nitrosation, oxidation or nitration. There is very little known about effects of C-nitroso compounds on human health, and although N-nitrosamines were studied in detail, the occurrence of C-nitroso compounds in food has not yet been studied. Any conclusion about the health risk arising from intake of non-volatile nitroso compounds in beer cannot be made without detailed knowledge of their chemical structure. Since the structures of non-volatile nitroso compounds are unknown and due to the expected diversity of their structures, it is practically not possible to find them in complex matrices by current methods. For these reasons, there is a requirement for a non-targeted method which will be able to find unknown non-volatile nitroso compounds as individual chromatographic peaks for subsequent detailed structural elucidation. A possible way to search for non-volatile nitroso compounds is to detect them by NCD after derivatization of polar groups and GC separation. However, the fundamental problem is that NCD is not selective enough and some peaks leading to false positives commonly appear in the chromatogram. These peaks could be confused with nitroso compounds. Therefore, the classical NCD is blind in non-targeted detection of unknown constituents of ATNC.

The chemical method for stepwise distinguishing N-nitrosamines from other NCD positive peaks was developed by Krull et al. [12]. However, due to stepwise exposure of a sample to different chemical agent (e.g. acetic acid solution or hydrobromic acid/acetic acid mixture) the method is laborious, some stages are not compatible with GC, and, N- and C-nitroso compounds are practically indistinguishable. In contrast, Hansen et al. [13] studied dependency of NCD response of N-nitrosamines, alkyl nitrites and nitro compounds on pyrolysis temperature of detector's pyrolytic tube in the range of 200–600 °C. They did not obtain any response to a single studied C-nitroso compound in the temperature range. Based on their results, authors suggested a pyrolytic response profile as a useful tool in distinguishing among NCD-positive compounds. In contrary to the Hansen's study, the temperature range in this study was extended to higher pyrolytic temperatures (500–800 °C), and C-nitroso compounds were also tested and detected. Based on these facts, the pyrolytic profiling NCD (PPNCD) method was designed and developed in this study.

The aim of this study was to develop a detection method for

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classification of nitroso compounds in a complex sample into different nitroso compound types (N- and C-nitroso group), and to distinguish them from interfering compounds, by GC with PPNCD. This method uses changes in peak intensity depending on NCD pyrolytic temperature and multivariate chemometric evaluation. The method will serve as a tool in a nitroso compounds identification procedure. The workflow of the method is schematically described in Fig. 1.

2. Experimental section

2.1. Reagents and chemicals

The following substances were used for construction of a classification function: methanolic solutions of N-nitrosamines, namely, N-nitrosodimethylamine, N-nitrosodiethylamine, N-nitrosodiisopropylamine, N-nitrosodipropylamine, N-nitrosodibutylamine, N-nitrosopiperidine, N-nitrosopyrrolidine, N-nitrosomorpholine, (100 μg mL^{-1}, Ultra Scientific, USA), N-nitrosodiethanolamine solution (100 μg mL^{-1}, Neochema, Germany), N-nitrosoproline, N-nitrososarcosine, N-nitrosopipecolic acid, and N-nitrosonornicotine (100 μg mL^{-1}, Isconlab, Germany); C-nitroso, nitro and other interfering compounds, namely, nitrosobenzene (95%, Merck, Darmstadt, Germany), nitrosotoluene (97%), N,N-dimethyl-4-nitrosoaniline (97%), 2-nitroso-1-naphtol (95%), methoxy-3-nitro-2-nitrosobenzene (98%), nitroethane (96%), nitropropane (98%), 2,4-dinitrophenol (95%), nitrobenzene (>99%), 2-nitroaniline (99%), eugenol (99%), 2-ethylphenol (99%), 2-allylphenol (99%), aniline (>99.5%), benzonitrile (99%), 2-furancarbaldehyde (99%), 3,4-dihydroxybenzoic acid (97%), pyridine (≥99.8%), D-(+)-glucose (≥99.5%), catechin (99%, Sigma-Aldrich, Steinheim, Germany), gramine (99%, Serva, Germany). Solution of N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1, v/v, Supelco, Steinheim, Germany) was used for derivatization of non-volatile compounds. Ammonium amidosulphonate (99%, Merck, Darmstadt, Germany) and sulphuric acid (96%, Lachner, Neratovice, Czech Republic) were used together in a solution as nitrite scavenger (see section Preparation of artificially nitrosated samples). The following solvents were also used: acetonitrile (≥99.9%) and methanol (≥99.9%, both from Sigma-Aldrich, Steinheim, Germany). Deionized water was produced by Milli-Q system. Hydrochloric acid (37%, Merck, Darmstadt, Germany) and sodium nitrite (≥97.0%, Sigma-Aldrich, Steinheim, Germany) were used for artificial nitrosation of beer samples.

N-nitramines were prepared by oxidation of corresponding N-nitrosamines (N-nitrosopyrrolidine, N-nitrosopiperidine) in a dichloromethane solution (2 mL, 50 μg mL^{-1}) by 1 mL of trifluoroacetic anhydride (Merck, Darmstadt, Germany) and 0.6 mL of hydrogen peroxide (33%, Merck, Darmstadt, Germany) at 65 °C for 60 min and the products were recrystallized in 5 mL of ethanol (96%, Lachner, Neratovice, Czech Republic) (after volume reduction by evaporation) [14].

All substances, before being derivatized and injected on GC-NCD, were solubilized in acetonitrile at three different concentrations levels based on the following rules: a) concentrations sufficient to detect them (peak height between 3 and 10 times of baseline noise), b) concentrations when peak height was tightly below the detector range (peak signal under 11750 mV) and c) concentrations approximately in the middle of the previous concentrations (measured at pyrolytic temperature where the particular group has the highest relative response, see Fig. 2a in Results and Discussion). These rules were selected for testing of a pyrolytic profile across the whole signal range of the detector. The range of concentrations differ among different groups of compounds, the lowest concentration was for N-nitrosamines (0.004–25.12 μmol L^{-1}) and the highest for aromatic interferences (0.17–83.4 mmol L^{-1}).

Due to the small number of tested C-nitroso and C-nitroso/nitro compounds, their pyrolytic profiles were measured at four concentration levels.

2.2. Instrumentation

Gas chromatographic-chemiluminescence detection analyses were carried out by the Thermo Trace 1310 gas chromatograph (Thermo Scientific, San Jose, USA) equipped with TG-200MS capillary column (30 m, 0.25 mm ID, 0.25 μm film thickness of trifluoropropylmethyl polysiloxane stationary phase). The injection of 1 μL of sample solution was performed by a split injection technique at split ratio 1:10 and 210 °C. The flow of argon (Messer, 99.996%) as a carrier gas was maintained at 0.6 mL/min.

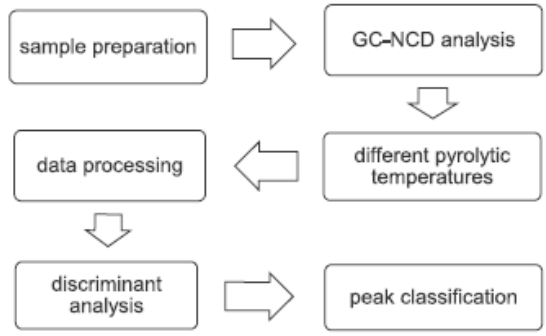

Fig. 1. Applied workflow for classification of nitroso compounds in beer.

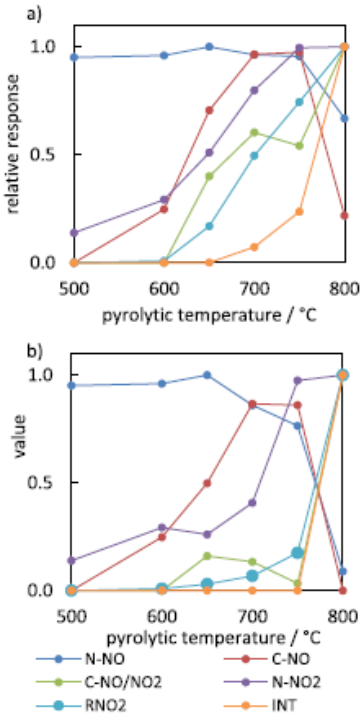

Fig. 2. Pyrolytic profiles of a) tested compound groups and b) pyrolytic patterns transformed by power functions.

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Programmed oven temperature during the analysis was set as follows: 50 °C (1.5 min) − 20 °C min^{-1} − 150 °C (5 min) − 10 °C min^{-1} − 210 °C (3 min) − 10 °C min^{-1} − 320 °C (10 min). Interface temperature was maintained at 250 °C. Chromatographic zones were detected by the Ellutia 820 TEA chemiluminescence detector (Ellutia, Ely, UK) in nitrosamine mode at different temperatures of pyrolytic tube to obtain pyrolytic profiles (500, 600, 650, 700, 750 and 800 °C). The flow of oxygen as a reagent gas for ozone generation in the detector was set at 3.2 mL min^{-1}.

After the method development, the selected pyrolytic temperatures (500, 650, 700, 750 and 800 °C) were used for real samples.

Gas chromatographic mass spectrometric analyses were carried out by the Agilent 7890B gas chromatograph equipped with the 7000D triple quadrupole mass spectrometric detector (Agilent Technologies, Santa Clara, USA). The chromatographic column, injection parameters and an oven temperature program remained the same as in the case of GC-NCD analysis. Helium as a carrier gas (Air Products, Czech Republic) was maintained at 0.56 mL min^{-1}. Interface temperature was maintained at 250 °C. Electron ionization was performed at two different conditions: first at 70 eV and 280 °C, second at 20 eV and 180 °C. The flow of nitrogen (Air Products, Czech Republic) as a collision gas was 1.5 mL min^{-1} and the quench flow of helium was 2.25 mL min^{-1}. A triple quadrupole mass spectrometric detector was used at different measurement modes: MS1 scan (40–600 m/z), neutral loss of 30 u, product and precursor ion mode. Collision energy for neutral loss scan, precursor ion and product ion mode were 6.0, 15.0 and 15.0 eV, respectively. A detailed list of MS/MS experiments can be found in Supporting information in Tables S–1.

2.3. Derivatization of standard solutions

Non-volatile standard compound solutions in acetonitrile were dried under a stream of nitrogen and 150 μL of derivatization reagent (BSTFA + TMCS) were added. After 15 min at 65 °C in a heat block, samples were directly analyzed by GC-NCD.

2.4. Preparation of artificially nitrosated samples

Real lager beer samples were treated by a) microbial contamination and b) chemical contamination by nitrite.

Beer samples for the microbial contamination experiment were contaminated by *Obesumbacterium proteus* (strain CCM 2806), known nitrate reducing bacteria and ATNC producer [15]. The blank beer sample was used for a comparison of the results.

The chemical contamination by nitrite was performed as follows: two lager beer samples (500 mL bottles), obtained directly from a local manufacturer, were acidified by 4 mL of hydrochloric acid and after that 2.0 g of sodium nitrite was added. The samples were stored in the dark at a laboratory temperature for one week. This procedure was performed in order to artificially prepare high nitroso compound concentrations in beer to easily detect them. The blank beer sample was treated in the same way without the nitrite addition. The sample preparation before the GC analysis was the same for all the samples respectively with some defined differences between the experiments a) and b). Five milliliters of a beer sample were mixed with 1 mL of 0.2 mol L^{-1} solution of ammonium amidosulphonate in 0.2 mol L^{-1} sulphuric acid and allowed to react for 15 min. The sample was then loaded on a SPE column (C-18E, 500 mg, Phenomenex), previously conditioned with 6 mL of methanol and 6 mL of deionized water. Then, the sorbent was washed with 6 mL of deionized water. In the experiment a), the sample poured through the SPE column was evaporated in a vacuum evaporator at 42 °C and 30 mbar, and in the experiment b), the SPE column was gently dried by vacuum and eluted by 5 mL of methanol and 5 mL of acetonitrile. The resulted extract was evaporated to dryness at 45 °C by a gentle stream of nitrogen. Residues were dissolved in 200 μL of acetonitrile and 200 μL of methanol. Methanolic and acetonitrilic parts were mixed together in a vial insert. Formed precipitate was separated by freezing for at least 30 min in a freezer. The liquid sample was withdrawn from solid precipitate and evaporated in a new insert under the stream of nitrogen at 45 °C. Residues were dissolved and derivatized by 150 μL of BSTFA:TMCS solution at 70 °C for 40 min. Then, the sample left to cool down and afterwards directly injected on GC.

A test mixture in a real sample was prepared in the same way as the blank beer sample in the experiment a). After the SPE clean-up, the sample was fortified with N-nitrososarcosine (100 μg L^{-1}), N-nitrosoproline (100 μg L^{-1}), nitrosobenzene (10 mg L^{-1}), N, N-dimethyl-4-nitrosoaniline (10 mg L^{-1}), N-nitropyrrolidine (~100 μg L^{-1}), D-glucose (80 mg L^{-1}) and gramine (80 mg L^{-1}). These compounds were selected according to their retention time to prevent excessive coelution of the peaks of the test mixture with peaks originating from the real sample. The standard compound for C-nitroso/nitro combination was not used due to coelution.

All samples were prepared and analyzed as blind test (in duplicate).

2.5. Data processing

Exploratory data analysis, analysis of variance (ANOVA) and linear discriminant analysis were performed in Statistica 8.0 (Statsoft. Inc, USA). The distribution of data was checked by a box-whisker and a normal probability plot, outliers were removed from the data set.

Relative peak areas (related to the highest area across the pyrolytic profile) and pairwise ratio between these areas of each standard compound (at least two different concentrations) were used as independent variables for linear discriminant analysis. Powers of relative peak areas were also calculated (more details in Results and discussion). Dependent variables in linear discriminant analysis were groups of compounds as follows: N-nitroso (31x), C-nitroso (16x), combination of C-nitroso and nitro (6x), N-nitro (4x) and interferences (46x) including nitro compounds and non-nitroso aromatic compounds. Pyrolytic profiles of each standard compound were measured in a triplicate and median was calculated from obtained relative values.

A forward stepwise selection method was used to choose the most discriminating variables for the final discriminant function (tolerance 0.01). These variables were further used as independent variables in discriminant classification. The data set (n = 103) was randomly divided into a training and a test set (1:1) with equal representation of each group of compounds. The training set was used to construct a discriminant function and variable selection, and its performance was checked by the test set, leave-one-out cross-validation method as well as by the test mixture in a real sample. A priori classification probability was set up the same for all groups at 0.2. Validation parameters (defined in Supporting Information) were calculated from obtained classifications of objects after a leave-one-out validation procedure, according to Ballabio and Todeschini [18].

The height of baseline noise was evaluated in a real sample analysis. The mean and 95% confidence interval were calculated. Upper limit of the confidence interval was multiplied by three and the peaks lower than this value were removed from the final evaluation.

2.6. Safety considerations

Substances used in this study, especially N-nitrosamines and C-

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nitroso compounds, are toxic and/or considered to be a potential carcinogen for a human. Safety precautions and protective equipment must be taken during the manipulation with these substances. Handling with volatile N-nitrosamines must be performed only in a fume hood to prevent exposure to these substances.

3. Results and discussion

At the beginning, effect of pyrolytic tube temperature on peak area was tested. Increasing the pyrolytic tube temperature from 500 to 800 °C was tested on a derivatized extract of a beer sample during the GC-NCD analysis. Several patterns of changes of the peak area with pyrolytic tube temperature were observed in the chromatogram. Within the changes, three main patterns were recorded. These observations led to the following hypothesis: as it must be a reflection of a different chemical structure and/or functional group of detected compounds, this phenomenon suggests using a pyrolytic profile for classification of NCD-positive peaks. These assumptions were also supported by a previously published study of Hansen et al. [13]. These facts and our interest in nitroso compounds analyses led us to the development of the method described in this paper.

3.1. Compound groups

At the beginning of the method development, the compound groups involved in the method were defined.

The following compound groups were selected for the purpose of the method: N-nitroso (N-NO), C-nitroso (C-NO), combination of C-nitroso and nitro (C-NO/NO_2), N-nitramine (N-NO_2), nitro (RNO_2) and other interfering compounds (INT - mainly unsaturated/aromatic compounds without nitroso and/or nitro group). These groups were selected based on the preliminary screening experiment of NCD-positive compound groups and the literature [12,13,16]. A pyrolytic profile of each group is projected in Fig. 2a. The C-NO/NO_2 group is the only combination of functional groups used in the method as this combination can occur during reactions of some phenolic compounds with nitrosation agents [9,17], and therefore, their occurrence is probable under nitrosation conditions in brewery matrices. Aromatic structure with the other defined nitrogenous functional groups is expected to be the most frequent combination. These compounds were also included in groups defined above (for example N-nitrosonornicotine in N-NO, nitrobenzene in RNO_2). In these types of compounds, the pyrolytic profile is governed by the nitrogenous functional group in the molecule, not by aromatic moiety, due to a different sensitivity of the NCD detector for these types of structures, see Table 1. The other group combinations were not tested and included in the method due to the unavailability of the standards and/or expected low probability of their occurrence in samples.

During the method development, the pattern of pyrolytic profile of RNO_2 group seems to be very similar to the group of unsaturated compounds, see Fig. 2a. Both of these two classes are considered to be interferences in nitroso compound analysis, therefore, they were coupled into a one group named as interferences (INT).

The pyrolytic profiles of each group were evaluated as independent on concentration by ANOVA ($\alpha = 0.05$), hence, a pyrolytic profile can be used as an indicator of a functional group, involved in an analyte molecule, over the entire range of the detector response. Molar sensitivities of NCD to functional groups were compared in Table 1. As can be seen, N-NO and RNO_2 groups have the highest sensitivity, due to the primary purpose of NCD for analyses of these types of compounds. Aromatic INT exhibits the lowest sensitivity, only 0.13% of N-NO, because their response is not produced by chemiluminescence of excited nitrogen dioxide and is also eliminated by a red optical filter (basic component of NCD) in front of photomultiplier tube.

Table 1
Molar sensitivities (relative to N-nitrosamines) of NCD for different compound groups at pyrolytic tube temperature 750 °C.

	N-NO	C-NO	RNO_2	INT
rel. sensitivity (%)	100.00	24.84	198.12	0.13

N-NO2 were not tested, but it is believed that it will be the same as for N-NO.

The number of objects was not consistent within the given groups due to the unavailability of pure compounds from some groups, however, the constructed model gives good awareness of behavior of each group for distinguishing N-NO and C-NO groups from other NCD-positive compounds (as can be seen in the following sections).

3.2. Construction of a classification function, variable selection and a model validation

The construction of a classification function was performed on the measured pyrolytic profiles of every compound in the defined compound classes. The selection of the most reliable variables and the validation of the function were necessary to be performed.

In order to construct the classification function, the relative peak areas were calculated as independent variables for the linear discriminant function. From Fig. 2a, it is obvious that the pattern of a pyrolytic profile, rather than absolute values of relative peak areas only, determines the compound group. Every pattern can be defined by ratios among each points of the profile, therefore, ratios between the relative areas were also calculated and used as independent variables. The training set was used for construction of the classification function by the forward stepwise method. Resulted preliminary model No. 1 was validated by the test set and also tested by the test mixture in a real sample (see section Preparation of artificially nitrosated samples and Data processing). The selected variables, model performance and validation parameters can be found in Tables S–2. This model shows good performance for the training and the test set, however, the classification of known standards of the test mixture in a complex real sample was not very successful (Supporting information Figure S-1 and Tables S–4). Misclassifications were observed for C-NO compounds which were confused with INT. Therefore, this model is not acceptable for the purposes of the method. Based on the posterior probability of classification, trimethylsilyl derivative of glucose was classified with approximately the same probability to the group of INT and N-NO_2. These misclassifications were probably caused by a higher variance of pyrolytic profiles in the real sample, as a result of imperfect resolution of chromatographic peaks. Since it is not always possible to obtain an excellent resolution of chromatographic peaks in complex sample analyses without excessive analysis time, the pyrolytic profile patterns need to be improved, especially between C-NO and INT groups, to increase discriminating ability and sensitivity of the method to C-NO group for complex samples. For this purpose, powers of relative peak areas were tested as discriminating variables. Power functions (mathematical operation usually used in statistics and chemometrics) were selected for their ability to reduce the powered number (numbers lower than 1 and higher than 0), and for the fact that the higher the number, the lower the relative reduction. The selection of power functions was based on the dependency of the differences between C-NO and INT groups, at given pyrolytic temperatures, on a power function in Supporting information in Figure S-2. The highest differences, at pyrolytic temperatures 500,

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600, 650, 700, 750 and 800 °C, were obtained at power 1, 1, 2, 4, 6, and 6, respectively. As a result, a gain in differences between pyrolytic profiles is obtained (see Figure S-3). The resulted pyrolytic patterns are projected in Fig. 2b and were also tested in the discriminant analysis with related ratios. The classification function No. 2 was built up through the training set by the forward stepwise method and validated by the test set and leave-one-out method. The model was also tested by the test mixture in a real sample. Projection of all pyrolytic pattern descriptors used as a starting point for model building is in Figure S-4 (training and test set together) – a smoothed line plot was chosen, rather than a bar plot, for better visual clarity of each compound group. Validation parameters are in Table 2 and properties of the model together with the selected discriminating variables are in Tables S–3 Projections of discriminant analysis to first three canonical variables are in Fig. 3. Validation exhibits satisfactory performance of the model No. 2. From the comparison of classification accuracy obtained by the test set (98.04%) and the leave-one-out method (96.12%) with accuracy obtained by random assignation of objects into one of the groups (67,53%; computed as 1 − random error rate [18]), it is obvious that classification by the developed model is not random. Moreover, misclassifications were observed only between groups neighboring in the canonical score plot.

Although the performance parameters of both models (No. 1 and No. 2) suggest very similar quality, the application of the test mixture in a real sample was successful only with the model No. 2. The resulted classification of the test mixture in a real sample shows only one misclassification, see Table 3. Trimethylsilyl derivative of D-glucose (INT1) was classified into C-NO/NO_2 group combination. Nevertheless, by inspection of the discriminant analysis canonical score plot, in Fig. 3, it can be concluded that it belongs to the INT group. The results suggest that using the power function to the relative peak area led to the classification function with the highest ability to discriminate among NCD-positive compounds in a complex chromatogram. Also, the clusters in model No. 2 are more separated from each other in comparison with model No. 1, as can be seen from canonical score plots and squared Mahalanobis distances (Fig. 3 and Fig. S-1 and Table 2 and Table. S-2).

As a conclusion, the resulted model No. 2 shows high total accuracy of classification and a very low error rate as well as a random error rate. The model also exhibits satisfactory performance for each of the compound group classes used in this study. The worst performance was observed for C-NO group in sensitivity and specificity, which is attributed to the overlap of C-NO and N-NO_2 groups in the canonical score plot. This phenomenon is also reflected in precision of N-NO_2 group. From this point of view, if the resulted classification of a given peak is C-NO and/or N-NO_2, the attention must be paid and the correct classification should be confirmed by inspection of pyrolytic pattern and the position of the point in the canonical score plot. On the other hand, the method has excellent performance for N-NO group (misclassifications were not observed) due to the unmistakable pyrolytic pattern of this group.

Table 2
Validation parameters of model No. 2 obtained by leave-one-out-method (the parameters are defined in Supporting information).

