Food Chemistry 365 (2021) 130522

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

A high throughput toolbox for comprehensive flavor compound mapping in mint

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ARTICLE INFO

Keywords:
Mentha
LC-MS
SIDA
3–nitrophenylhydrazine
Glycidyltrimethylammonium chloride
APCI

ABSTRACT

Essential oils of the genus *Mentha* are extensively used as flavor ingredients in the industry. To overcome the time consuming and laborious traditional flavor analysis, a new quick, high–throughput toolbox based on a bead–beater homogenization followed by a UHPLC–MS/MS analysis has been developed and validated. While terpenes could be directly detected using atmospheric pressure chemical ionization (APCI), carbonyl compounds and alcohols required derivatization by 3–nitrophenylhydrazine (3-NPH) and glycidyltrimethylammonium chloride (GTMA) to ensure sufficient sensitivity for analysis of a single leaf. Using this approach, in total, 59 flavor-active metabolites representing the characteristic flavor of mint were quantified in leaves as well as in distilled oils using fast and robust UHPLC–MS/MS methods. The application of this toolbox enables a mapping of key pathways of mint flavor biosynthesis and can therefore support extensive breeding studies and the monitoring of chemosensate changes, depending on factors such as growth stages and environmental conditions.

1. Introduction

The genus *Mentha* is an important member of the Lamiaceae family and is represented by 18 species and 11 natural hybrids (Tucker & Naczi, 2007). It grows wild throughout the temperate regions of Europe, Asia, Africa, Australia and North America, and tolerates a wide range of agroclimatic conditions. Commercially the most important mint species are peppermint (*Mentha* × *piperita*), spearmint (*Mentha spicata* L.) and cornmint (*Mentha arvensis*). *Mentha* × *piperita* is a triple cross of *Mentha aquatica* L. and *Mentha spicata* L., whereas *Mentha spicata* L. is a cross of *Mentha suaveolens* and *Mentha longifolia* and known as native spearmint. Scotch spearmint is a hybrid of *Mentha arvensis* and *Mentha spicata L.* (Gobert et al., 2002; Morton, 1956).

In 2016, a total of 42,000 tons of mint were produced and it is increasing from year to year due to its economic importance for the food, cosmetic, confectionary and pharmaceutical industries. Because of climate change, diseases and pests, stagnant yields and increasing demand for natural ingredients, there is a need to ensure a sustainable and robust supply of this plant. For this reason, it is more important than ever to develop new methods which allow us to identify and quantify the most important aroma compounds in mint quickly and precisely. These methods can be used to support the breeding of new varieties and to monitor, optimize and identify differences during production, in order to help manage the enormously increasing demand of such a natural ingredient.

The traditional flavor approach starts with separation of the volatile aroma compounds from non-volatiles by steam distillation, solvent assisted flavor evaporation (SAFE) extraction (Engel et al., 1999) or simultaneous distillation–extraction (SDE) (Schultz et al., 1977) of a few grams of food material, before identification and quantitation of the volatile compounds by gas chromatography–mass spectrometry (GC–MS).

Research carried out in recent years has shown that only ~ 230 odorants out of a total of 10,000 reported volatiles (using bioresponse-guided techniques like aroma extract dilution analysis (AEDA) (Grosch, 2001; Schieberle, 1995)) are key aroma compounds, with no more than 3–40 key food odorants and 15–40 key tastants required to mimic the authentic flavor of specific food items (Dunkel et al., 2014).

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https://doi.org/10.1016/j.foodchem.2021.130522
Received 4 May 2021; Received in revised form 29 June 2021; Accepted 1 July 2021
Available online 4 July 2021

In recent years, research groups have repeatedly dealt with the subject of aroma analysis in mint, to identify and quantify the key aroma substances. Compounds which are primarily responsible for the refreshing and cooling sensation of peppermint include (–)–menthol, menthone, menthyl acetate and (+)–menthofuran, whereas (*R,S*)–carvone, 1,8–cineole, limonene, (*E,Z*)-2,6-nonadienal, (*E*)–β–damascenone and (3*E*,5*Z*)–1,3,5-undecatriene are the major flavor contributors to the aroma of spearmint (Kelley & Cadwallader, 2017; Rohloff, 1999; Schmidt et al., 2009).

To achieve an accurate quantitation of individual compounds in different concentration ranges present in e.g. fresh strawberry juice (Schieberle & Hofmann, 1997), stable isotope dilution analysis (SIDA) for highly volatile and structurally similar aroma compounds is the method of choice for both liquid chromatography–mass spectrometry (LC–MS) and GC–MS analysis using stable isotope labelled standards to compensate for any work-up losses (Schieberle & Grosch, 1987; Stark et al., 2011).

Because of high volatility, the chirality and the structural similarity of terpenes, the traditional analysis is performed by gas chromatography–flame ionization detector (GC–FID) and GC–MS (Giese et al., 2015; Jirovetz et al., 2002; Kelley & Cadwallader, 2017) but there are already first approaches to analyze volatile compounds like limonene, terpinolene, α-pinene, myrcene, linalool, α–humulene and β–caryophyllene by means of liquid chromatography and atmospheric pressure chemical ionization in cannabis products (Hyland et al., 2016).

Bhutani et *al.* used derivatization with dansyl chloride for the phenolic groups to quantitate vanillic acid and the aroma-active compound vanillin in guinea pig plasma by means of LC–MS/MS (Bhutani et al., 2018). Glycidyltrimethylammonium chloride (GTMA) was already established to derivatize dihydrobenzoic acids and non-reducing sugars through a nucleophilic reaction of the hydroxy group with the epoxide of the glycidyl of the reagent before analysis by means of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF-MS) (Gouw et al., 2002).

In 2019, Hofstetter et *al.* established a highly accurate, sensitive, high-throughput SIDA approach, which allows the quantitation of tastants and odorants in different apple juices in one method, without time-consuming and labor-intensive workups, using ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). By derivatization of the carbonyl compounds with 3–nitrophenylhydrazine (3–NPH), which was already used for sugars (Han et al., 2016) and short chain fatty acids (Han et al., 2015), they could develop an approach for small, hard to ionize and volatile compounds within a wide concentration range from nmol/L found for propyl–2–methylbutanoate up to mmol/L for fructose in apple juices (Hofstetter et al., 2019). Based on this broad applicable approach, a method for quantitation of odor-active and volatile 2–acetyl azaheterocycles was developed and validated (Bösl et al., 2021).