	C-NO	N-NO	INT	C-NO/NO_2	N-NO_2	total
sensitivity	0.813	1.000	0.977	1.000	1.000	
specificity	0.897	1.000	0.950	1.000	1.000	
precision	1.000	1.000	1.000	0.857	0.500	
accuracy						0.961
error rate						0.039
random error rate						0.325

3.3. Artificially nitrosated beer sample application

The artificially nitrosated beer sample was used for the demonstration of the presented method. The results obtained from this sample were also used for the identification of selected compounds by GC-MS/MS in the next subsection.

The method was demonstrated on beer samples a) contaminated by *O. proteus* and b) artificially nitrosated by nitrite. The samples were treated according to the procedure described in the section Preparation of artificially nitrosated samples, and peaks were classified by model No. 2. To verify the classification results and distinguish eventual nitroso compounds from original beer, the chromatogram of nitrosated beer samples was compared to the chromatogram of original beer sample, Fig. 4 and S-5. Chromatograms of treated samples show increasing number of peaks classified into groups of N-NO, C-NO and C-NO/NO_2 combination. These results give the evidence that the method is able to detect and classify nitroso compounds in real samples. Both experiments gave peaks with pyrolytic patterns identical to those obtained from standard compounds involved in the method development, Figure S-6. This result suggests that the classification method comprises the most common groups of NCD-positive compounds presented in beer samples. The classification of peaks is recorded in Table 4, scores of canonical variables are also projected in Fig. 3b and S-7.

As can be seen in Fig. 3b, the chemical nitrosation by nitrite led mainly to a formation of N-NO compounds, few C-NO compounds, and also some unexpected pyrolytic patterns were observed (marked as a question mark in the plot). These unexpected pyrolytic patterns may be caused by a molecule with NCD-positive functional group not involved in the method development (pyrolytic patterns which were not used in construction of discriminant function), combination of the groups in one compound (for example N-NO and C-NO in a molecule) and/or peak coelution of compounds with different NCD-positive functional group. On the other hand, the contamination by *O. proteus* led to the sample where only C-NO and C-NO/NO_2 combination groups were detected.

3.4. GC-MS/MS

The beer sample artificially contaminated by nitrite was also analyzed by GC-MS/MS to elucidate structural characteristics of some of nitroso compounds detected and classified by NCD.

The attention was paid to the most intense peaks in the GC-NCD chromatogram (retention time 14.67, 15.1 and 15.27 min, all classified as N-NO) and one peak classified as C-NO (retention time 19.02 min). Firstly, retention time of the peaks at GC-MS/MS were calculated from the equation in Figure S-8 – due to the observed retention time shift (calculated retention time of N-NO peaks were 14.06, 14.48 and 14.64 min and C-NO 18.27 min). This time shift was probably caused by different hardware equipment on the output from the chromatographic column and/or different vacuum status and thereby increased pressure drop (for more details see Supporting Information). Measured retention time of studied peaks at GC-MS/MS were 13.9, 14.4, 14.7 and 18.3 min and their MS1 full scan mass spectrum was recorded, Figure S-9. For each peak, the neutral loss scan of 30 u (loss of NO.), a selected precursor and a product ion scan were also performed, Tables S–1. Since neutral loss of 30 u is not fully specific for loss of nitrosyl radical from the molecule containing a nitroso group, the obtained neutral loss scans were used only for the peaks previously classified to nitroso groups (it is the main reason why the PPNCD method presented in this paper is highly valuable even if MS/MS instrumentation is available) and interpreted with caution. The electron ionization was

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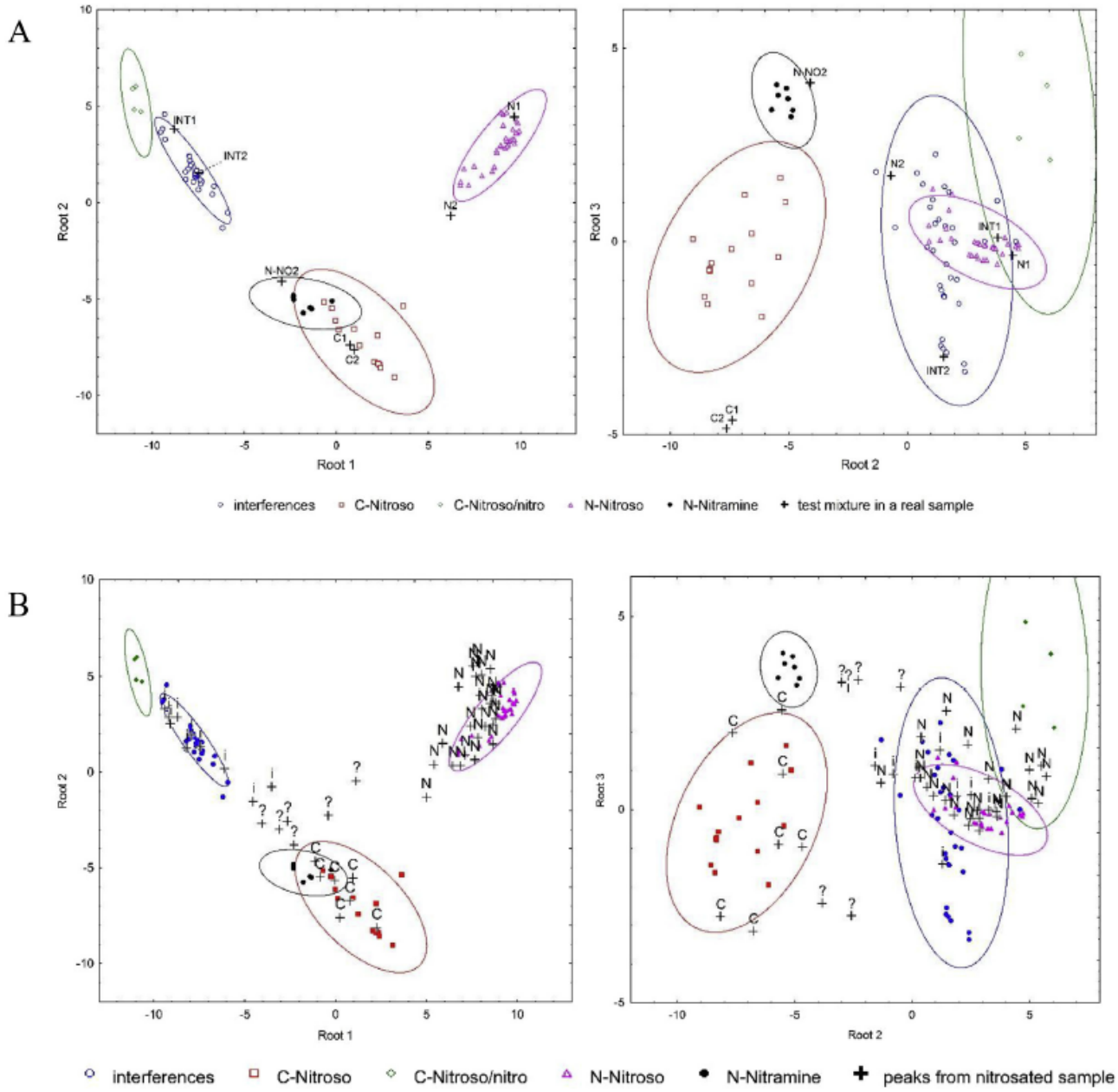

Fig. 3. Projection of discriminant analysis to canonical variables and classification results of peaks from A) the test mixture in a real sample (black crosses – peaks from test mixture in a real sample) and B) artificially nitrosated beer sample by nitrite (black crosses – peaks from nitrosated beer sample).

Table 3
Classification of the test mixture standards in a real sample by model No. 2 (Misclassifications are highlighted by asterisks).

indication	analyte (trimethylsilylated)	classification
INT1	D-glucose	C-NO/NO2*
N1	N-Nitrososarcosine	N-NO
N2	N-Nitrosoproline	N-NO
INT2	gramine	INT
C1	nitrosobenzene	C-NO
C2	N,N-dimethyl-4-nitrosoaniline	C-NO
N-NO2	N-Nitropyrrolidine	N-NO2

performed under different conditions, firstly, standard condition at 70 eV and 280 °C, and secondly, softer ionization condition at 20 eV and 180 °C. The softer ionization was selected for reduction of a fragmentation rate of analyte molecules which in some cases leads to more intense molecular ion. In practice, softer ionization was used for most of the MS experiments. Neutral loss of 30 u was revealed as a very useful tool in molecular ion identification of nitroso compounds due to the loss of nitrosyl radical from molecular ion and improved signal to noise ratio, see neutral loss scans in Supporting information Figure S-11, S-14 and S-16.

Tandem mass spectrometry was used for the elucidation of structural information of the selected nitroso compounds

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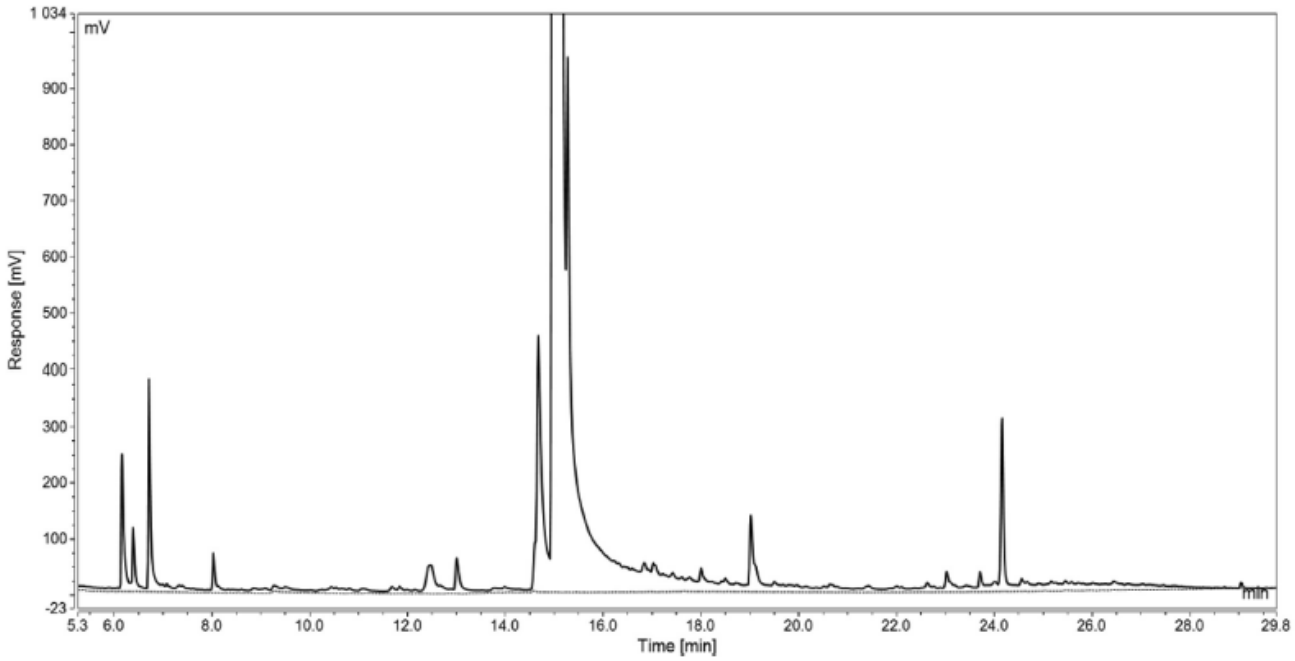

Fig. 4. GC-NCD chromatogram of artificially nitrosated beer sample by nitrite (full line) and original beer sample (dashed line). Chromatograms are recorded at pyrolytic temperature of 750 °C.

Table 4
Classification of peaks of artificially nitrosated beer sample by nitrite (possible unknown combination of groups based on inspection of canonical plots are highlighted by asterisks).

retention time/min		class.
GC-NCD	GC-MS	
6.16	5.81	N-NO
6.39	6.03	N-NO
6.71	6.35	N-NO
8.03	7.62	N-NO
9.26	8.81	INT
11.68	11.15	N-NO
11.83	11.30	N-NO
12.43	11.88	INT
12.50	11.95	N-NO
13.00	12.44	N-NO
14.67	14.06	N-NO
15.11	14.48	N-NO
15.27	14.64	N-NO
18.00	17.28	N-NO
19.02	18.27	C-NO
19.09	18.34	N-NO2*
22.62	21.76	N-NO2*
23.02	22.15	N-NO2*
23.71	22.81	C-NO
24.16	23.24	C-NO
24.56	23.63	C-NO
24.68	23.75	N-NO
29.05	27.98	C-NO

previously detected and classified by GC-NCD in the beer sample artificially contaminated by nitrite.

The most dominant peak at 14.4 min (at GC-MS/MS and 15.1 min at GC-NCD) matched the retention time with trimethylsilylated standard of N-nitrosoproline. Comparison of the mass spectra of the unknown peak and NPRO standard is in Fig. S10. Both have the assumed molecular ion at m/z 216. The neutral loss scan reveals the loss of nitrosyl radical from m/z 216 and 201, molecular ion and $[M\text{-}CH_3]^+$. The expected fragmentation of N-nitrosoproline trimethylsilylester based on product ion scans of m/z 99 and 142 (Fig. S11) is shown in Fig. S12. Based on the GC-NCD and GC-MS/MS analyses, it is obvious that the dominant peak in GC-NCD chromatogram is the only one previously detected and identified non-volatile N-nitroso compound in beer [6,19] (N-nitrosoproline in form of trimethylsilylester).

The peak at retention time 14.7 min (at GC-MS/MS and

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15.3 min at GC-NCD) shows a very similar fragmentation pattern like N-nitrosoproline but 14 units higher ($M^{+\cdot}$ at m/z 230). From this point of view, the N-nitrosopipecolic acid is offered as the first choice during the identification. Therefore, the retention was compared to trimethylsilylated standard of N-nitrosopipecolic acid, Fig. S13. Although mass spectra are similar, the retention differs. Some differences are also obvious from the product ion spectra of m/z 156 obtained from an unknown peak and trimethylsilylated N-nitrosopipecolic acid in Figs. S13 and S13x. From these results it could be assumed that the peak at 14.7 min is represented by a compound with a very similar structure like N-nitrosopipecolic acid trimethylsilylester, probably by its positional isomer, however, the pyroglutamic acid structure should be also considered.

The compound represented by the peak at 13.9 min (at GC-MS/MS and 14.7 min at GC-NCD) is not derivatized by trimethylsilyl group because of the lack of m/z 73 (characteristic fragment of trimethylsilyl derivates), as it is obvious from MS1 spectrum in Fig. S9. The proposed molecular ion, based on MS1 spectrum, the neutral loss scan and the precursor ion scan of m/z 99, is at m/z 172. The neutral loss scan reveals the loss of nitrosyl radical from the molecular ion and a fragment at m/z 99. The fragment at m/z 99 has the same product ion spectrum as the fragment from N-nitrosoproline trimethylsilylester, Figs. S14 and S11. Therefore, the nitrosated pyrrolidine structure in the molecule is assumed. Based on the product and precursor ion spectra, the proposed structure and fragmentation pathway of the analyte molecule is described in Fig. S15. This compound was identified as N-nitrosoproline ethyl ester – probably formed by reaction of N-nitrosoproline with ethanol in beer after acidification of beer sample during artificial nitrosation of the sample.

The last peak selected for a detailed MS analysis with retention time 18.3 min (at GC-MS/MS and 19.0 min at GC-NCD) was classified to C-NO group by PPNCD. The MS1 spectrum and the neutral loss scan suggest m/z 327 as a molecular ion, Figs. S9 and S16. The peak classification and the odd molecular mass suggest an odd nitrogen atom in the unknown analyte molecule. Since the classification described in this paper is not able to express detailed information about a number of nitroso groups in a molecule, only one C-NO group is presumed in the molecule of analyte represented by the peak at 18.3 min. For this peak, several product ion spectra were recorded, Fig. S16. In the MS1 spectrum, a few evenly spaced fragments ($\Delta = 15$ amu) at higher m/z were observed, which is analogous to mass spectra of trimethylsilylated methoxy phenolic acids (e.g., vanillic, ferulic and syringic acid). The fragmentation pattern of TMS methoxy phenolic acids is represented by the loss of a methyl radical ($[M\text{-}15]^+$) and a neutral molecule of formaldehyde ($[M\text{-}30]^{+\cdot}$) [20]. Although the mass spectrum of the studied peak shows the same losses as methoxy phenolic acids, the presence of nitrogen atom follows from the expected odd molecular mass. Therefore, the mass spectrometry analysis supports the previous classification result of the peak to nitrogen containing group (C-NO). Based on these facts, it is assumed that the losses discussed above were caused by methyl and nitrosyl radical. MS2 fragmentation of m/z 135 reveals benzoyl structure of m/z 105 due to the loss of carbon monoxide (loss of 28) and acetylene (loss of 26). Phenolic structure derivatized by trimethylsilyl group and the presence of nitrogen atom is evident from obtained mass spectra, however, further mass spectrometric experiments should be performed for detailed structural characterization of the molecule.

After the mass spectrometric analyses, the selected peaks were identified as follows: N-nitrosoproline ethyl ester at 14.7 min, N-nitrosoproline trimethylsilyl ester at 15.1 min, derivative of N-nitrosopipecolic acid trimethylsilyl ester at 15.3 and phenolic compound with the presence of nitrogen (probably C-NO group) at 19.0 min.

The structural mass spectrometric analyses performed at selected peaks are consistent with its classification by PPNCD. Some of the compounds were firstly detected and described in beer but a detailed study of their structure needs to be done in the future.

4. Conclusions

It can be concluded that the developed method provides a powerful tool in detection and differentiation of N-NO and C-NO compounds in complex samples. Together with MS/MS instrument, the application potential of the method can be fully exploited. As a result, the method will be applicable to non-targeted detection of N- and C-NO compounds in any sample, not only in brewery samples as it is described in the previous section, prior their structural identification. Nevertheless, the method provides reliable results only after sufficient peak separation (without excessive coelutions) and correct peak integration. The method will be used by the authors for identification of non-volatile nitroso compounds in beer and malt and the results will be published in the future. A practical impact of the presented method is in a valuable contribution to the identification tasks of nitroso compounds in complex samples. It could be also beneficial in research of the presence and identification of this type of compounds in many types of products. Together with possible toxicological studies of nitroso compounds identified by the method in the future, it could significantly contribute to uncovering the impact of non-volatile nitroso compounds exposure to human health resulting from their occurrence in food and other products.

Author contributions

All authors have given the approval to the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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Supporting information

for publication

Pyrolytic Profiling Nitrosamine Specific Chemiluminescence Detection Combined with Multivariate Chemometric Discrimination for Non-targeted Detection and Classification of Nitroso Compounds in Complex Samples

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Definition of parameters [18] from Table 2 and S-2 and S-3:

Accuracy – represents the percentage of correctly assigned objects, it is also called *classification rate* or *non-error rate;*

Error rate – represents percentace of wrongly assigned objects; also defined as *1 – accuracy;*

Random error rate – represents the error obtained with a random assignation of objects to one of the defined classes;

Sensitivity – the model ability to correctly recognize objects belonging to the given group; *sensitivity* is equal to 1, if all objects from given group are correctly classified;

Specificity – the ability of the given group to reject the objects of all the other groups; *specificity* is equal to 1, if none of objects from other groups are classified to given group;

Precision – the capability of a model not to classify objects of other groups in the given group; *precision* is equal to 1, if all the objects classified to given group are correctly classified.

Calculation of these parameters were performed according to Ballabio D., Todeschini R. Multivariate Classification for Qualitative Analysis. In Infrared Spectroscopy for Food Quality Analysis and Control. Sun D.-W. Amsterdam, Elsevier 2009, 83–104.

Retention time shift between GC-NCD and GC-MS/MS:

Before the analysis it was necessary to transfer the chromatographic method from GC-NCD to GC-MS/MS in order to obtain comparable retention times of each peak. Since both

methods use different carrier gas, the helium velocity in GC-MS/MS was necessary to be set up at the same value as in GC-NCD. Carrier gas velocity of argon (GC-NCD) and the corresponding helium flow (GC-MS/MS) were calculated from experimental conditions at GC-NCD by Agilent Technologies Method Translator, see section Instrumentation. The translated GC-MS/MS method was compared to the GC-NCD method by retention times of standard solution of N-nitrosopiperidine and trimethylsilylated N-nitrososarcosine, N-nitrosoproline, and N-nitrosohydroxyproline (each 500 µg/L). Retention times between the methods (paired t-test $p = 0.008$) differed significantly. The relation of retention times from both methods showed a linear function, Figure S8. Although carrier gas velocity was calculated for both methods, the retention differs. It can be explained by different hardware equipment on the output from the chromatographic column; the NCD has additional tubing and pyrolytic tube in comparison with the MS detector (these parts were not included in the calculation during the method translation due to unknown diameters and indefinable temperature of tubing). Therefore, the flow and pressure gradient along the column were different between the methods, both methods resulted in different retention times. In spite of these discrepancies of retention times, the function from Figure S8 was used for further prediction of retention times of peaks at GC-MS/MS.

Table S-1: Parameters of MS/MS experiments

retention window / min	product ion	precursor ion	CIDa / eV
13.0 - 15.0	142		15
	127		15
	114		15
	99	99	15/15b
13.5 - 15.5	99		15
	142		15
13.6 - 15.6	156	156	15/15b
17.3 - 19.3	312		15
	297		15
	208		15
	179		15
	135		15
	163		15
5.5 - 30.0		neutral loss of 30	6c

a – colision energy for product ion mode; b – colision energy for precursor ion mode; c – colision energy for neutral loss mode

Table S-2: Properties of discriminant function *No. 1*

Constants of Classification Functions

	INT	C-NO	C-NO/NO2	N-NO	N-NO2
500	181,58	140,21	199,67	343,87	112,27
650^2	31,50	75,94	13,15	67,32	34,31
800^6	2,77	22,49	-5,87	-18,19	28,90
800/700	19,03	11,25	20,19	13,90	9,73
800/650	9,14	9,34	10,87	9,89	6,26
700/500	34,36	10,39	53,02	16,33	14,20
750/500	19,13	16,36	13,49	17,84	11,19
800^6/500	-1,08	-8,44	1,55	-3,99	-5,22
700^4/500	-28,71	-8,88	-40,29	-12,61	-11,29
750^6/500	-15,26	-2,16	-19,80	-4,75	-4,27
Constant	-160,06	-102,04	-228,30	-204,22	-58,78

Chi-Square Tests with Successive Roots Removed

roots removed	Eigenvalue	Canonical R	Wilks' Lambda	Chi-Sqr.	df	p-level
0	57,23	0,99	0,00	373,51	40	0,0000
1	18,07	0,97	0,01	192,65	27	0,0000
2	1,15	0,73	0,25	61,45	16	0,0000
3	0,85	0,68	0,54	27,35	7	0,0003

Standardized Coefficients for Canonical Variables

variable	Root 1	Root 2	Root 3	Root 4
500	0,773	1,240	-0,269	0,172
650^2	0,621	-0,607	-1,005	1,085
800^6	-0,272	-0,902	0,278	-0,279
800/700	-0,717	1,229	-1,485	0,509
800/650	0,091	0,217	-0,437	1,820
700/500	-3,193	4,236	1,996	4,111

750/500	-0,077	0,518	-3,768	-0,244
800^6/500	-0,424	1,168	0,295	-0,546
700^4/500	2,222	-2,632	-0,176	-1,934
750^6/500	1,432	-1,695	0,525	-0,234
Eigenvalue	57,229	18,072	1,152	0,849
Cum.Prop.	0,740	0,974	0,989	1,000

Squared Mahalanobis Distances

	INT	C-NO	C-NO/NO2	N-NO	N-NO2
INT					
C-NO	152,92				
C-NO/NO2	49,30	312,27			
N-NO	270,14	148,67	408,58		
N-NO2	105,44	40,37	226,07	203,72	

	sensitivity	specificity	precision	accuracy	error rate
C-NO	0,978	0,034	1,000		
N-NO	0,938	0,044	0,938		
INT	1,000	0,000	0,857		
C-NO/NO2	1,000	0,000	1,000		
N-NO2	0,667	0,030	0,667		
				0,971	0,029

Table S-3: Properties of discriminant function *No. 2*

Constants of Classification Functions

	INT	C-NO	C-NO/NO2	N-NO	N-NO2
500	15,23	24,22	28,64	149,14	33,52
700	-529,41	-431,53	-704,91	-473,99	-465,13
700/750	505,00	443,48	683,98	483,91	467,00
750	308,80	268,92	396,91	284,28	289,19
800	20,66	16,18	22,64	7,88	20,31
650	-35,24	-48,36	-42,81	-45,70	-49,20
650/700	22,48	33,14	23,90	27,19	31,69
constant	-166,38	-148,22	-287,11	-216,32	-162,71

Chi-Square Tests with Successive Roots Removed

roots removed	Eigenvalue	Canonical R	Wilks' Lambda	Chi-Sqr.	df	p-level
0	30,48	0,98	0,00	258,82	28	0,0000
1	3,04	0,87	0,11	100,14	18	0,0000
2	1,04	0,71	0,46	35,89	10	0,0001
3	0,07	0,26	0,93	3,11	4	0,5400

Standardized Coefficients for Canonical Variables

variable	Root 1	Root 2	Root 3	Root 4
500	-11,618	3,211	0,672	-1,702
700	-3,611	-31,627	8,069	0,920
700/750	1,452	25,245	-14,874	-1,614
750	1,763	13,909	-5,901	-5,399
800	1,053	0,632	0,152	-2,562
650	0,555	1,987	3,511	3,695
650/700	-0,147	-2,042	-2,255	-1,630
Eigenval	30,480	3,042	1,040	0,070
Cum.Prop	0,880	0,968	0,998	1,000

Squared Mahalanobis Distances

	INT	C-NO	C-NO/NO2	N-NO	N-NO2
INT					
C-NO	17,03				
C-NO/NO2	40,35	70,55			
N-NO	138,59	106,86	172,79		
N-NO2	11,49	3,71 *	59,54	98,06	

- - statistically insignificant distance

Table S-4: Classification of the test mixture standards in a real sample by model No. 1

indication	analyte	classification	posterior probabilities				
			INT	C-NO	C-NO/NO_2	N-NO	N-NO_2
INT1	D-glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)	INT	0.50	0.02	0.00	0.00	0.48
N1	N-Nitrososarcosine, trimethylsilylester	N-NO	0.00	0.00	0.00	1.00	0.00
N2	N-Nitrosoproline, trimethylsilylester	N-NO	0.00	0.00	0.00	1.00	0.00
INT2	gramine, N-(trimethylsilyl)	INT	1.00	0.00	0.00	0.00	0.00
C1	nitrosobenzene	INT *	1.00	0.00	0.00	0.00	0.00
C2	N,N-dimethyl-4-nitrosoaniline	INT *	1.00	0.00	0.00	0.00	0.00
N-NO2	N-Nitropyrrolidine	N-NO_2	0.01	0.11	0.00	0.00	0.88

Misclassifications are highlighted by asterisks.

Table S-5: Classification of the original and bacterially contaminated beer samples (classification by model *No.2*)

original beer							*Obesumbacterium proteus contamination*						
posterior probabilities							posterior probabilities						
rt / min	INT	C-NO	C-NO/NO2	N-NO	N-NO2	class.	rt / min	INT	C-NO	C-NO/NO2	N-NO	N-NO2	class.
6,36	1,00	0,00	0,00	0,00	0,00	INT	6,42	1,00	0,00	0,00	0,00	0,00	INT
6,64	1,00	0,00	0,00	0,00	0,00	INT							
							6,88	0,00	0,71	0,00	0,00	0,29	C-NO
7,47	1,00	0,00	0,00	0,00	0,00	INT	7,51	1,00	0,00	0,00	0,00	0,00	INT
8,27	1,00	0,00	0,00	0,00	0,00	INT							
							8,58	1,00	0,00	0,00	0,00	0,00	INT
8,67	1,00	0,00	0,00	0,00	0,00	INT	8,68	1,00	0,00	0,00	0,00	0,00	INT
9,03	0,00	0,00	1,00	0,00	0,00	C-NO/NO2	9,03	0,00	0,00	1,00	0,00	0,00	C-NO/NO2
							9,21	1,00	0,00	0,00	0,00	0,00	INT
9,49	1,00	0,00	0,00	0,00	0,00	INT							
9,81	1,00	0,00	0,00	0,00	0,00	INT							
11,63	1,00	0,00	0,00	0,00	0,00	INT							
11,87	1,00	0,00	0,00	0,00	0,00	INT	11,84	1,00	0,00	0,00	0,00	0,00	INT
12,06	1,00	0,00	0,00	0,00	0,00	INT	12,06	1,00	0,00	0,00	0,00	0,00	INT
12,40	1,00	0,00	0,00	0,00	0,00	INT	12,45	1,00	0,00	0,00	0,00	0,00	INT

12,53	0,00	0,00	1,00	0,00	0,00	C-NO/NO2	12,54	1,00	0,00	0,00	0,00	0,00	INT
12,80	1,00	0,00	0,00	0,00	0,00	INT							
13,00	0,71	0,00	0,29	0,00	0,00	INT	13,01	1,00	0,00	0,00	0,00	0,00	INT
13,47	1,00	0,00	0,00	0,00	0,00	INT	13,49	1,00	0,00	0,00	0,00	0,00	INT
13,69	1,00	0,00	0,00	0,00	0,00	INT	13,68	1,00	0,00	0,00	0,00	0,00	INT
13,80	1,00	0,00	0,00	0,00	0,00	INT	13,79	1,00	0,00	0,00	0,00	0,00	INT
14,04	0,88	0,00	0,12	0,00	0,00	INT	14,05	0,98	0,00	0,02	0,00	0,00	INT
14,19	1,00	0,00	0,00	0,00	0,00	INT	14,21	1,00	0,00	0,00	0,00	0,00	INT
14,37	1,00	0,00	0,00	0,00	0,00	INT	14,30	1,00	0,00	0,00	0,00	0,00	INT
							14,56	1,00	0,00	0,00	0,00	0,00	INT
14,67	0,00	0,00	1,00	0,00	0,00	C-NO/NO2	14,67	0,24	0,00	0,76	0,00	0,00	C-NO/NO2
							14,83	1,00	0,00	0,00	0,00	0,00	INT
							14,88	1,00	0,00	0,00	0,00	0,00	INT
							15,00	1,00	0,00	0,00	0,00	0,00	INT
15,17	1,00	0,00	0,00	0,00	0,00	INT	15,16	1,00	0,00	0,00	0,00	0,00	INT
15,24	0,04	0,00	0,96	0,00	0,00	C-NO/NO2	15,23	0,49	0,00	0,51	0,00	0,00	C-NO/NO2
15,37	1,00	0,00	0,00	0,00	0,00	INT							
							15,47	0,00	1,00	0,00	0,00	0,00	C-NO
15,68	1,00	0,00	0,00	0,00	0,00	INT	15,70	1,00	0,00	0,00	0,00	0,00	INT
15,82	1,00	0,00	0,00	0,00	0,00	INT							

15,97	1,00	0,00	0,00	0,00	0,00	INT							
16,08	0,89	0,00	0,11	0,00	0,00	INT	16,09	0,00	0,00	1,00	0,00	0,00	C-NO/NO2
16,17	1,00	0,00	0,00	0,00	0,00	INT	16,19	1,00	0,00	0,00	0,00	0,00	INT
16,35	0,01	0,00	0,99	0,00	0,00	C-NO/NO2	16,37	0,11	0,00	0,89	0,00	0,00	C-NO/NO2
16,76	0,00	0,00	1,00	0,00	0,00	C-NO/NO2	16,78	1,00	0,00	0,00	0,00	0,00	INT
16,98	1,00	0,00	0,00	0,00	0,00	INT	16,99	1,00	0,00	0,00	0,00	0,00	INT
							17,14	1,00	0,00	0,00	0,00	0,00	INT
17,25	0,03	0,00	0,97	0,00	0,00	C-NO/NO2	17,27	0,07	0,00	0,93	0,00	0,00	C-NO/NO2
17,48	1,00	0,00	0,00	0,00	0,00	INT	17,50	1,00	0,00	0,00	0,00	0,00	INT
17,59	0,41	0,00	0,59	0,00	0,00	C-NO/NO2	17,60	1,00	0,00	0,00	0,00	0,00	INT
17,64	0,00	0,00	0,00	0,00	1,00	N-NO2	17,66	0,00	0,11	0,00	0,00	0,89	N-NO2
17,97	1,00	0,00	0,00	0,00	0,00	INT							
18,29	1,00	0,00	0,00	0,00	0,00	INT	18,29	1,00	0,00	0,00	0,00	0,00	INT
18,45	1,00	0,00	0,00	0,00	0,00	INT	18,45	0,00	1,00	0,00	0,00	0,00	C-NO
18,73	1,00	0,00	0,00	0,00	0,00	INT	18,73	1,00	0,00	0,00	0,00	0,00	INT
19,04	1,00	0,00	0,00	0,00	0,00	INT	19,06	1,00	0,00	0,00	0,00	0,00	INT
19,43	0,01	0,00	0,99	0,00	0,00	C-NO/NO2	19,42	1,00	0,00	0,00	0,00	0,00	INT
19,53	1,00	0,00	0,00	0,00	0,00	INT	19,55	1,00	0,00	0,00	0,00	0,00	INT
19,74	1,00	0,00	0,00	0,00	0,00	INT	19,74	1,00	0,00	0,00	0,00	0,00	INT
20,39	1,00	0,00	0,00	0,00	0,00	INT	20,40	1,00	0,00	0,00	0,00	0,00	INT

20,62	1,00	0,00	0,00	0,00	0,00	INT	20,63	1,00	0,00	0,00	0,00	0,00	INT
21,20	1,00	0,00	0,00	0,00	0,00	INT	21,19	1,00	0,00	0,00	0,00	0,00	INT
21,56	1,00	0,00	0,00	0,00	0,00	INT	21,59	1,00	0,00	0,00	0,00	0,00	INT
22,31	1,00	0,00	0,00	0,00	0,00	INT	22,31	1,00	0,00	0,00	0,00	0,00	INT
23,59	1,00	0,00	0,00	0,00	0,00	INT							
23,72	0,00	1,00	0,00	0,00	0,00	C-NO	23,73	0,09	0,91	0,00	0,00	0,00	C-NO
							24,14	0,00	0,00	1,00	0,00	0,00	C-NO/NO2
25,17	1,00	0,00	0,00	0,00	0,00	INT							
							25,51	0,00	1,00	0,00	0,00	0,00	C-NO
							25,67	0,00	1,00	0,00	0,00	0,00	C-NO
							25,79	1,00	0,00	0,00	0,00	0,00	INT
							25,96	1,00	0,00	0,00	0,00	0,00	INT
26,16	0,22	0,00	0,78	0,00	0,00	C-NO/NO2	26,15	0,00	1,00	0,00	0,00	0,00	C-NO
26,41	0,48	0,00	0,52	0,00	0,00	C-NO/NO2	26,41	1,00	0,00	0,00	0,00	0,00	INT
26,60	1,00	0,00	0,00	0,00	0,00	INT	26,58	0,00	1,00	0,00	0,00	0,00	C-NO
							26,72	0,00	1,00	0,00	0,00	0,00	C-NO
26,75	1,00	0,00	0,00	0,00	0,00	INT							
							26,87	0,00	1,00	0,00	0,00	0,00	C-NO
27,04	0,88	0,00	0,12	0,00	0,00	INT	27,06	0,00	1,00	0,00	0,00	0,00	C-NO
27,22	1,00	0,00	0,00	0,00	0,00	INT							

							27,40	0,00	1,00	0,00	0,00	0,00	C-NO
							27,74	0,00	1,00	0,00	0,00	0,00	C-NO
27,82	1,00	0,00	0,00	0,00	0,00	INT	27,82	0,00	0,99	0,00	0,00	0,01	C-NO
28,06	1,00	0,00	0,00	0,00	0,00	INT							
							28,16	0,00	1,00	0,00	0,00	0,00	C-NO
28,27	1,00	0,00	0,00	0,00	0,00	INT	28,24	0,00	1,00	0,00	0,00	0,00	C-NO

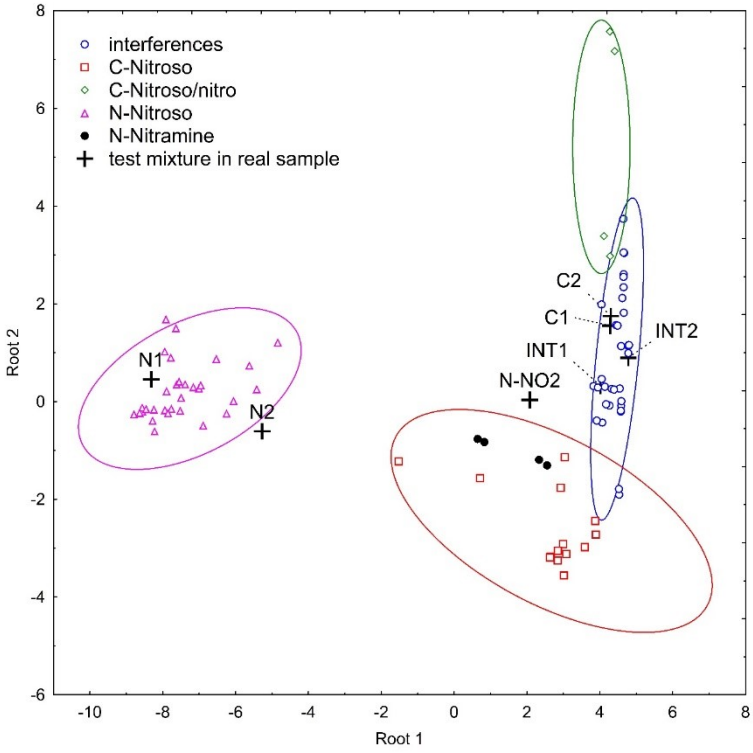


Figure S-1 Canonical variables of discriminant function of model *No. 1*

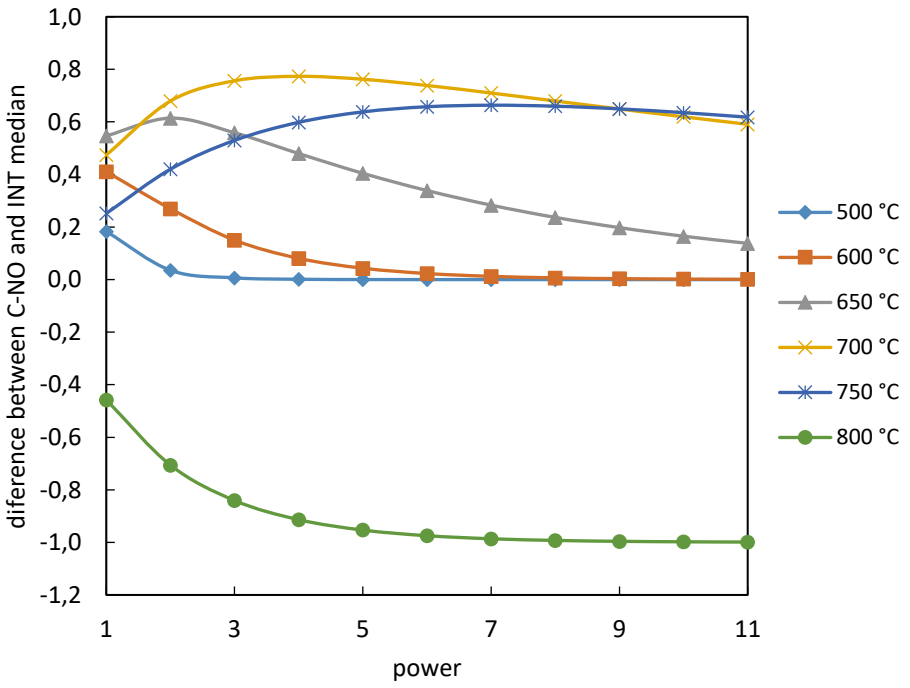


Figure S-2 Dependency of the difference between C-nitroso and interference group median on a power function

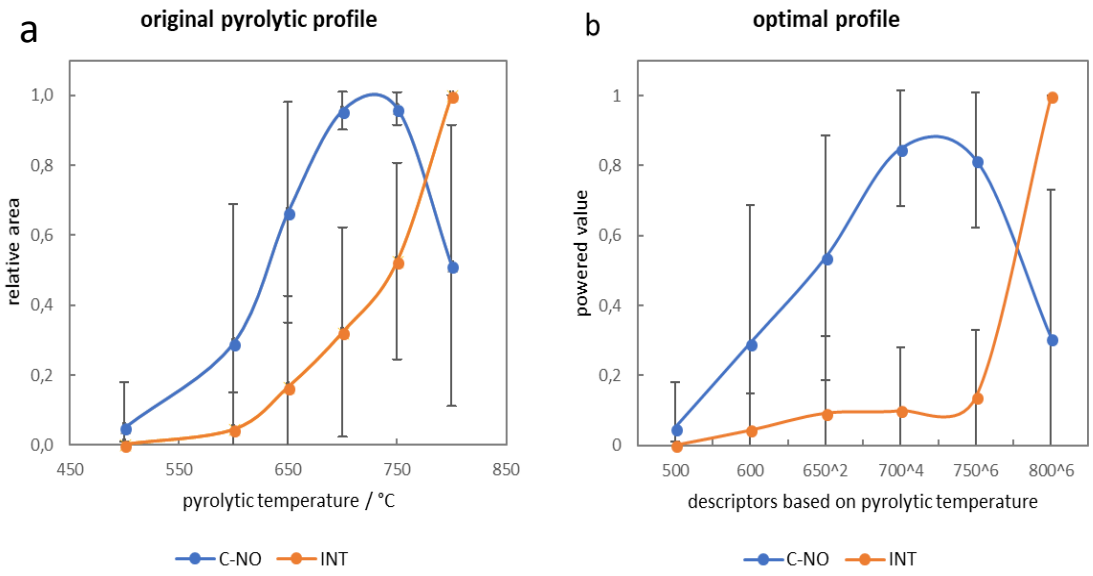


Figure S-3: Comparison of pyrolytic profiles of C-nitroso and interference group a) before and b) after power functions

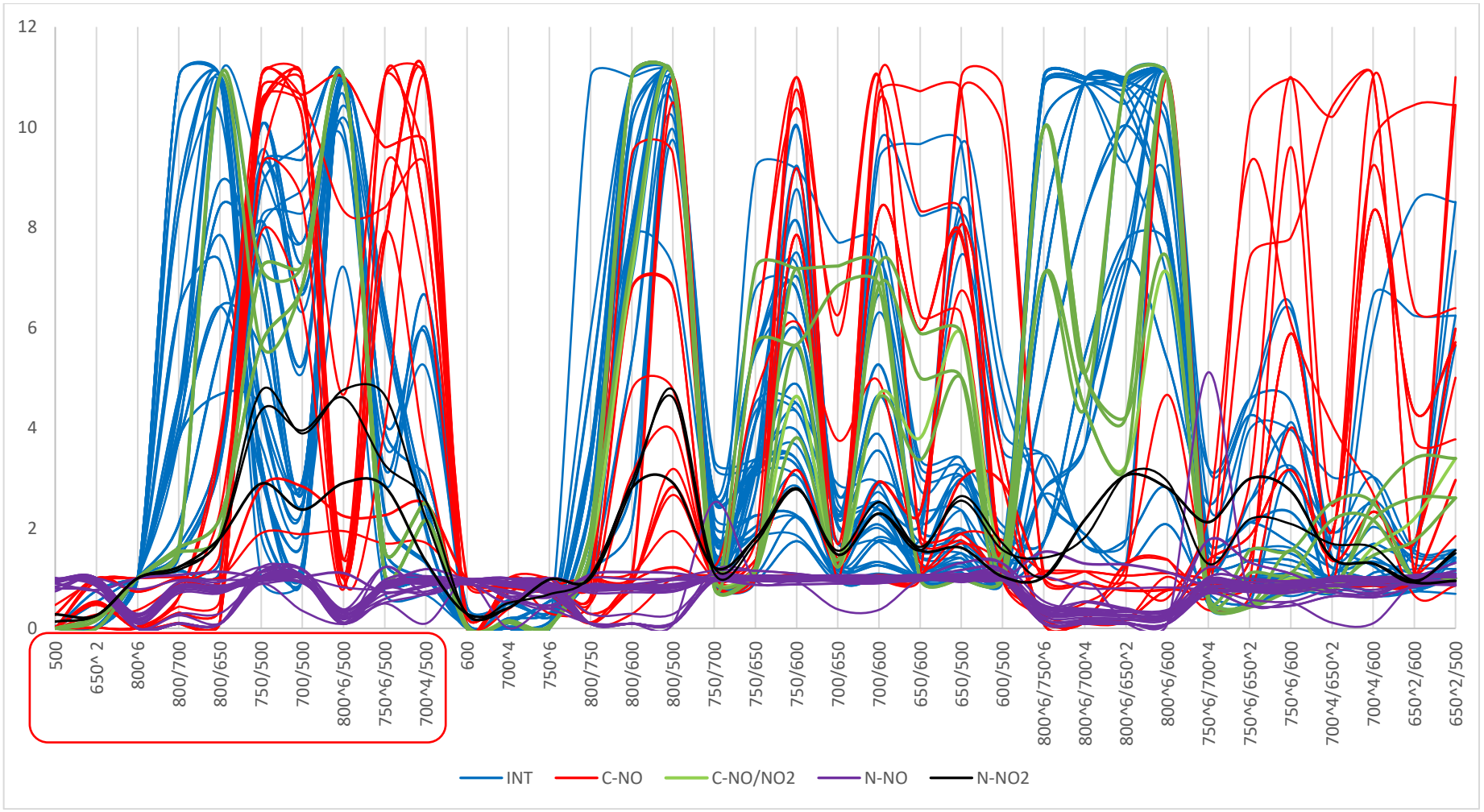


Figure S-4: Pyrolytic paterns of whole data set (test and training set). Variables at x-axis are relative responses at given temperature and their ratios (selected variables for model No. 2 are highlighted by red frame).

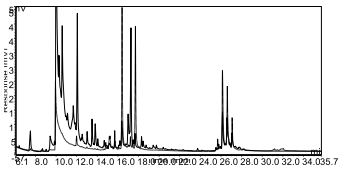


Figure S-5: GC-NCD chromatogram of bacterially contaminated (*O. proteus*) beer sample (full line) and original beer sample (dasched line). Chromatograms are recorded at pyrolytic temperature of 750 °C.

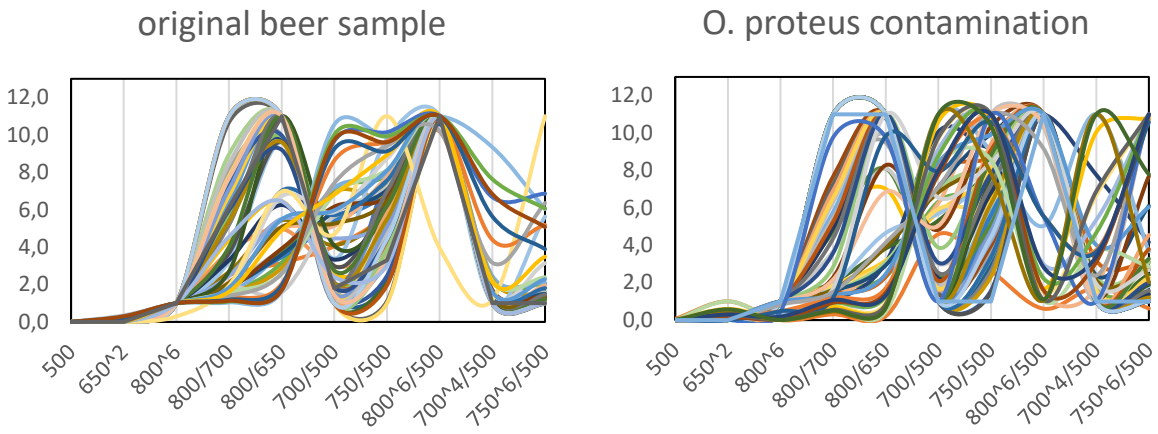


Figure S-6: Pyrolytic patterns of original and O. proteus contaminated beer sample

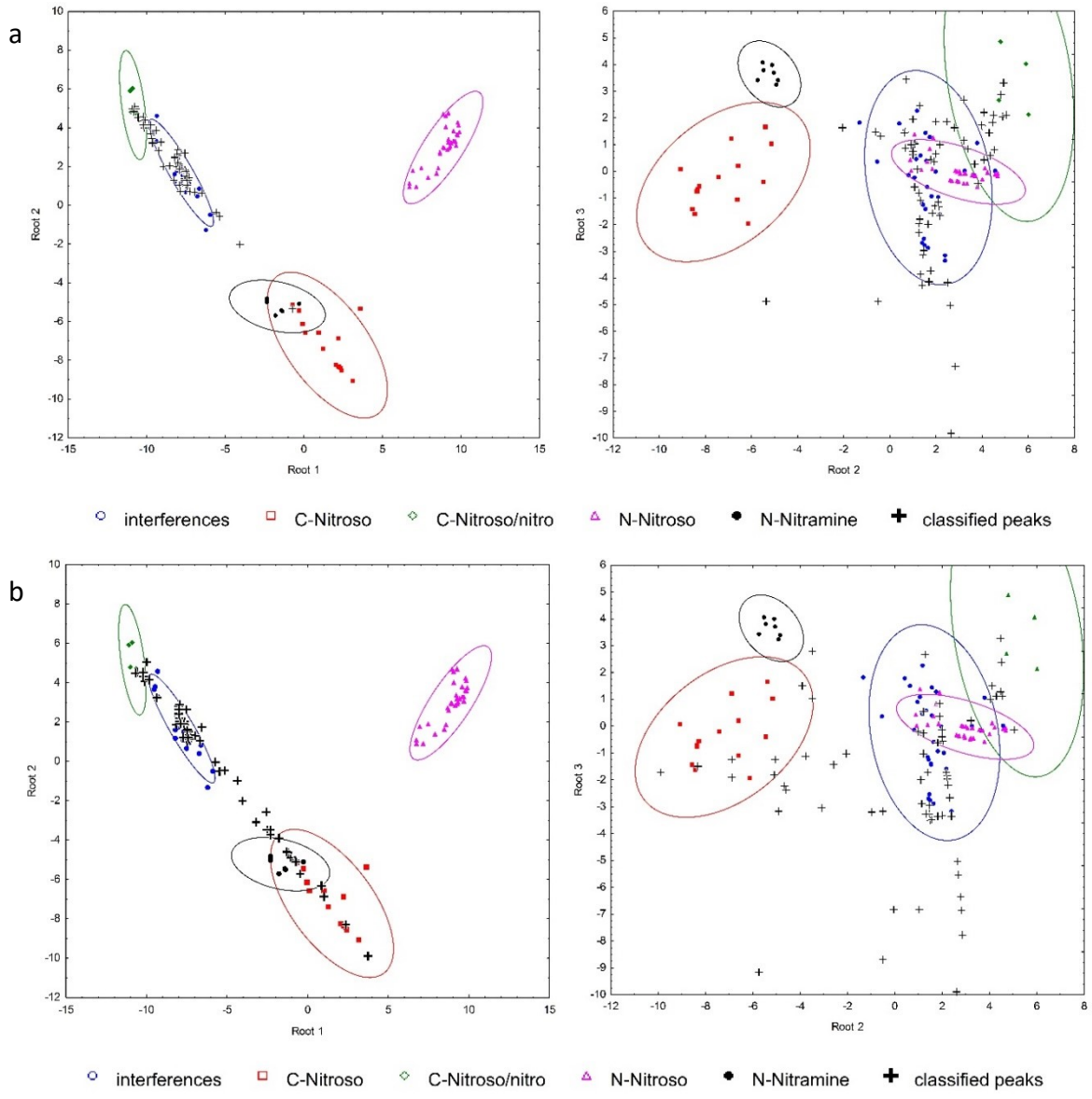


Figure S-7: CDA of a) original and b) O. proteus contaminated beer sample

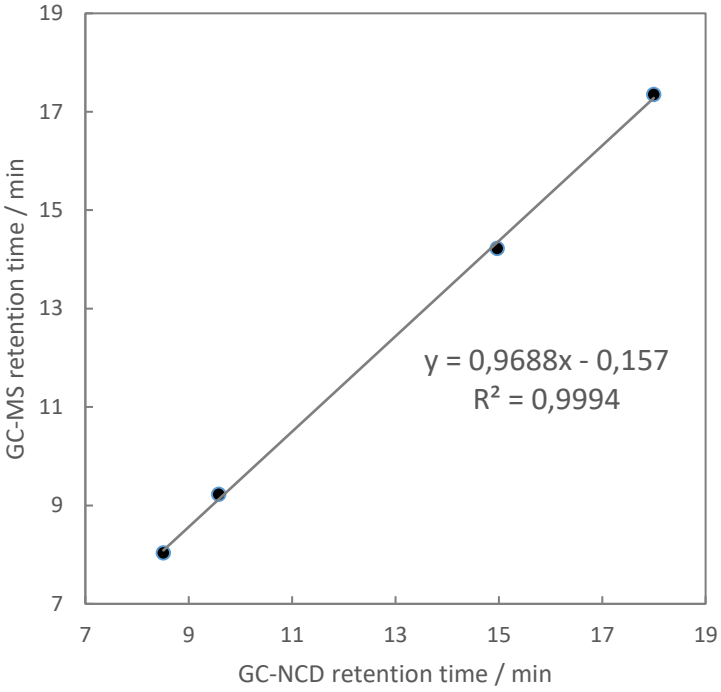


Figure S-8: Relationship between retention times on GC-NCD and GC-MS/MS

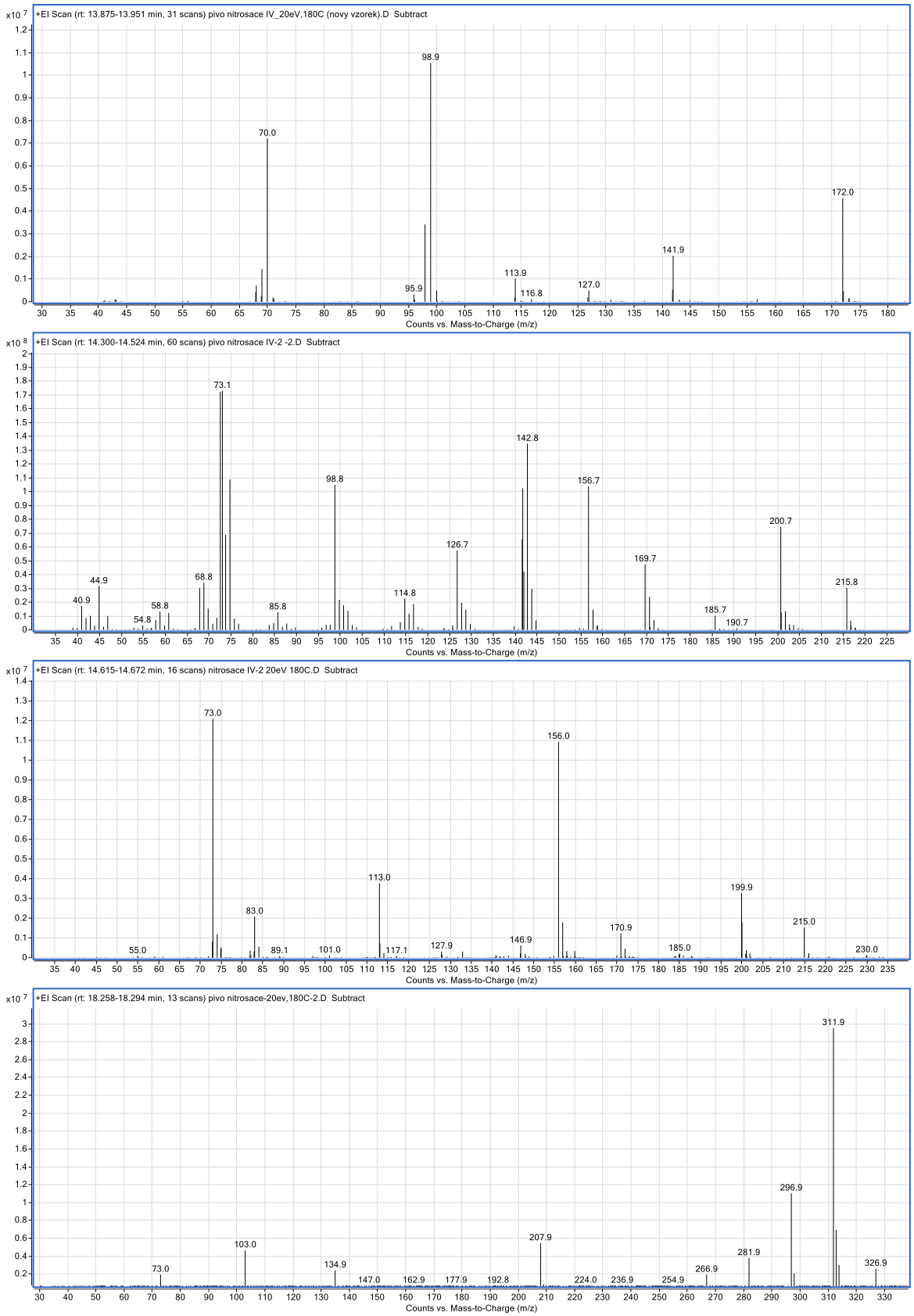


Figure S-9: MS1 full scan mass spectra of the selected peaks. Electron ionization was performed at 20 eV and 180 °C, with exception of peak at 14.5 min (70 eV, 280 °C)

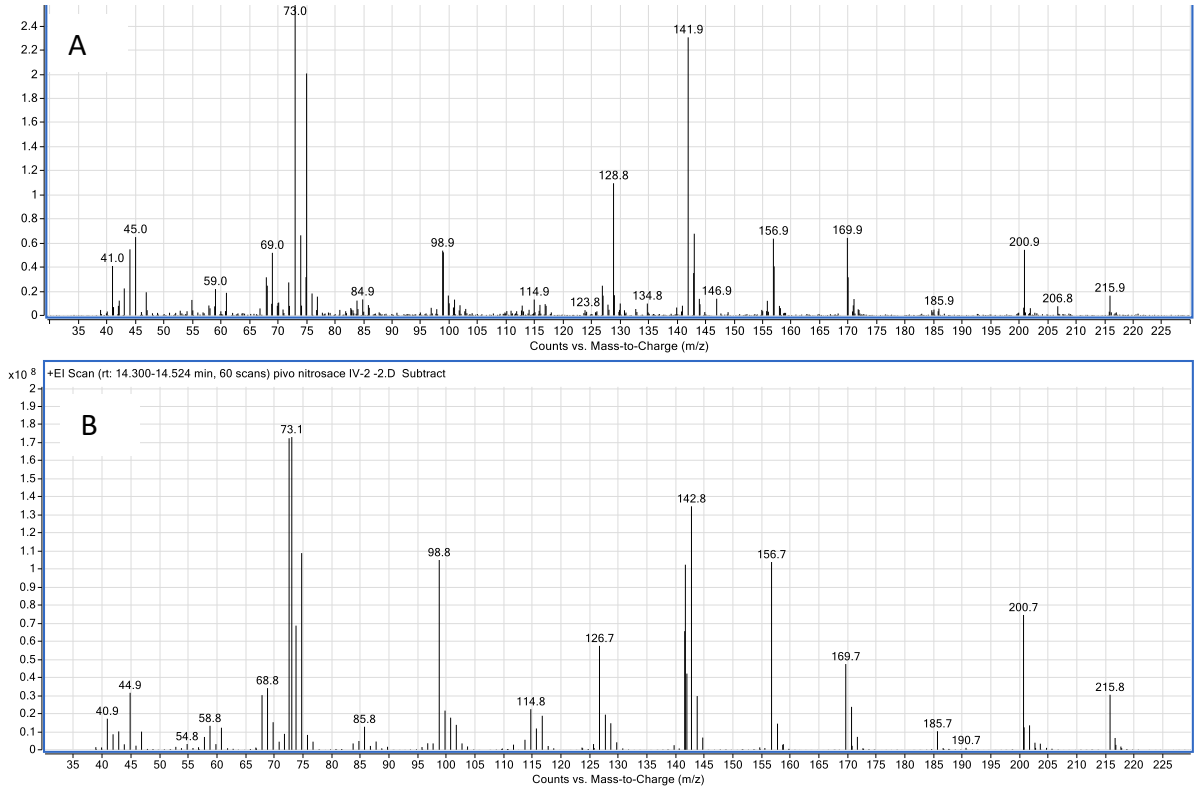


Figure S-10: Comparison of mass spectra (A) N-nitrosoproline trimethylsilylester and (B) unknown peak at 14.4 min

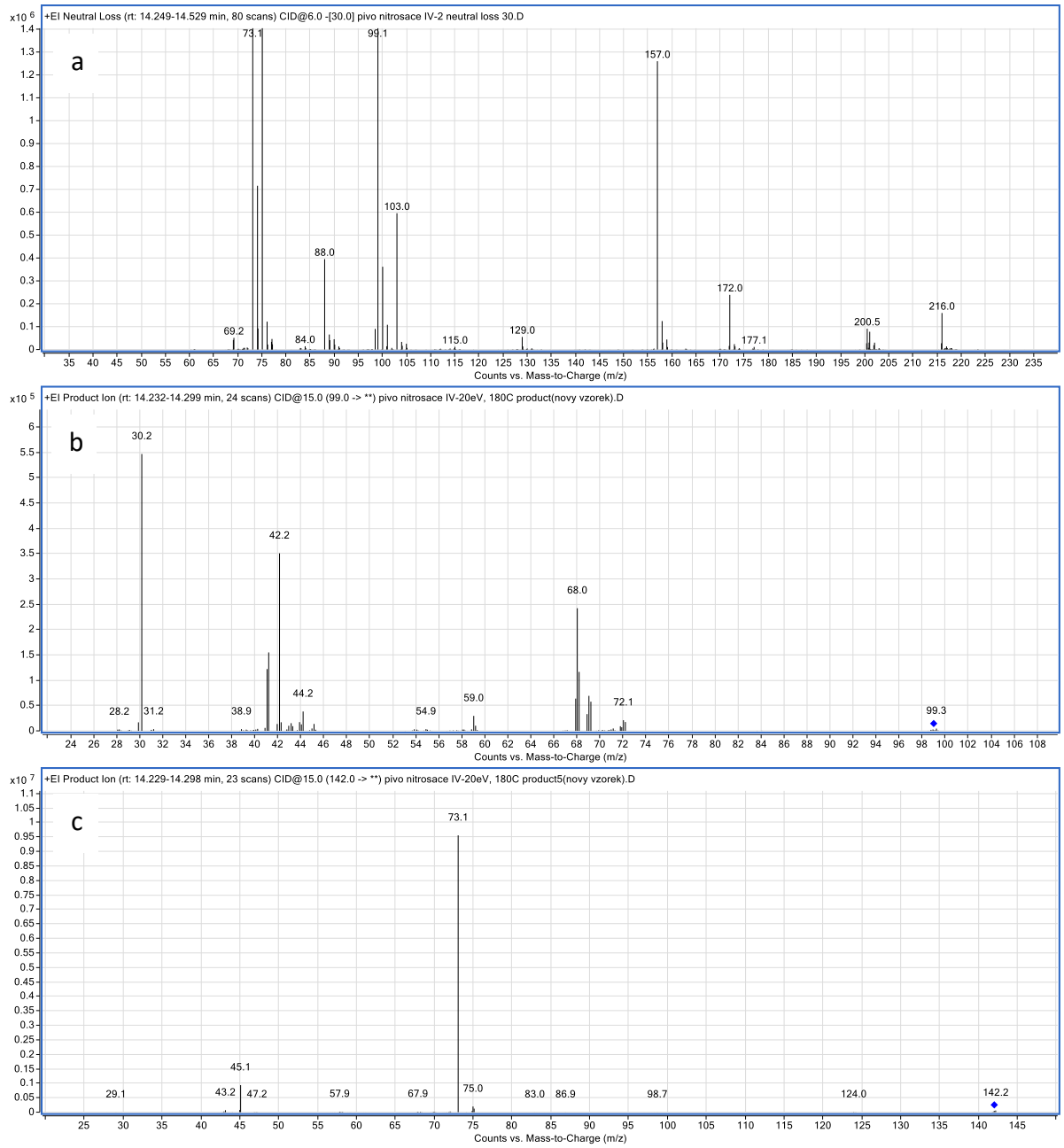


Figure S-11: CID based mass spectra of peak at 14.4 min: a) neutral loss of 30 amu; product ion mass spectra of b) m/z 99 and c) m/z 142.

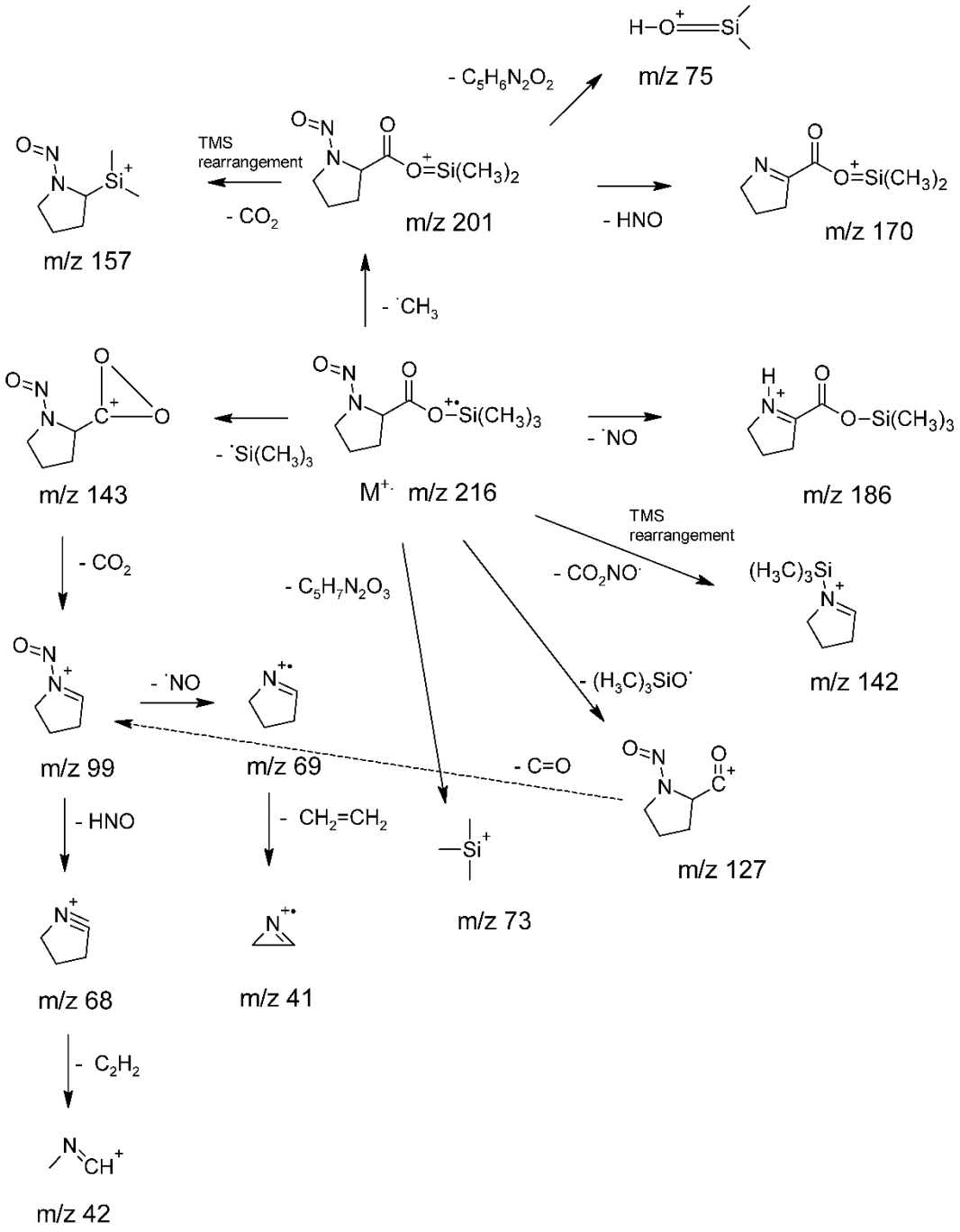


Figure S12: Mass fragmentation of N-nitrosoproline trimethylsilyl ester

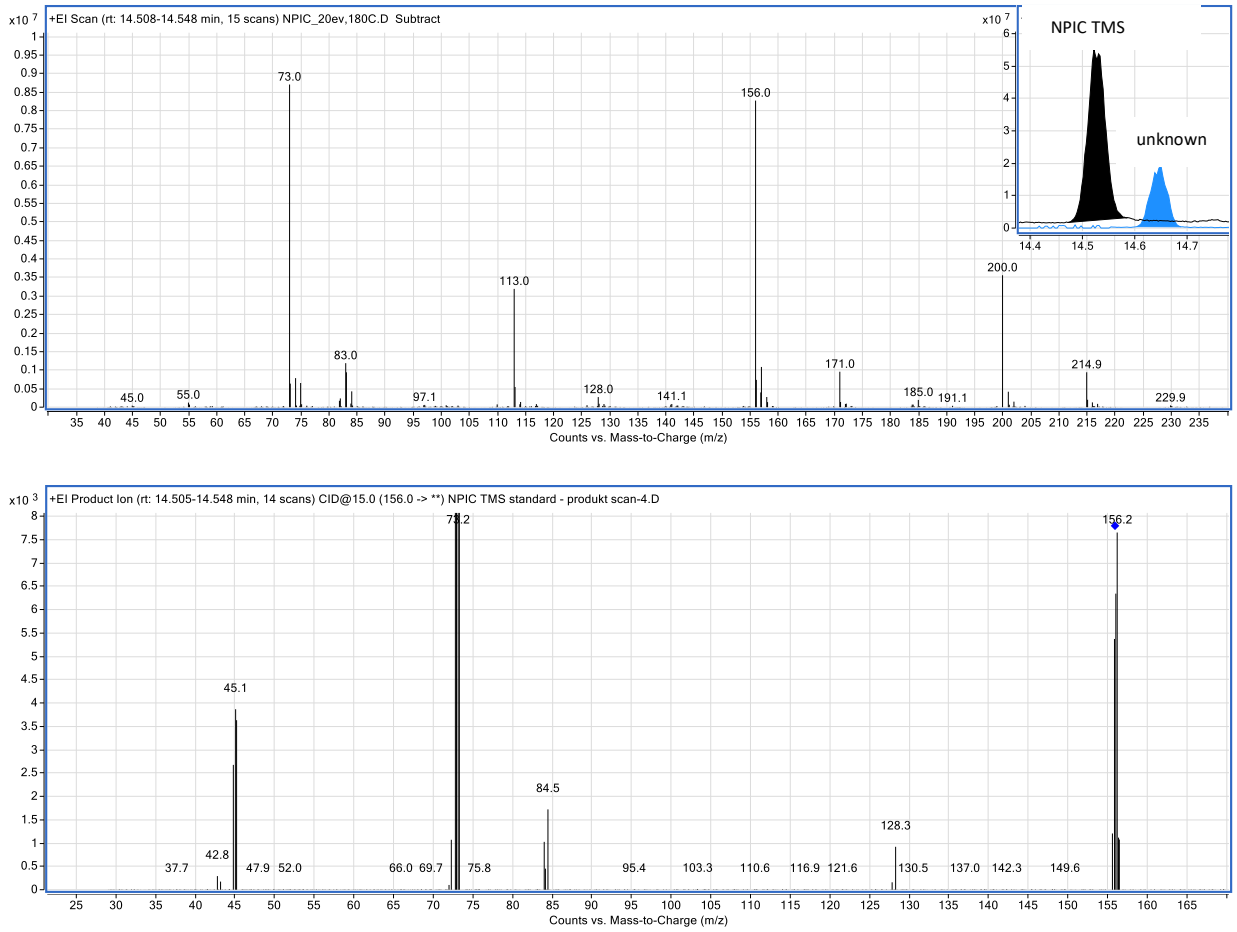


Figure S-13: Mass spectrum of N-nitrosopipecolic acid thimethylsilylester (NPIC TMS) standard and its product ion scan from m/z 156. Retention time comparison of NPIC TMS and unknown peak at 14.6 min is highlighted.

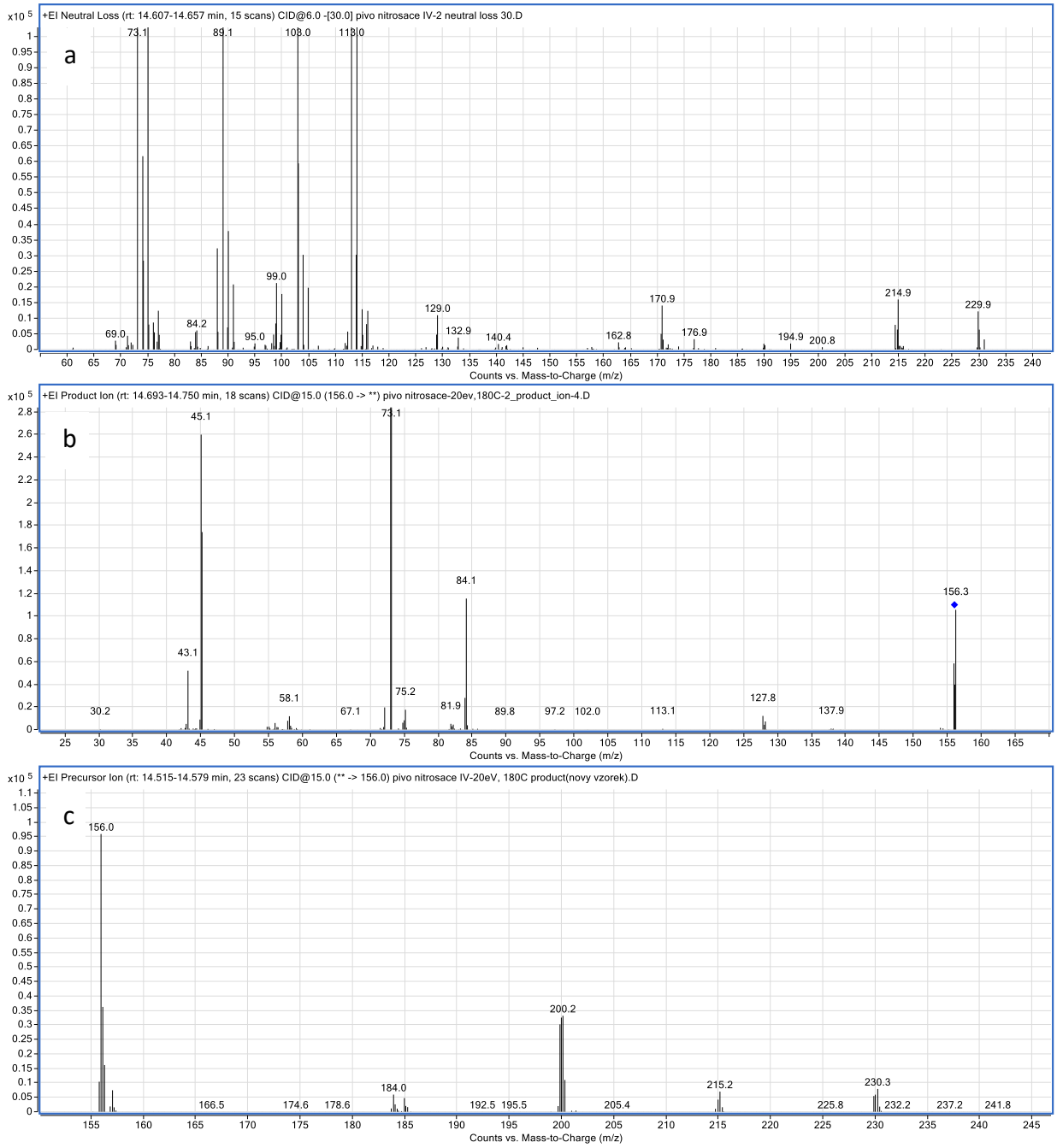
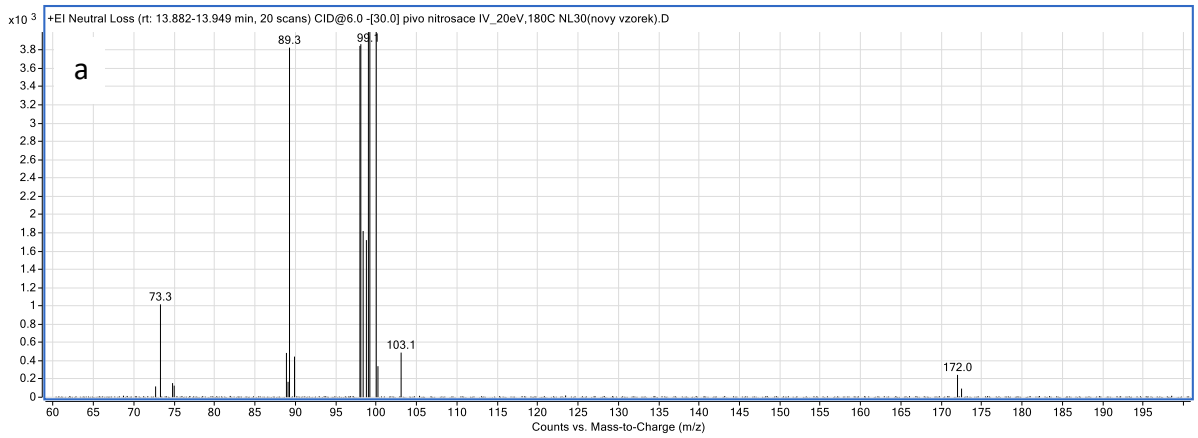
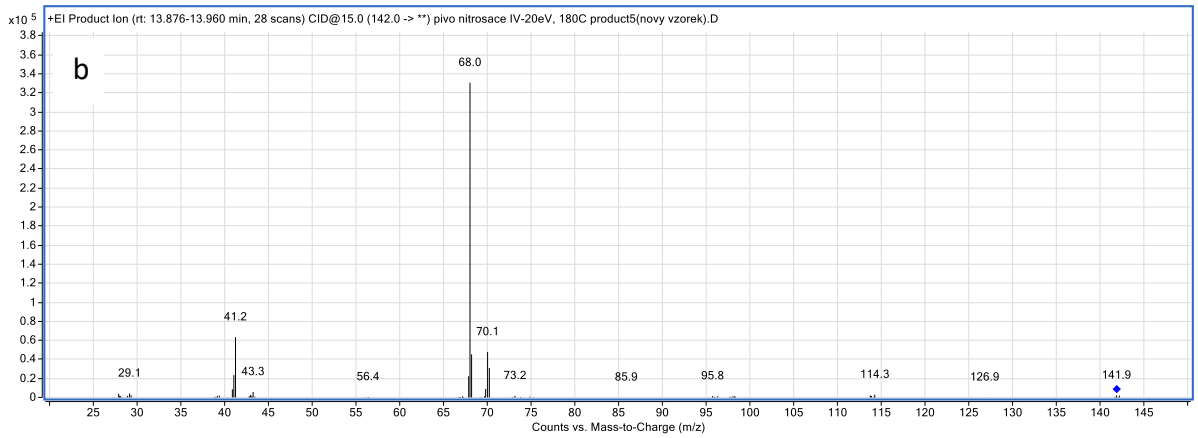
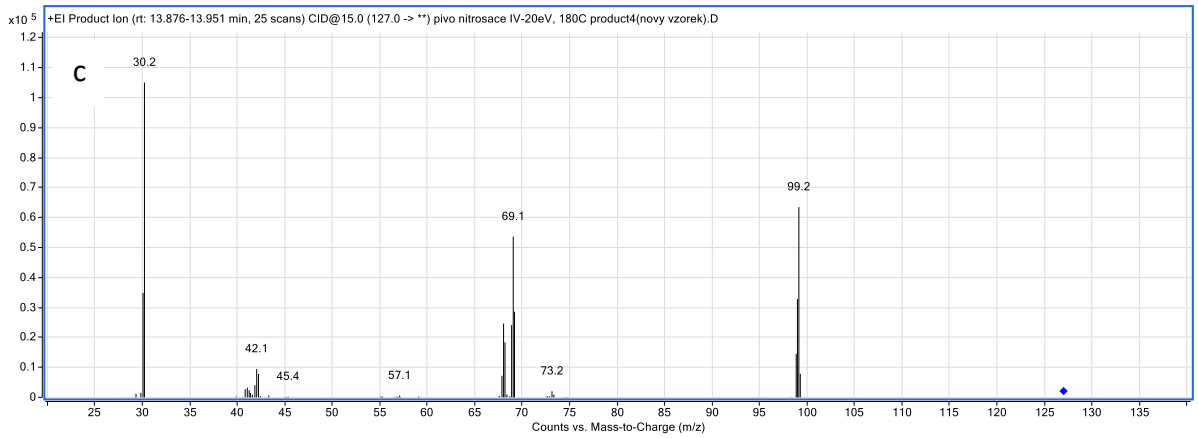


Figure S-13x. CID based mass spectra of peak at 14.6 min: a) neutral loss of 30 amu; b) product ion mass spectrum of m/z 156 and c) precursor ion mass spectrum of m/z 156.


x10 3
+EI Neutral Loss (rt: 13.882-13.949 min, 20 scans) CID@6.0 -[30.0] pivo nitrosace IV_20eV,180C NL30(novy vzorek).D
a
89.3
99.1
73.3
103.1
172.0
Counts vs. Mass-to-Charge (m/z)


x10 5
+EI Product Ion (rt: 13.876-13.960 min, 28 scans) CID@15.0 (142.0 -> **) pivo nitrosace IV-20eV, 180C product5(novy vzorek).D
b
68.0
41.2
70.1
29.1
43.3
56.4
73.2
85.9
95.8
114.3
126.9
141.9
Counts vs. Mass-to-Charge (m/z)


x10 5
+EI Product Ion (rt: 13.876-13.951 min, 25 scans) CID@15.0 (127.0 -> **) pivo nitrosace IV-20eV, 180C product4(novy vzorek).D
c
30.2
99.2
69.1
42.1
45.4
57.1
73.2
Counts vs. Mass-to-Charge (m/z)

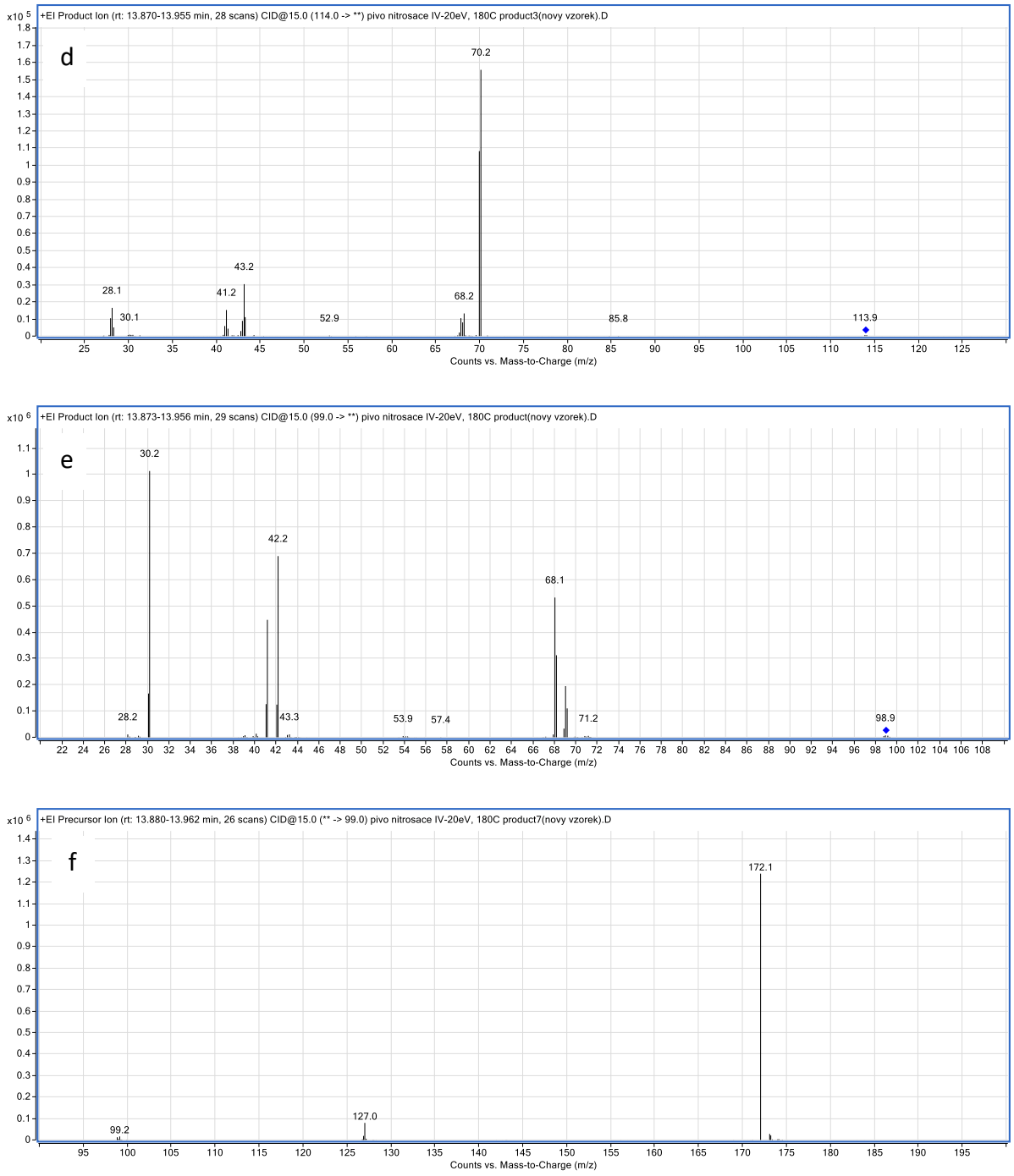


Figure S-14: CID based mass spectra of peak at 14.0 min: a) neutral loss of 30 amu; product ion mass spectra of b) m/z 142, c) m/z 127, d) m/z 114, e) m/z 99 and f) precursor ion mass spectrum of m/z 99.

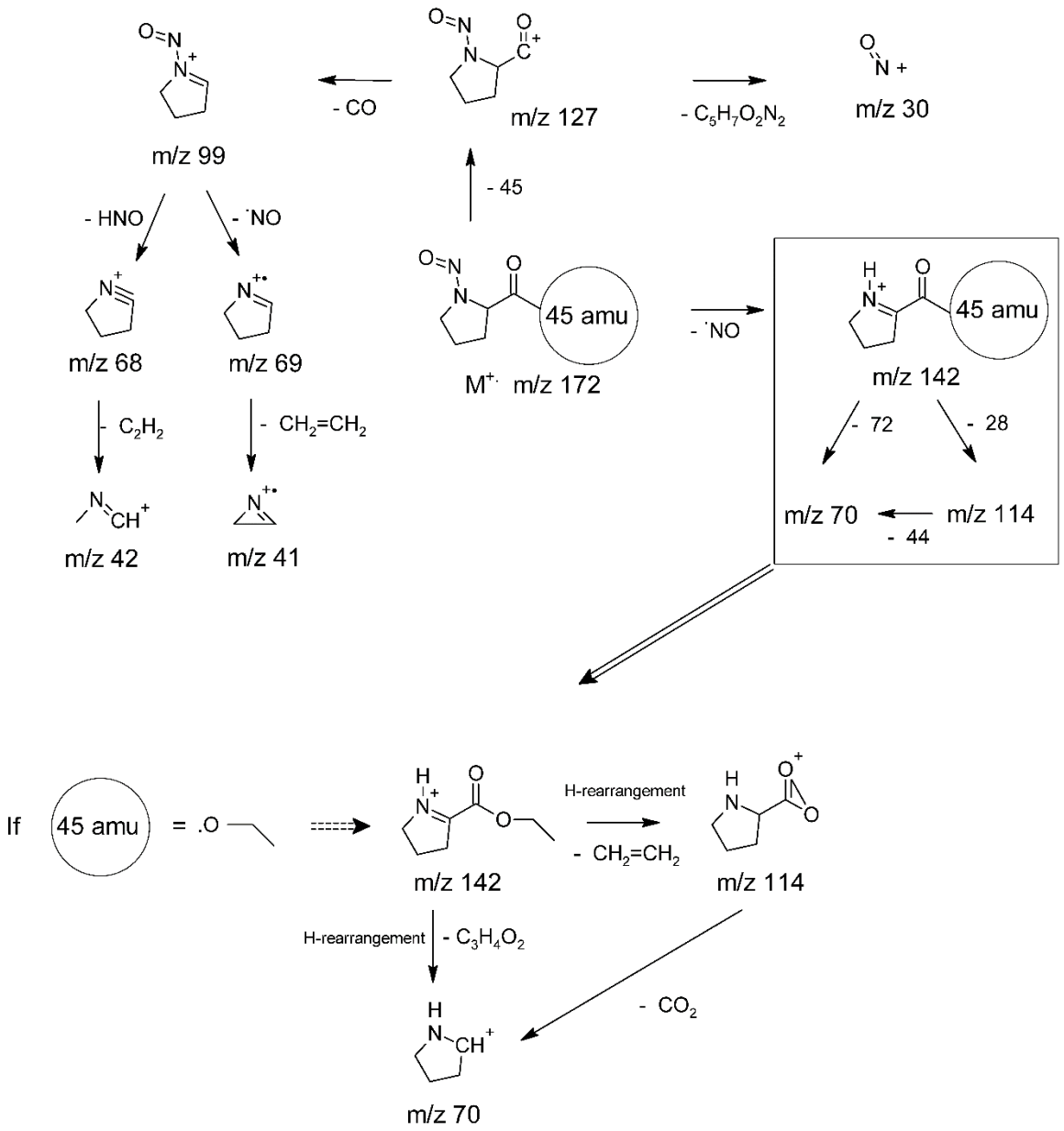
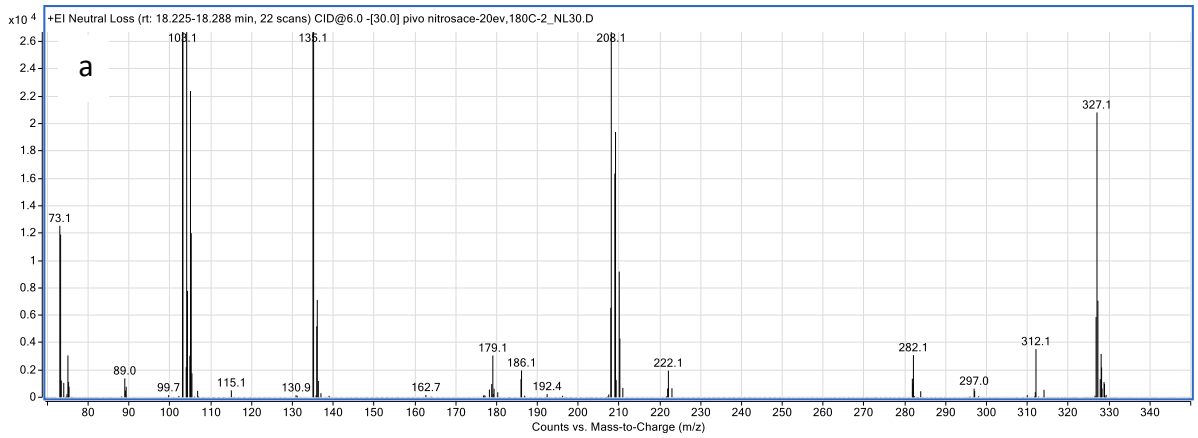
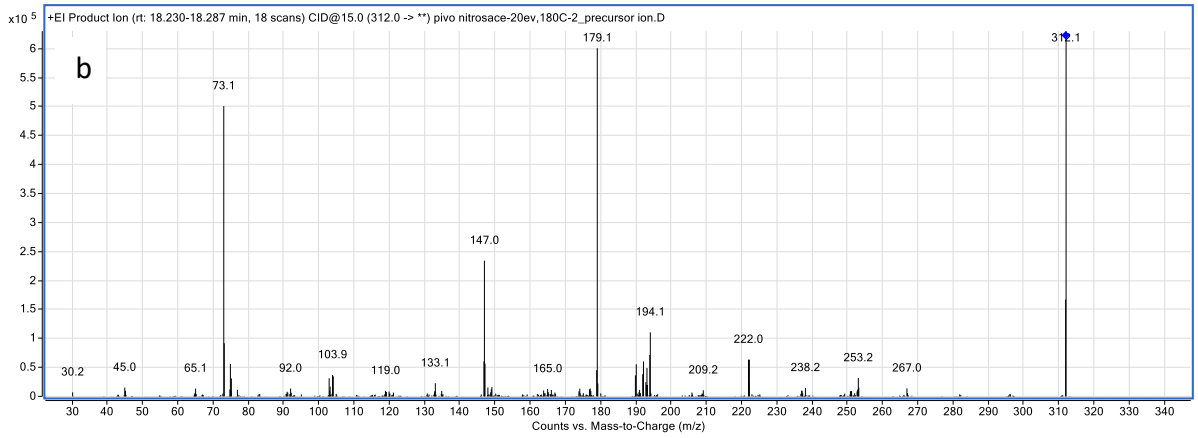
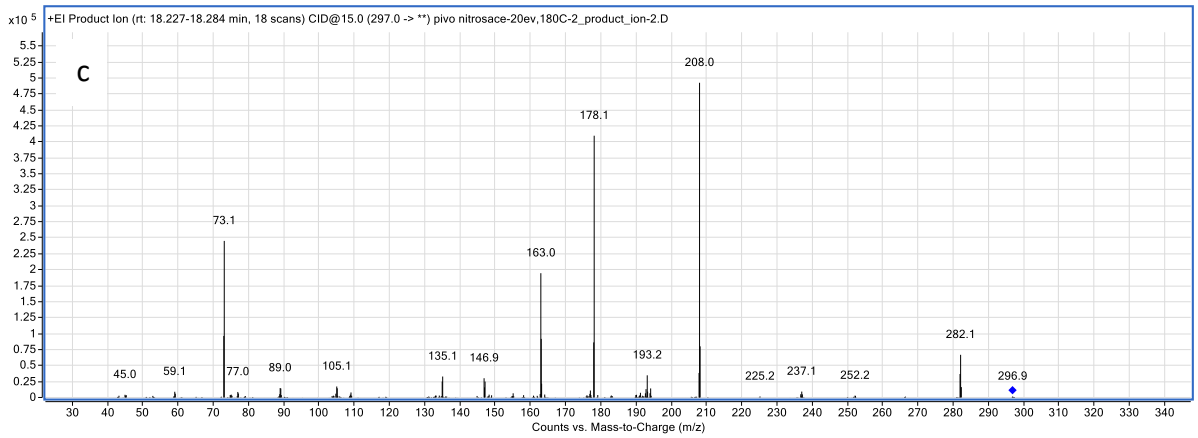


Figure S15: Proposed mass fragmentation of peak at 14.0 min and possible identification of a molecle as N-nitrosoproline ethylester


x10 4
+EI Neutral Loss (rt: 18.225-18.288 min, 22 scans) CID@6.0 -[30.0] pivo nitrosace-20ev,180C-2_NL30.D
a
103.1
135.1
208.1
327.1
73.1
179.1
186.1
89.0
99.7
115.1
130.9
162.7
192.4
222.1
282.1
297.0
312.1
Counts vs. Mass-to-Charge (m/z)


x10 5
+EI Product Ion (rt: 18.230-18.287 min, 18 scans) CID@15.0 (312.0 -> **) pivo nitrosace-20ev,180C-2_precursor ion.D
b
179.1
312.1
73.1
147.0
194.1
222.0
253.2
103.9
133.1
30.2
45.0
65.1
92.0
119.0
165.0
209.2
238.2
267.0
Counts vs. Mass-to-Charge (m/z)


x10 5
+EI Product Ion (rt: 18.227-18.284 min, 18 scans) CID@15.0 (297.0 -> **) pivo nitrosace-20ev,180C-2_product_ion-2.D
c
208.0
178.1
73.1
163.0
282.1
135.1
146.9
193.2
45.0
59.1
77.0
89.0
105.1
225.2
237.1
252.2
296.9
Counts vs. Mass-to-Charge (m/z)

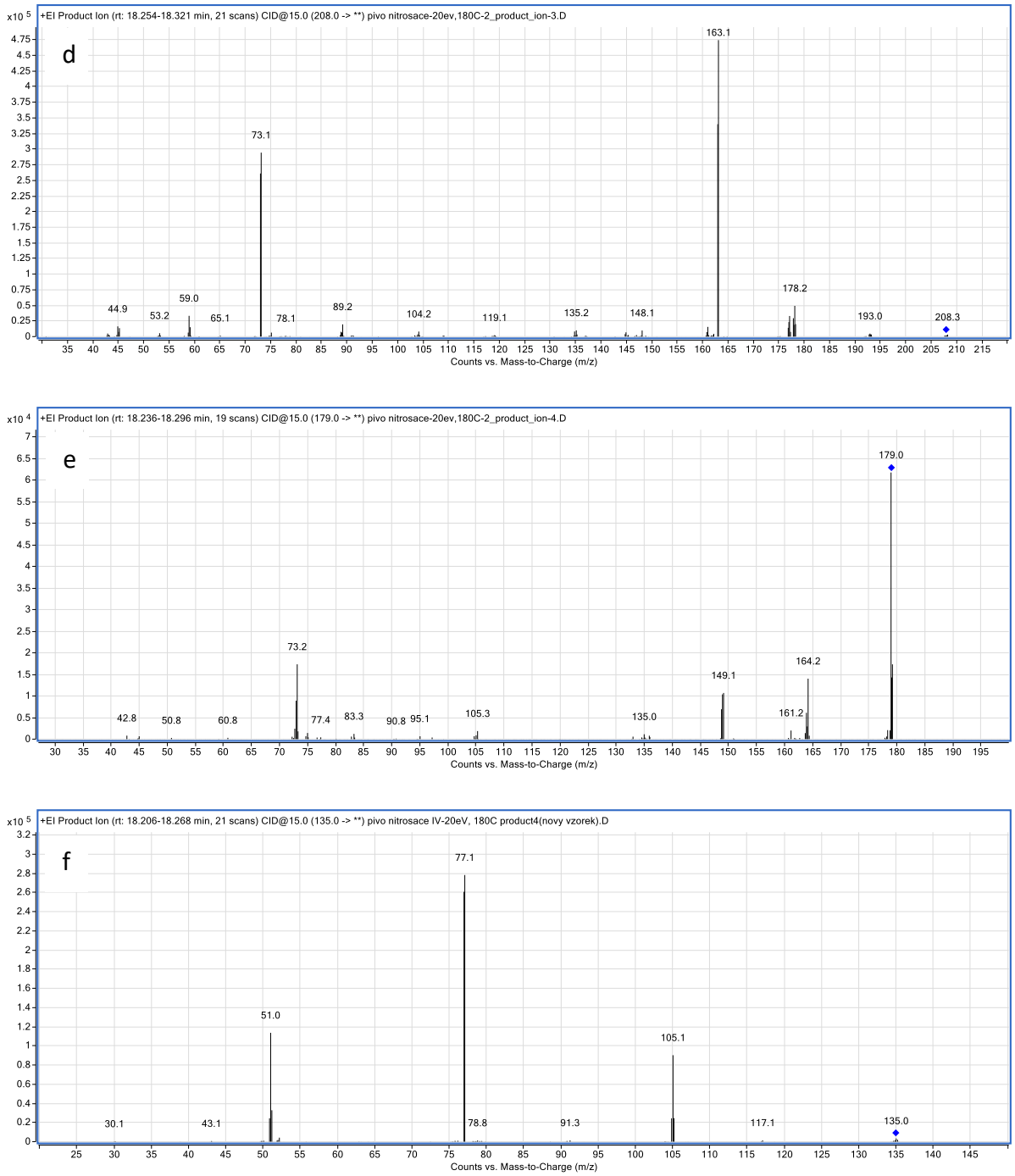


Figure S-16: CID based mass spectra of peak at 18.3 min: a) neutral loss of 30 amu; product ion mass spectra of b) m/z 312, c) m/z 297, d) m/z 208, e) m/z 179 and f) m/z 135.

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The usage of a reflectometric method for 5-(hydroxymethyl)furan-2-carbaldehyde determination as a stale flavor sensor for beer

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ABSTRACT

A rapid, mobile and objective method for *in situ* determination of a degree of sensory deterioration of beer caused by the aging process during storage was developed and validated. The novel method uses current reflectometric method of 5-(hydroxymethyl)furan-2-carbaldehyde (HMF) determination for the purpose of taste sensor. It requires only few minutes and an easy-to-use mobile reflectometer with test strips. According to our previous study, HMF is a very good indicator of a stale flavor in beer determined by a sensory analysis (correlation higher than 0.96). The reflectometric signal is transformed to the degree of the stale flavor using specified algorithm, which was derived in this study. The method provides results comparable with the sensory analysis by a trained sensory panel for lager beers and also provides objective reflection of beer deterioration for other beer styles.

1. Introduction

Beer is one of the most commonly consumed beverages in the world and is an integral part of diet in many countries. Also, culture in some countries, especially in Germany, the Czech Republic or Ireland is strongly tied to a tradition of beer and these countries regularly possess the first few rankings in annual beer consumption per capita (Bamforth, 2004). From the point of view of a consumer, one of the most important quality parameters of beer is the flavor of the particular beer brand. Hence, beer should be delivered to a consumer in the possibly best sensory quality. Unfortunately, beer can be sensory deteriorated during the way from a brewery to a consumer in the way of a change of organoleptic character of beer in a negative manner.

The sensory beer aging is a phenomenon that considerably reduces sensory quality of beer and can have a negative influence on a consumer's perception of beer. The aging process considerably changes an organoleptic character of beer which is coupled with the development of the stale flavor of beer usually described as a honey-bitter-astringent flavor (EBC Analysis Committee, 2012). An acceleration of the beer aging process is caused by an inappropriate storage and transport conditions. Therefore, unfortunately, the sensory beer aging often occurs before the declared beer shelf-life. Besides the wrong storage and transport conditions, the level of the sensory aging could be also influenced by a chemical composition of beer. Many reaction mechanisms are coupled with the beer aging including oxidation of higher alcohols, the Strecker degradation of amino acids, the aldol condensation, a degradation of hop bitter acids, an oxidation of unsaturated fatty acids, the Maillard reaction, the Fenton reaction, and many others (Vanderhaegen, Neven, Verachtert, & Derdelinckx, 2006).

It is well-known that the most important compounds forming the stale flavor of the beer are carbonyl compounds formed by the Maillard reaction, the Strecker degradation, and alcohol oxidation (Baert, De Clippeleer, Hughes, De Cooman, & Aerts, 2012). Specifically, the most relevant stale flavor indicators appear to be compounds with the furan-2-carbaldehyde structure (particularly 5-(hydroxymethyl)furan-2-carbaldehyde (HMF) and furan-2-carbaldehyde (FA)), whose role in the beer aging was previously published (Čejka, Čulík, Horák, Jurková, & Olšovská, 2013; Olšovská, Štěrba, Vrzal, Jurková, & Čejka, 2016). Their concentration in beer appear to be proportional to a sensory deterioration of beer which was thermally loaded during the transport and the storage.

The degree of the sensory deterioration is possible to be determined by a sensory analysis performed by trained assessors or by an instrumental analysis of carbonyl compounds, so called "aging indicators", usually by gas or liquid chromatography (Ortiz, 2015; Wu, Shi, & Feng, 2009). However, these methods do not allow a field control, such as in on-trade and off-trade, because the sampling and the sample transport to a laboratory can significantly affect the results.

Recently, Rico-Yuste et al., developed a mobile method for the determination of FA in beer (Rico-Yuste et al., 2016). However, this

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method requires a one-hour long incubation of a disposable polymer film in the beer sample. Moreover, this method is usable for the determination of FA (with an interference of HMF) using a chemical analysis which replaces the HPLC method, but finally does not give information about the stale flavor.

The aim of this study was the development of a fast, easy-to-use, objective, mobile and cost-effective method for an *in situ* determination of the stale flavor intensity in beer. The method was designed for control purposes in order to guarantee beer quality in the on-trade (draught beer) and off-trade (bottled beer), but is usable also in a routine laboratory control or for anyone interested in sensory quality of beer. A mobile, commercially available reflectometer (RQflex 10 and RQflex 20 Merck) was used for the determination of a color intensity (red-violet) due to product formation by a reaction of HMF with a barbituric acid and aminophenazone derivative in the range of HMF concentration 1–60 mg/L. Simultaneously, the dependence between results of the sensory and chromatographic analyses as well as reflectometric signals was found, and an algorithm for a conversion of reflectometric signal to a sensory scale was derived.

2. Materials and methods

2.1. Reagents and chemicals

The standards of 5-(hydroxymethyl)furan-2-carbaldehyde (~99%) and furan-2-carbaldehyde (99%) were purchased from Sigma-Aldrich (Germany). The other chemicals were 3-fluorobenzaldehyde (97%), sodium thiosulphate pentahydrate (99.5%), PFBHA (o-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride (≥98%)), hexane (≥99%) all from Sigma-Aldrich (Germany), sulphuric acid (96%) and phosphoric acid (85%) from Merck (Germany), ethanol (96%) and ascorbic acid (99.7%) both from Lach-Ner (Czech Republic), ultrapure water was prepared by MilliQ system (Millipore, USA). The blank and test strips for the reflectometric determination of 5-(hydroxymethyl) furan-2-carbaldehyde were purchased from Merck (Germany).

2.2. Beer samples

The fresh samples of lager (A – E, G – L and N1 – N5), ale (M and O), IPA (P and Q), wheat beer (R) and non-alcoholic beer (F produced by a special strain of yeast, V and W produced by interrupted fermentation) were obtained directly from Czech manufacturers. The other European lager beers (S – U) were purchased from the local market. The other 34 samples of lager and ale obtained from the local markets were used for screening of occurrence of sensory deteriorated beers in off-trade.

Five brands of the lager beer samples (A – E) and one non-alcoholic beer (F) produced in the Czech Republic were used for a method development. The beer samples were stored at 0, 20 and 30 °C in thermostatic boxes for six months and gradually analyzed in the monthly intervals. This experiment was performed in two consecutive years. The experiment is explained in more detail in our previous study (Olšovská et al., 2016).

Further, the beer samples (A – L, M, O, P, Q, R, V, and W) were exposed to a heat shock of 45 °C for one week. Next, five lager beer samples (N1 – N5) were stored at a laboratory temperature (approx. 20 °C) for a minimum of half a year and a maximum of two years for simulating natural beer aging. All samples were in glass half-liter bottles placed in the dark during the storage.

The all fresh and aged samples were analyzed in duplicate by HPLC, GC–MS, reflectometry and the sensory analysis.

2.3. Reproducibility test

Reproducibility of the method was performed using three reflectometric instruments (one RQflex10, two RQflex20) in the same time on the same place at room temperature. Forty-four lager samples (original gravity 10–12% w/w) of different age were used: 2month-old (3 samples), 5month-old (4 samples), 6month-old (13samples), 7month-old (3 samples) 9month-old (7 samples), 10month-old (7 samples), 11month-old (5 samples), 28month-old (1 sample), 12years-old (1 sample) stored at 20 °C. The results obtained from three tested reflectometers were compared by *t*-test and standard deviation among all reflectometers was calculated.

2.4. Sample preparation

Prior to determination of FA by GC–MS, 5 mL of the beer samples were acidified using 0.1% phosphoric acid at pH = 4.4, then 20 µL of an internal standard solution (3-fluorobenzaldehyde, 120 mg/L), and 50 µL of 0.1 mol/L sodium thiosulphate pentahydrate were added. The sample was subsequently derivatized using 500 µL of the PFBHA solution (0.017 mol/L) for one hour at laboratory temperature. Then, 50 µL of 9 mol/L sulphuric acid were added to stop the derivatization reaction, and the solution was shaken with 1 mL of hexane. The bottom layer was discarded and the organic layer was three times shaken with 5 mL of 0.05 mol/L sulphuric acid. The organic layer was then transferred to vials, and FA was determined by GC–MS. The blank sample (5% (v/v) ethanolic water solution) was treated in the same way.

The samples containing HMF and analyzed by HPLC were firstly degassed by ultrasonication for 2 min (until the foam collapse), and filtered through a cellulose microfilter with 0.2 µm pores.

The reflectometric analysis does not require any sample pre-treatment.

2.5. GC–MS

The GC–MS analysis was performed on the Trace GC Ultra chromatograph equipped with the DSQ II mass spectrometric detector (Thermo Electron Corp.) and the TG-WAX MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The injection of 1 µL of the sample was carried out by a splitless injection at 250 °C. The oven temperature was programmed as follows: 60 °C (2 min) – 6 °C/min – 235 °C (0 min). The transfer line and the ion source temperature were 250 and 200 °C, respectively. The electron impact ionization at 70 eV was used; the characteristic ion for PFBHA derivates at m/z 181 was detected in SIM mode. FA was identified by a comparison of the retention time and mass spectrum with the standard, and was determined by the internal standard method. The limit of quantification of FA was 0.3 µg/L.

2.6. HPLC

The determinations of HMF were carried out on the Ultimate 3000 (Thermo Fisher Scientific) liquid chromatograph with the Aminex Fast Acid Analysis HPLC column (100 mm × 7.8 mm, 9 µm particle size) and the Cation H Micro-Guard precolumn. The isocratic analysis was performed at 65 °C with 5 mmol/L sulphuric acid in ultrapure water as a mobile phase with the flow rate of 1.2 mL/min and 10 µL sample injection. The analyte was detected at 283 nm. The determination was performed by using the calibration curve method.

2.7. Reflectometric analysis

The reflectometric analysis was carried out by the mobile reflectometer RQflex (model 10 and 20, Merck, Germany) and the blank and test strips for the HMF determination (Merck, Germany). The newly developed method was carried out as follows. The reflectometer was recalibrated using a recalibration barcode together with the blank strip soaked into the beer sample. Using this procedure, an influence of beer color was eliminated. The analysis was then performed by soaking the HMF test strip into the beer sample and pressing the START button of the reflectometer at the same time. After a 3-minute reaction, the test

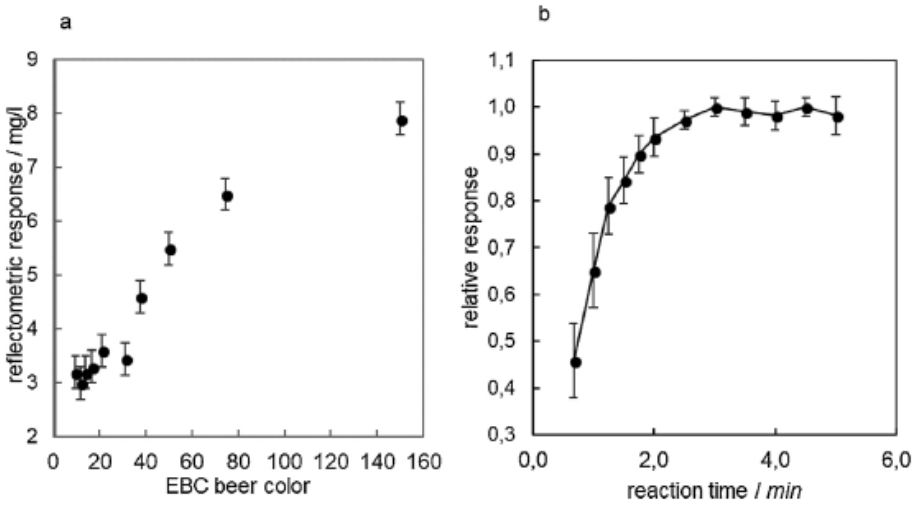

Fig. 1. Reflectometric response dependency on beer color intensity (a) and reaction time (b).

strip was placed into the measuring cell of the reflectometer and the measurement was triggered by pressing the START button. The quality of the results was checked by the analysis of a quality control (QC) sample consisting of an aqueous solution of HMF at accurate concentration approximately 5 mg/L. The QC standard samples were prepared every day to be fresh. The obtained signal of the reflectometer, expressed as the HMF concentration (mg/L), was used as reflectometric a response in this study.

2.8. Sensory analysis

The sensory analysis was carried out by a 12-member sensory panel of the Research Institute of Brewing and Malting in Prague. Assessors were selected and trained according to ISO 8586:2015 and ISO 11132:2012. The performance of the assessors was checked by comparison of their evaluation with the committee (Čejka, Olšovská, Štěrba, Slabý, & Vrzal, 2018). The test was conducted in the sensory laboratory, equipped according to EBC 13.2 (EBC Analysis Committee, 2012) and ISO 8589:2008. The samples were served in duplicate in glasses, tempered to 10 ± 2 °C.

During the sensory evaluation, the main emphasis was placed on the development of the beer stale flavor, and the assessors evaluated this character using marks ranging from 0 to 4 (0 – none, 1 – weak, 2 – medium, 3 – strong, 4 – very strong). The stale flavor was defined as combination of honey-bitter-astringent. The assessors were acquainted and trained with this flavor through one-year-old samples. The trimmed mean from the obtained values was calculated by omitting of the maximum and minimum values.

2.9. Data processing

The reflectometric responses of the beer samples in particular batches were corrected using the QC reflectometric response by Eq. (1). The only constant error was observed during the method development (not proportional error).

$$R_{corr.} = R_{sample} - (R_{QC} - C_{QC}) \quad (1)$$

Where $R_{corr.}$ is the corrected reflectometric response of the sample in mg/L, R_{sample} is the reflectometric response of the sample in mg/L, R_{QC} is the reflectometric response of the QC sample in mg/L and C_{QC} is the known accurate concentration of HMF in the QC sample in mg/L.

The results from GC–MS, HPLC, reflectometric and sensory analyses were processed by the regression analysis and a paired *t*-test at the 95% confidence level.

3. Results and discussion

3.1. Method development

The first step in the method development was a selection of the most reliable indicator of the lager beer aging strongly correlating with the stale flavor. A formation of particular carbonyl compounds during lager beer storage was recently partially published by our research team (Olšovská et al., 2016), however, with the exception of the HMF behavior. Based on this study, the correlation coefficient between an HMF formation and the storage time was obtained and was higher than 0.96 (data not shown). Further, the dependency of HMF concentrations in time was linear at all studied temperatures (as well as in case of FA). Moreover, both, FA and HMF, showed the tightest dependency between their concentrations and the sensory stale flavor intensity, the correlation coefficients were 0.87 and 0.83, respectively. Hence, both compounds could be used as the beer aging indicators and/or the beer stale flavor intensity indicators. A similar result was obtained and previously published by Shimizu et al. (2001). The relatively high concentrations of HMF in beer and a necessity of a simple, fast, objective, and cost-effective method finally led to choosing HMF as an aging indicator.

The reflectometer (RQflex, Merck) meets our basic requirements of the method – it allows a fast, easy and mobile analysis through test strips for the HMF determination. Currently, the reflectometric application for the determination of HMF in an aqueous solution of juice or honey is commercially available (Merck application note - a; Merck application note - b). However, it is not applicable for beer samples. Therefore, we have used the principle of reflectometric measurement and have developed a procedure for beer samples and a specific algorithm converting the reflectometric response to the stale flavor intensity.

Firstly, it was necessary to eliminate a blank signal because of beer the color interference. Therefore, the blank strip soaked into a beer sample must be used during the reflectometer recalibration and the signal of the beer is subtracted. Nevertheless, the method is limited by beer color since a color higher than 32 EBC units significantly influences the reflectometric analysis (Fig. 1a). In other words, the method is usable for pale and amber beers, not for the dark ones.

Next, the length of the reaction time was optimized. The reflectometric response development in time is described in Fig. 1b. The beer sample with HMF concentration of 5.1 mg/L was used in triplicate for this purpose. The maximal reflectometric response was obtained at the third minute and consistent results were obtained between the third and fifth minute (longer reaction time was not tested). Therefore, the

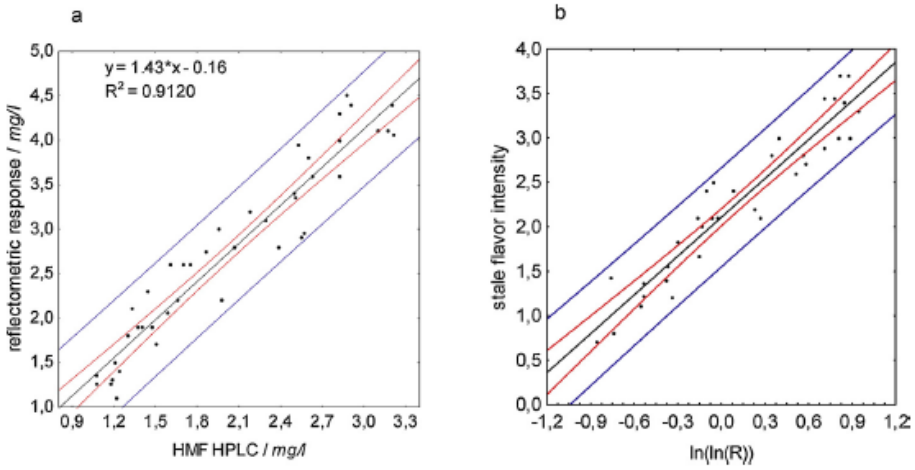

Fig. 2. Correlation between reflectometric responses and HMF concentrations obtained by the HPLC analysis (a) and correlation between stale flavor intensity and the reflectometric response of the aged beer samples.

reaction time of 3 min was selected as optimal.

3.2. Correlation between the HPLC and reflectometric responses

Reflectometric responses and HMF concentrations obtained by the HPLC analysis of aged beer samples were correlated by the regression analysis, see Fig. 2a. The reflectometric results correlate linearly with the HPLC data, however, a proportional error is evident (a slope is statistically different from 1 and an intercept is insignificant). The most likely explanation is the existence of other compounds in beer samples which contribute, as well as HMF, to the resulting reflectometric response, especially at a higher degree of the sensory deterioration. For that reason, a comparison of the reflectometric sensitivity of HMF and FA was performed (based on a known increase of FA during beer aging) (Olšovská et al., 2016; Vanderhaegen et al., 2006). As seen in Fig. 3, the sensitivity of the reflectometric method for FA is 2.2 times higher than for HMF but concentrations of FA in beer is usually many times lower than HMF (FA concentration is in the order of hundreds of µg/L and HMF in units of mg/L). Therefore, FA could contribute to the reflectometric response very little but it still could be significant in aged beers. To evaluate a possible contribution of FA to the reflectometric response, an expected sum of reflectometric responses of HMF and FA in analyzed beers (obtained as a sum of HMF concentration and a theoretical signal of FA calculated from the equation in Fig. 3) were compared with the reflectometric response obtained experimentally. Comparison made by the paired *t*-test did, however, not show statistically significant differences ($p = 0.452$).

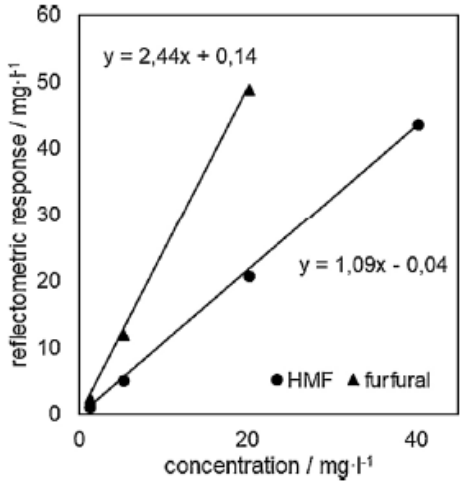

Fig. 3. Comparison of reflectometric sensitivities of FA and HMF.

As it follows from the above comparison, FA likely causes the proportional error in the reflectometric analysis in comparison with the HPLC analysis of HMF and the resulting reflectometric response can be characterized as an apparent sum of both furan-2-carbaldehydes, FA and HMF, in beer sample. It must be highlighted however that FA does not acts as an interfering compound in this method, conversely, we use it for determination of stale flavor together with HMF. FA would only interfere for specific HMF determination. Moreover, formation of HMF and FA, which are both aging indicators of beer, correlate together ($r = 0.72$). Therefore, HPLC can be replaced by the reflectometry for the purpose of the developed method.

3.3. Relationship between the reflectometric and the sensory analysis

The data collected from the reflectometric and the sensory analysis of aged beers were used for a construction of a predictive model for the determination of the stale flavor intensity in beer. The dependency of the stale flavor intensity on reflectometric response exhibits a logarithmical function, see Fig. 2b, Table 1 and Eq. (2).

$$\text{stale flavor intensity} = 1.45\ln(\ln(R_{corr.})) + 2.11 \quad (2)$$

Where $R_{corr.}$ is corrected reflectometric response of the sample in mg/L from Eq. (1).

The resulted model with the coefficient of determination 0.9026 was tested by a residual analysis. This showed a constant variance of the residuals around zero, no dependency of residuals on dependent and independent variable and a normal distribution of residuals. Analysis of variance of the resulting model showed mean square of the model, residual mean square, and Fisher F-value 21.538, 0.073, and 295.041 ($p < 0.01$), respectively. Therefore, we can assume that the

Table 1
Parameters of linearized function of stale flavor intensity on reflectometric response.

parameter	value
slope	1.45 ± 0.17
intercept	2.11 ± 0.10
R^2	0.9026
s.d.	0.25
F	296.46
p	$1{\cdot}10^{-17}$

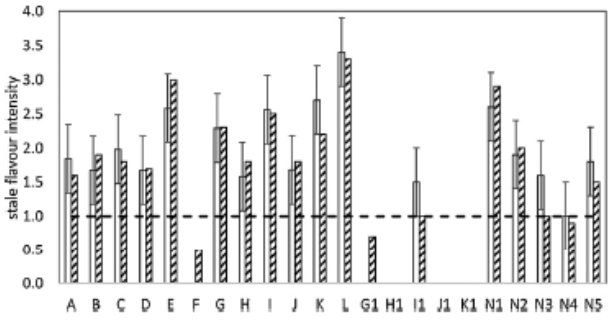

Fig. 4. Comparison of stale flavor intensity determined by the reflectometric method (white bars) with results of the sensory analysis (striped bars) of lagers.

data was evaluated by the function correctly, and it can be used for the determination of the stale flavor intensity.

3.4. Method testing and validation

Before the method testing itself, the limit of quantification of the stale flavor intensity of beer was determined as 1.0 and uncertainty of predicted values ± 0.5 (based on the parameters of the linear dependency of the stale flavor intensity on the logarithmic reflectometric response).

The final method was tested and validated on 42 beer samples (see Section 2.2. Beer samples). The stale flavor intensity was evaluated by the sensory panel and the reflectometric analysis according to the developed method and the results were compared. Results were in a good agreement for lager beer produced in the Czech Republic (samples A-L and N1 – N5) (paired *t*-test p = 0.571), Fig. 4. In contrast, other beer styles (samples M and O – W) show a statistically significant difference between the method results (p = 0.001), Fig. 5, only some samples exhibit an accordance. However, all these samples show values, emerging from the sensory analysis that are lower than the one from the reflectometric response. These significant differences between the sensory and the reflectometric method are likely caused by masking the stale flavor in beer (especially in more aromatic beer styles as ale, IPA and wheat beer; samples M and O – R), by using a very different brewing technology (non-alcoholic beers made by interrupted fermentation; samples V and W), or higher adjuncts use (lager beer samples S, T and U). Nevertheless, our method can be used as a measure of thermal load of beer during the storage for a broad range of beer styles. However, this does not apply to non-alcoholic beers made by interrupted fermentation because HMF formed during a boiling is not degraded

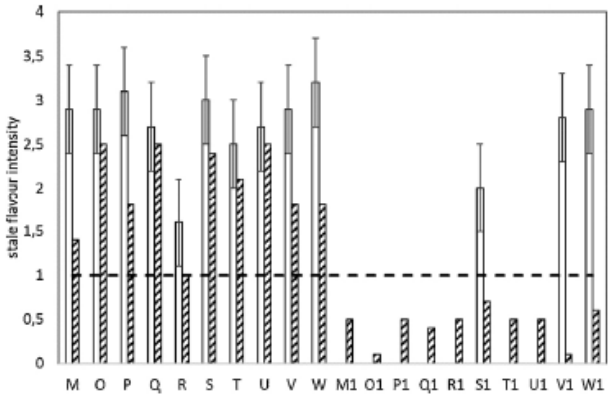

Fig. 5. Comparison of stale flavor intensity determined by the reflectometric method (white bars) with results of the sensory analysis (striped bars) of ales, wheat beers, lagers with a high degree of subrogation and non-alcoholic beer (made by interrupted fermentation).

Table 2
Relative response development of a quality sample with a different amount of ascorbic acid in time at 21 °C.

time/day	REF[a]	ascorbic acid/HMF molar ratio				
		0	115	659	1289	2921
0	1.00	1.00	1.00	1.00	1.00	1.00
1	0.98	1.02	1.00	1.00	1.00	1.00
2	1.00	0.52	0.96	0.99	1.01	1.01
3	1.00	< 0.2	0.96	1.00	1.02	1.03
5	1.00	< 0.2	0.84	1.02	1.00	1.03
10	1.00	< 0.2	–	0.98	1.14	1.43
12	1.00	< 0.2	–	0.85	1.15	1.50

[a] Reference sample stored at 4 °C; values are within uncertainty ± 0.03.

during the fermentation and concentrations of HMF in fresh beer is very high in contrast to other beers (Akıllıoglu, Mogol, & Gökmen, 2011).

3.5. Reproducibility

We can conclude based on *t*-test that there are not find statistically different results among the three individual reflectometers (p < 0,05). The reproducibility of the method expressed as relative deviation is 7.3%.

3.6. Quality control sample stabilization

The stability of a QC sample is a crucial parameter for avoiding false positives/negatives. The instability of the QC sample would limit *in situ* utilization of the method. The degradation of HMF in the QC sample at the laboratory temperature is a function of time, as it was evident during the method development, (see Table 2). The degradation is most likely caused by oxidation of HMF (Girisuta, Janssen, & Heeres, 2006), therefore, the ascorbic acid which has an antioxidative effect was added to the QC sample to improve the HMF stability. Five levels of molar ratios of the ascorbic acid and HMF (ascorbic acid/HMF at 0, 115, 659, 1289 and 2921) were tested and the results of the HMF stability at the laboratory temperature (21 ± 2 °C) are shown in Table 2. A molar ratio of 659 exhibits a good stability of the QC sample for ten days at the laboratory temperature (corresponding concentration of the ascorbic acid to HMF concentration of 5 mg/L is 4.6 g/L). Higher concentrations of ascorbic acid cause an increase of the reflectometric response because the ascorbic acid is thermally degraded to FA. Nevertheless, addition of the ascorbic acid in selected tested concentrations did not affect the reflectometric response (even though that Merck states the ascorbic acid can interfere with the color reaction at concentration higher than 1 g/L).

3.7. Screening of real beer samples

To outline a real situation of sensory deterioration of beer by its inappropriate storage, 34 beer samples from local supermarkets were analyzed by the method, see Table 3. Nearly 56% of the tested samples were positive, meaning that the degree of measured deterioration was higher than the limit of quantification (the stale flavor intensity higher

Table 3
Screening results of real beer samples from local markets.

	number	%
N	34	
positive	19	55.88
weak – medium (1–2)	14	41.18
medium – strong (2–3)	5	14.71
strong – very strong (3–4)	0	0.00

than 1.0). Weak/medium deterioration was noted in 41% of the samples and 15% of samples were damaged significantly (medium/strong).

With this simple experiment we demonstrated, how necessary the newly developed method is. Fifteen percent of tested samples was sensorially deteriorated, which implies that the high-quality level and the reputation of brands which were damaged, could be threatened. Moreover, if a brewery would apply this method directly during the final quality check, it would not be any problem to prove that the beer was not stored or transported at the recommended temperature, when deteriorated beer is found in on-trade or off-trade.

4. Conclusions

Until better technology is available to slow down the aging process of beer, which is very unrealistic, the issue of good storage will be the limiting factor in order to maintain beer quality in the market. Therefore, there is a need for an effective tool to check the sensory quality of beer. The newly developed method can enable and facilitate *in situ* monitoring of the stale flavor in beer. The analysis is not only rapid, cheap and easy-to use, but also objective, because it is based on chemical measurement of a beer aging indicator. The correlation was derived from hundreds of measurements for 3 years, as many types of beers were monitored during aging.

Acknowledgements

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Research Article

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The chemical profiling of fatty acids during the brewing process

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Abstract

BACKGROUND: Although fatty acids have a beneficial effect on yeast growth during fermentation, their effect on foam and sensory stability of beer is negative. In general, long-chain fatty acids originate from raw materials, whereas short-chain acids are produced by yeast during fermentation. If the concentration of short-chain fatty acids, especially isovaleric and butyric acid, overreaches a sensory threshold, then an unpleasant aroma, such as cheesy or sweaty feet, can be formed in beer.

RESULTS: The distribution of fatty acids, from the preparation of sweet wort to the final beer, was studied using chemometric evaluation. Differences were observed between the decoction and infusion system using four barley varieties. Attention was paid to the behavior of short-chain fatty acids, namely isovaleric acid. The concentration of isovaleric acid in commercial beers brewed in infusion and decoction systems was approximately 1.4 and 1.0 mg L^{-1}, respectively. The same trend was observed in experimental samples (1.3 and 0.5 mg L^{-1}, respectively). This phenomenon was confirmed experimentally; based on the results, this possibly explains why, during the fermentation, isovaleric acid is coupled with the redox state of yeast cell, which is given by the wort composition (i.e. by the mashing process).

CONCLUSION: The formation of isovaleric acid is not only caused by microbiology infection or by oxidized hops, but also is influenced by the mashing process.

Keywords: chemical profiling; fatty acids; isovaleric acid; mashing

INTRODUCTION

Despite recent studies concerning the role and fate of fatty acids (FA) during the malting and brewing process, not all questions about them have been answered. Lipids and FA represent approximately 1 to 3 g kg^{-1} of the grain weight.[1] FA have a beneficial effect on yeast growth during the fermentation, although their influence on beer aroma, namely during beer staling,[2] and the stability of beer foam is strongly negative.[3] These compounds and their oxidized forms cause an unpleasant off-flavour of beer, such as rancid, cheesy, soapy, fatty, isovaleric, butyric and other characteristics.

Anness *et al.*[4] measured lipids (phospholipids, monoglycerides, diglycerides, triglycerides, free FA and steryl esters) in beer and raw materials as the total concentration of long-chain fatty acids (16:0, 18:0, 18:1, 18:2 and 18:3) and found that the brewing process as a whole is extremely efficient in eliminating lipids. Lipids in wort and beer are reduced in quantity at each of major process steps: mash separation, wort boiling, trub removal and filtration. It was concluded that very little of the lipids originating from raw materials survive the brewing process. Only 0.03% remains in the finished beer because spent grains, hot breaks and cropped yeast account for the effective removal of lipids during the brewing process.

Clarke *et al.*[5] targeted their study on medium-chain FA (C6, C8, C10) because, according to Clapperton and Brown,[6] octanoic and decanoic acids are significant contributors to the flavour of many lager beers. Clarke *et al.*[5] also summarised and evaluated the effects that influence the concentration of medium-chain FA in beer, namely the effect of yeast strain, pitching rate, fermentation time, pressure and fermentation temperature, as well as aeration and wort composition.

Clapperton and Brown[6] found that the concentrations of FA within particular beer types vary significantly and that top fermented beers contain a lower concentration of FA than bottom fermented beers.

Bravi *et al.*[7] paid great attention to lipids in the brewing process. They evaluated the influence of both technological steps and yeast biomass in the lipid composition of beer. Lipid contents and their FA profile were evaluated in brewing raw materials, wort and beer. In comparison with Anness *et al.*,[4] they analysed a broader spectrum of FA (from C8 to C24, saturated and unsaturated) using a high-resolution gas chromatography with a flame ionization detector (GC-FID). They concluded that the main technological steps influencing the lipid content of beer are a whirlpool and filtration. Furthermore, they found that the use of an infusion mashing system could result in lower lipid content compared

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to a decoction system. Moreover, the presence of metabolically active yeast cells was found to have a great influence on the FA composition of lipids. In the next study, Bravi *et al.*[8] found that not only the brewing process, but also the composition of the raw materials affects the composition of lipids in final beer. They studied the lipid content of five micro-malted Italian barley varieties and the influence of these varieties on the changes in FA profile during the malting process. They found that different barley varieties possess different FA contents and different FA patterns. The correlation between the lipid content of barley and the quality of the resulting malt confirmed the negative influence of lipids. Bravi *et al.*[9,10] finally described an analytical method for determination of FA in wort using GC-FID and its subsequent determination in beer. A very similar method was reported by Horák *et al.*[11]

Some studies concerning a slight contribution of lipids/FA derived from hops are also available. Hop seed oil is a rich source of triglycerides and esters of long-chain alcohols, both of which can release FA after hydrolysis.[4] According to Bravi *et al.*,[7] the lipid content of hop is more than 5.5 g kg^{-1}, from which the most abundant FA are 10:0, 12:0, 16:0, 18:2 (*n*-6) and 18:3 (*n*-3). However, the total amount of these FA is negligible compared to the amount of FA from malt.

The present study aimed to monitor the fate of FA during the brewing process depending on the mashing system used. Detailed attention was paid to the origin of short-chain FA, namely isovaleric acid, which has not been studied previously in this context.

MATERIALS AND METHODS

Chemicals

Standards of fatty acids: isobutyric (99%), butyric (99.5%), isovaleric (99%), valeric (99%), caproic (99%), caprylic (99.5%), nonanoic (97%) and capric (99.5%); standards of methyl esters of FA: methyl dodecanoate (99.5%), methyl myristate (99%), methyl palmitate (99%), methyl stearate (99%), methyl oleate (99%), methyl linoleate (99%) and methyl linolenate (98.5%); and internal standards: heptanoic acid (99%), tridecanoic acid (99%), pentadecanoic acid (99%) and heptadecanoic acid (99%) were purchased from Sigma-Aldrich (Prague, Czech Republic).

Other chemicals used were hexane (99%) (Merck, Darmstadt, Germany), ethanol analytical grade (Lachner, Neratovice, Czech Republic), methanol (99.9%) (Merck), HCl (37%) (Merck), chloroform analytical grade (Merck) and BF_3-methanol (14%) (Sigma-Aldrich). Ultrapure water was prepared by MilliQ (Millipore, Billerica, MA, USA). Solid phase extraction of FA was performed on LiChrolut EN 200 mg columns (Merck).

Beer preparation

Four varieties of barley (Malz, Bojos, Overture and Francin) from the 2013 and 2016 harvests were malted in three independent malt houses (to extend malt variability across malt houses) into Pilsner malt. Malt houses are equipped with pneumatic malting devices (two malt houses with a Saladin malting box; one with a Lausmann malting system). The eight malts obtained were used to prepare 16 beers by two different technologies, namely decoction and infusion. The experiments were performed repeatedly in 2013 and 2016.

An amount comprising 33 kg of malt with 81.5% extract (weights of malts with different extract were recalculated) was used for both technologies; the volume of final wort was 180 L. Double decoction mashing started at an initial temperature of 37 °C. Infusion mashing had an initial temperature of 52 °C and the sample was heated to reach both saccharification temperatures within the process. Subsequent process steps in the production of beer were identical. Hop extract (NATECO2, Wolnzach, Germany) and SAAZ hop pellets (Bohemia Hop, Žatec, Czech Republic) were dosed during the dynamic overpressure hop boiling for 80 min (first batch at the beginning of the boiling: 50% of bitter acids in form of hop extract; second batch at 30 min of boiling: 35% bitter acids in form of pellets; third batch at 65 min of boiling: 15% bitter acids in form of pellets); final bitterness of beer was 20 international bitterness units. Fermentation was performed by yeast strain RIBM 95 at a maximum temperature of 12 °C until the concentration of diacetyl was lower than 150 µg L^{-1}. Maturation lasted for 21 days at 2 °C.

Bottled beer samples from market

Real samples of lager beer were obtained from the Czech and German market. Ten samples were produced using decoction mashing and eight samples were produced by infusion mashing.

Preparation of beer fortified with glutamic acid

Wort prepared in accordance with the procedure described for Beer preparation (decoction mashing) was used to investigate the influence of glutamic acid in wort on the concentration of isovaleric acid in the final beer. In total, 1 L of the original wort sample and 1 L of the wort sample with the addition of glutamic acid (100 mg L^{-1}) were fermented in the laboratory with yeast strain RIBM 95 (pitching rate 15×10^6 cells mL^{-1}) at 10 °C for 6 days. Maturation performed in polyethylene terephthalate bottles took 21 days at 5 °C. The process was performed in duplicate. The concentration of isovaleric acid in the final beer was analysed by GC. The final beer was also evaluated by sensory analysis.

Preparation of internal standards

Internal standard 1 (ISTD 1) was prepared by dissolving 0.05 g of heptanoic acid in 25 mL of ethanol. Internal standard 2 (ISTD 2) was prepared by dissolving 0.05 g of tridecanoic, pentadecanoic and heptadecanoic acid in 25 mL of ethanol. In calibration, ISTD 1 was used for the determination of short-chain FA (up to C10); tridecanoic acid was used as internal standard for the determination of dodecanoic acid; pentadecanoic acid was used as an internal standard for determination of myristic and palmitic acid; and heptadecanoic acid was used as an internal standard for determination of the rest of FAs.

GC measurement

FA were determined using GC-FID as described by Horák *et al.*[11]; analyses of both short- and long-chain FAs were carried out using a Chrompack CP 9001 gas chromatograph (Chrompack, Inc., Raritan, NJ, USA) with a split injection, DB-WAX 20 m × 0.18 mm × 0.18 µm column (Agilent Technologies Inc., Santa Clara, CA, USA) and FID under the same chromatographic conditions. The limit of quantification 0.01 mg L^{-1} is the same for all FA. The uncertainty of the method is 15% and 20% for FA up to C10 (short-chain) and longer than C10 (long-chain) FA, respectively.

Volatile compounds, namely 3-methylbutanol and coeluting 2-methylbutanol, were determined in accordance with EBC 9.39.[12]

Determination of amino acids

Amino acids were analysed by high-performance liquid chromatography with fluorescence detection on an AQ-C18 column (250 × 4 mm, 5 µm) (Watrex, Prague, Czech Republic) after derivatization using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (ACQ) in accordance with the manufacturer's instructions.[13] The preparation of the sample was optimized for wort and beer. Next, 5 mL of filtered sample was pipetted into a 50-mL volumetric flask; 5 mL of internal standard (L-2-aminobutyric acid) and water to a volume of 50 mL was added. After intensive shaking by hand, the sample was filtered through a centrifugal filter (modified polyethersulfone 10 K, 500 µL) (VWR Scientific, Radnor, PA, USA) to remove proteins. Then, 50 µL of filtrate was diluted ten times (addition of 450 µL of water). After mixing, the derivatization was conducted in accordance with the original method.

Sample preparation

SPE column was washed with 2.5 mL of methanol, 5.0 mL of water and 20 mL of sample with 10 µL of ISTD 1 and ISTD 2, and 1 mL of HCl (1 mol L^{-1}) was immediately applied. After loading of sample, the column was washed with 5.0 mL of water and dried out with a flow of nitrogen. Fatty acids were eluted twice with 0.5 mL of chloroform. For analysis of free FA, 3 µL of chloroform extract was injected on the GC column. The rest of the extract was dried out with a flow of nitrogen, dissolved in 0.1 mL of BF_3 in methanol and heated for 60 min at 85 °C. Then, 0.2 mL of water was added after derivatization. Fatty acid methyl esters were extracted using 0.2 mL of hexane and 3 µL of hexane extract was injected on the GC column.

Sensory analysis

The sensory analysis was carried out by a 12-member sensory panel of the Research Institute of Brewing and Malting in Prague. Accessors were selected and trained according to ISO 8586:2015[14] and ISO 11132:2012.[15] The performance of the assessors was checked by comparison of their evaluation with the committee.[16] The test was conducted in the sensory laboratory, equipped in accordance with EBC 13.2[17] and ISO 8589:2008.[18] The samples were served in duplicate in glasses, tempered to 10 ± 2 °C.

During the sensory evaluation, the main emphasis was placed on the detection of isovaleric acid flavor, and the assessors evaluated this character using marks ranging from 0 to 5 (where 0 = none and 5 = very strong). The assessors were acquainted and trained using certified beer flavor standard of isovaleric acid (Aroxa; Cara Technology Limited, Leatherhead, UK). Because isovaleric acid flavor was not detected in any of the tested beer samples, data were not subsequently processed.

Statistical analysis

Statistical analysis, data treatment and preparation of graphs was performed using R, version 3.4.0 (R Foundation for Statistical Computing, Vienna, Austria) with the *muma*, *mixOmics* and *pheatmap* packages.[19,20] Computed concentrations of FA from two independent batches (from years 2013 and 2016) were integrated by a multivariate integrative method (MINT)[21] to minimize systematic unwanted variation between batches. Data were then scaled by *z*-score and visualized in a heat map where the columns were clustered by hierarchical clustering based on Euclidean distances and the Ward method.

A comparison of the concentration of compounds in the decoction and infusion systems was performed using a *t*-test. Detailed relationships between selected compounds in wort and beer were studied using principal component analysis (PCA).

RESULTS AND DISCUSSION

The chemical profiles of FA of 16 beers and their intermediates (sweet wort and wort) were statistically processed using several approaches. The first involved the construction of a heat-map (Fig. 1) from which it is obvious that the most significant factor influencing FA profile is the mashing system. On the other hand, the influence of barley variety and malt house is negligible. Three groups of FA were obtained based on the individual profiles of FA between sweet wort, wort and beer. The first group includes long-chain, saturated and unsaturated FA, namely linoleic, oleic, linolenic, palmitic and myristic acid, with the highest concentration in sweet wort (sum of saturated long-chain FA 2.0–4.8 mg L^{-1}; sum of unsaturated long-chain FA 0.9–3.6 mg L^{-1}). A significant decrease of these FA in wort is evident, and the concentration of long-chain FA in the final beer remains the same (sum of saturated long-chain FA 0.5–2.3 mg L^{-1}; sum of unsaturated long-chain FA < 0.01–0.9 mg L^{-1}). A detailed profile of the sum of long-chain FA is shown in Fig. 2(a, b). The second group of FA includes short-chain fatty acids, namely butyric, isobutyric, isovaleric, caprylic and caproic acid. Initially, their concentration in sweet wort and wort is low (sum of short-chain FA 1.2–2.2 mg L^{-1}) and it then increases during fermentation (sum of short-chain FA 2.3–8.1 mg L^{-1}). A detailed profile of the sum of short-chain FA is shown in Fig. 2(c).

The third group is formed by FA with an unspecified profile, namely valeric, pelargonic, capric, lauric and stearic acid. No trend in the route from sweet wort to final beer was observed with valeric, pelargonic and lauric acids because of their very low concentration (near to the limit of quantification) in each sample (valeric and pelargonic acid around 0.05 mg L^{-1}; lauric acid around 0.6 mg L^{-1}).

These results are in accordance with previously published data and complement them. Annes *et al.*[4] who mainly studied long-chain FA, also observed a rapid decrease of lipids and namely long-chain FA (16:0, 18:0, 18:1, 18:2 and 18:3) during wort separation; the majority of these FA are removed with the spent grains.

Similar to Bravi *et al.*,[7] in the present study, differences were also observed in the FA amount in beers prepared by infusion and decoction mashing. Bravi *et al.*[7] found a higher total amount of FA in sweet wort and beer produced in the decoction system, whereas a higher amount of FA in pitched wort was found in the infusion system. Accordingly, it was assumed that the decoction system causes extensive proteolysis and solubilization.

Although Bravi *et al.*[7] described differences between infusion and decoction systems in terms of the total amount of FA, the present study focused on differences between the two systems in more detail. Differences in FA chemical profiles between samples brewed using infusion (eight samples) and decoction system (eight samples) are evident from the heat-map shown in Fig. 1. The major differences were found in the concentration of butyric and isovaleric acids in final beer; their amount was significantly higher when infusion mashing was used (Fig. 2d, e). The same feature was observed with isovaleric acid in a measurement of commercial lager beers that were brewed using infusion (eight samples) and decoction mashing (10 samples) (Fig. 3). The concentrations of isovaleric acid in commercial beers brewed in infusion and decoction systems were approximately 1.4 and 1.0 mg L^{-1}, respectively. This

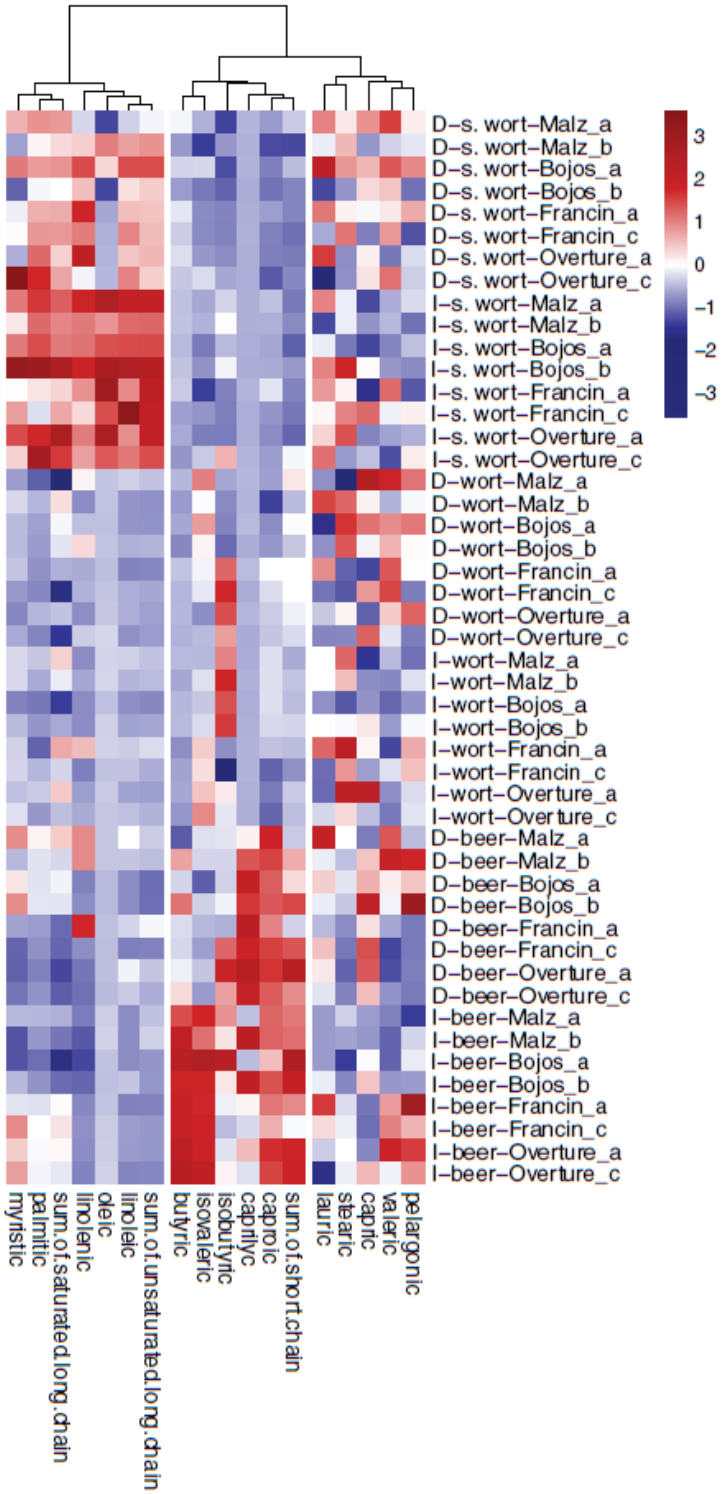

Figure 1. Heat map of fatty acids in different brewery matrices. Numbers depict the same batches. D, decoction; I, infusion; a, b, c, different malt-houses.

is in accordance with our experimental samples, where isovaleric acid was found in infusion and decoction beers at concentrations of approximately 1.3 and 0.5 mg L^{-1}, respectively.

Isovaleric acid has an unpleasant aroma, which is usually described as old cheese, rancid or sweaty, such that its presence in beer in a higher concentration is undesirable. The measured concentration of isovaleric acid in almost all studied beers was higher than the sensorial threshold, which is 0.3–0.9 mg L^{-1} according to the literature.[22] It should be noted that isovaleric acid was not sensorially detected in experimental beers (data not shown),

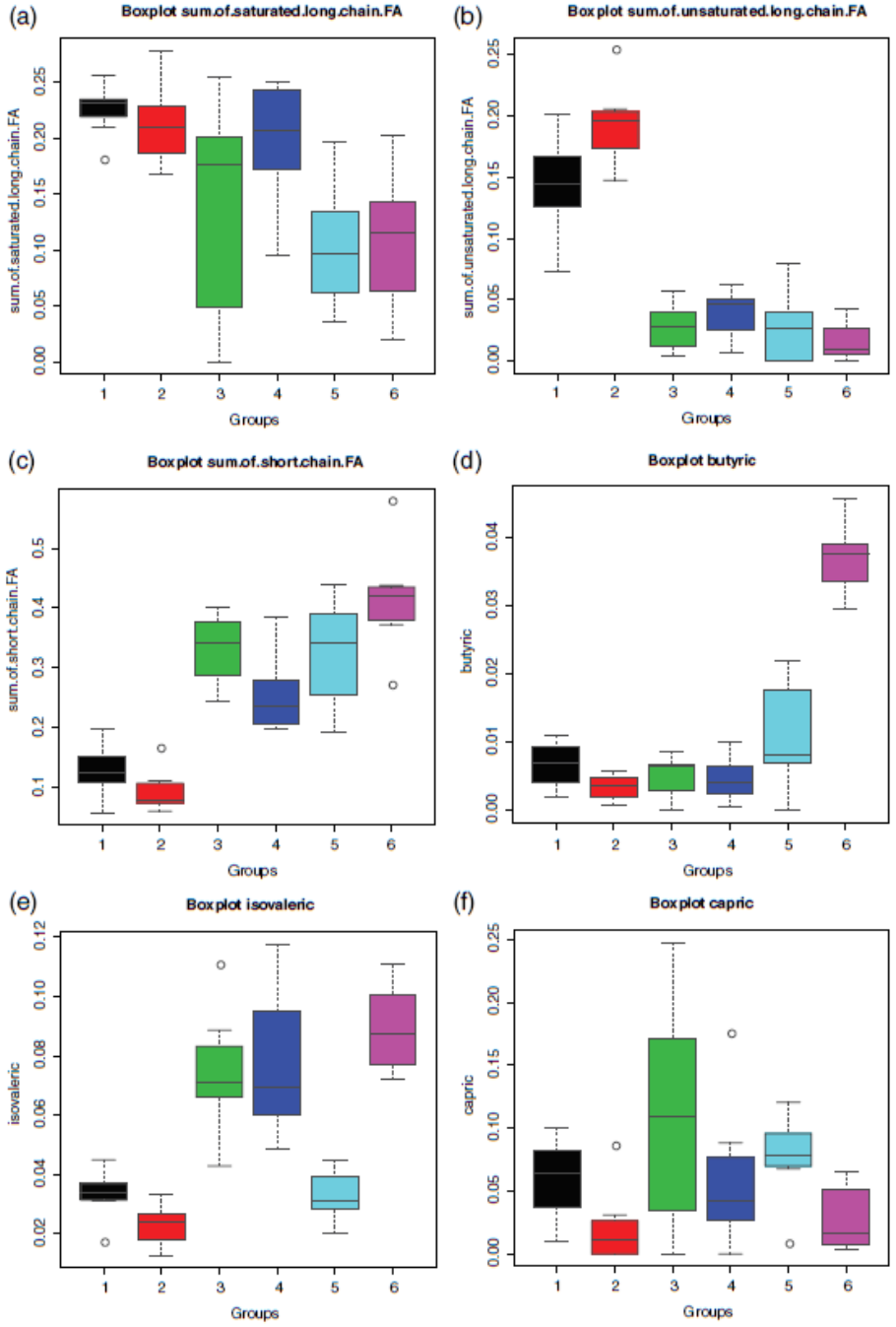

Figure 2. Boxplots of concentration of selected FA in mg L^{-1}. *x*-axes are described according to the matrix and mashing method. 1, sweet wort/decoction; 2, sweet wort/infusion; 3, wort/decoction; 4, wort/infusion; 5, beer/decoction; 6, beer/infusion. a) Sum of saturated long-chain FA; b) Sum of unsaturated long-chain FA c) Sum of short-chain FA; d) Butyric acid; e) Isovaleric acid; f) Capric acid.

although it can contribute to the overall organoleptic character of beer.

A significant difference between the infusion and decoction systems was also found in the concentration of oleic acid, where a higher concentration was found in sweet wort prepared by infusion. However, after boiling of wort, its concentrations in both systems were similar up to the final beer. The distribution of capric acid should also be noted. It follows from Fig. 2(f) that the concentration of this FA is higher in the decoction system and remains almost the same across all the observed steps, ranging from 0.8 to 1.0 mg L^{-1}. The concentration of capric acid in infusion system also remains at the same value, ranging from 0.1 to 0.3 mg L^{-1}.

In general, on inspecting the profiles of majority FA during the process, they are seen to have a similar trend in both the infusion and decoction systems; they differ only in absolute amount, although the trend is similar. For example, the sum of short-chain FA is increasing during the brewing process (Fig. 2c), whereas the

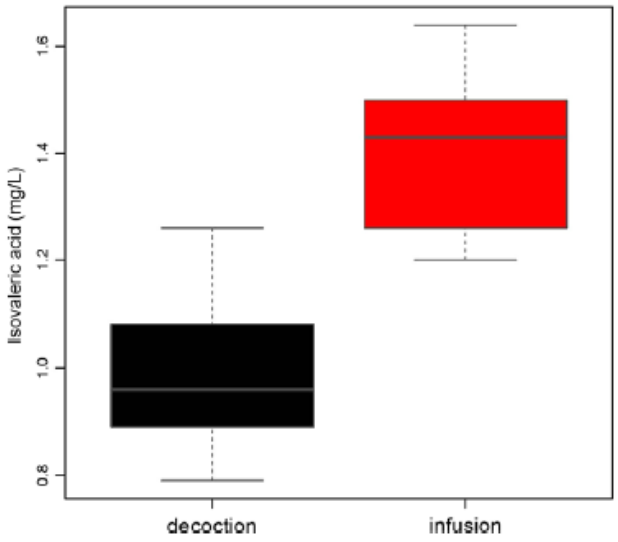

Figure 3. Comparison of isovaleric acid concentration in commercial lager beers brewed using decoction (ten samples) and infusion (eight samples) mashing.

sum of long-chain FA slightly decreases (Fig. 2a, b). However, there is one interesting exception, namely the trend of isovaleric acid. Although its concentration slightly decreases from wort to final beer when decoction mashing was used, it rapidly increases in the infusion system. To explain this result, the data were analysed in more detail.

As can be seen from Fig. 2(e), the initial amount of isovaleric acid in sweet wort (approximately 0.4 mg L^{-1}) originates from malt, increasing further (to approximately 0.6 mg L^{-1}) after the addition of hop. Its concentration in decoction beer slightly decreased after fermentation, probably as a result of utilization by yeast, and a rapid increase of the amount of isovaleric acid (approximately 1.2 mg L^{-1}) was observed in infusion brews after the fermentation step. Furthermore, it is well known that the formation of isovaleric acid during fermentation is likely coupled with leucine metabolism (Fig. 4).[23,24] Such findings prompted a detailed study of the composition of the original wort, namely with respect to amino acid content. Wort after infusion mashing contained a significantly ($P < 0.05$) higher concentration of most monitored amino acids (aspartic acid, glutamic acid, serine, histidine, cysteine, methionine, isoleucine, glycine, threonine, valine, phenylalanine). Leucine was not found in a significantly higher concentration, and PCA analysis revealed no correlation between isovaleric and butyric acid in beer and leucine in wort (Fig. 5). However, as can be seen in Fig. 5, the concentration of glutamic acid in wort, which is involved in many metabolic pathways of cells, correlates with the final amount of isovaleric acid in beer. In sum, changes in wort composition affect the course of fermentation, as described previously,[25] and the difference in the isovaleric acid concentration in final beer was not caused by different concentrations of leucine in wort.

As follows from the formation of isovaleric acid from leucine by yeast metabolism (Fig. 4), leucine initially is deaminated, which is followed by decarboxylation and a reduction or oxidation of not only the resulting isovaleric acid but also 3-methylbutanol (3-methylbutanol and 2-methylbutanol were determined as the sum of both alcohols because of their coelution; hereafter, only 3-methylbutanol is mentioned). This pathway primary takes the path directed to higher alcohol, when coenzyme NADH is

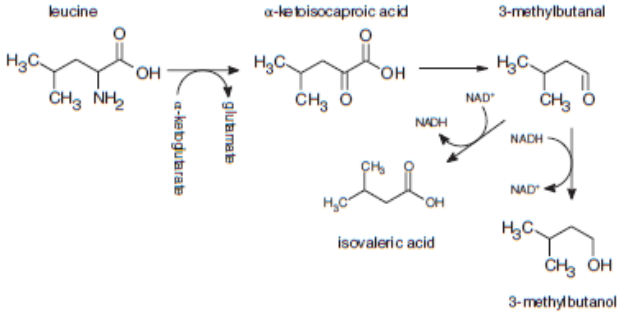

Figure 4. Catabolic pathway of leucine leading to isovaleric acid and 3-methylbutanol formation.

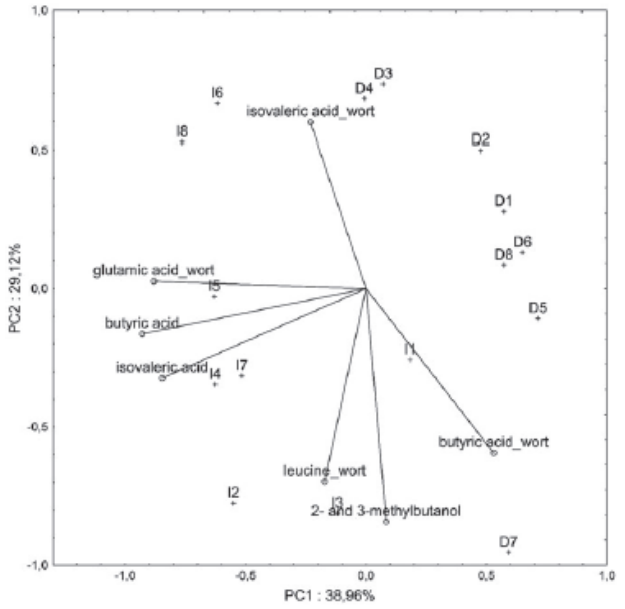

Figure 5. Principal component analysis biplot of selected compounds from wort and beer. The points represent beer samples (I, infusion; D, decoction).

regenerated back to NAD^+. Conversely, the oxidized form NAD^+ is required for isovaleric acid formation. It is assumed that the isovaleric acid/3-methylbutanol ratio formed during fermentation is given by the available NADH/NAD^+ ratio and that both of these pathways could contribute to the maintenance of redox balance in the cell.[26] The molar ratio of isovaleric acid to 3-methylbutanol in our beer samples was lower in decoction beer (Fig. 6). Because a significant difference in leucine concentration between infusion and decoction wort was not found, it can be assumed that the resulting difference in isovaleric acid concentration in the final beers is the consequence of a different redox state (NADH/NAD^+) in yeast cells. This was probably caused by the different or differently intensive metabolic pathways of yeast based on different composition of wort and the availability of nutrients. It can be assumed that the same metabolic change could be responsible for higher concentration of butyric acid in infusion beer.

The effect of glutamic acid on the resulting isovaleric acid concentration was tested in a laboratory fermentation experiment. Glutamic acid was added to wort (100 mg L^{-1}) and fermentation was performed in a 1-L special fermentation cylinder according to patent No. 303042.[27] The concentration of isovaleric acid

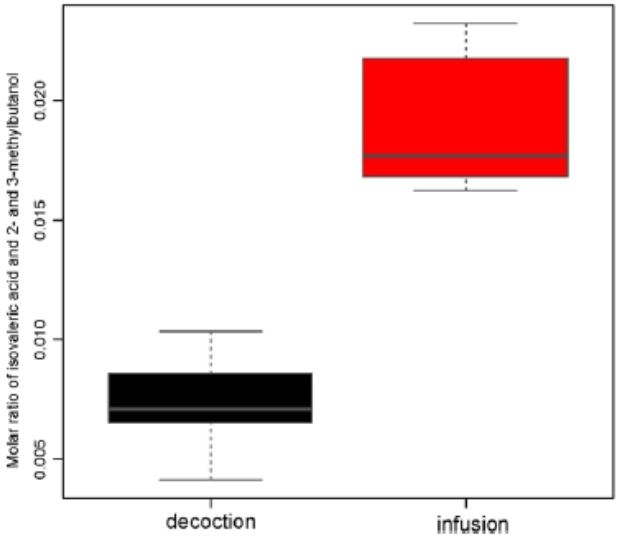

Figure 6. Molar ratio of isovaleric acid and 2- and 3-methylbutanol in beer from the decoction and infusion systems.

in the related final beers was compared with the concentration of isovaleric acid in control beers (without the addition of glutamic acid). No statistical difference was found between compared beers ($P < 0.05$; data not shown). Sensory analysis also found no statistical difference between the samples (the flavor of isovaleric acid was not detected by the sensory panel). Thus, the assumption that only glutamic acid can influence final concentration of isovaleric acid in beer was not confirmed. This result may be discussed in conjunction with the findings of the study by Yin *et al.*,[28] where glutamic acid was found to have a negative effect on total amino acid absorption by yeasts. Also, it can be assumed that glutamic acid addition lowered the absorption of amino acids, including leucine, the main agent of the Ehrlich pathway.

Naturally, the higher concentration of isovaleric acid could be caused by the use of oxidised hops[29] and alternatively by infection by *Dekkera bruxellensis* (earlier *Brettanomyces* species).[30,31] These sources were excluded because both types of experimental beer (decoction and infusion) were hopped in an identical way and the hops was controlled prior to the experiment. Furthermore, microbiological tests were performed during the experiment.

CONCLUSIONS

In conclusion, in the present study, the chemical profiles of FA during the brewing process were investigated. By contrast to barley variety and malt house, the mashing technology significantly influenced these profiles. Differences between infusion and decoction technology were found and these were compared using chemometric evaluation. These profiles differed with respect to the content of isovaleric and butyric acids in experimental beer and oleic acid in sweet wort (with all of them being higher for the infusion system). Although the concentrations of isovaleric acid were higher than the sensory threshold, its presence was not observed by sensory analysis. The difference in the amount of isovaleric acid was verified in commercial beers. These differences were studied in more detail by comparing the composition of wort. It is assumed that the production of isovaleric acid during fermentation is coupled with the redox state of yeast cell, which is given by the wort composition and therefore by the mashing process.

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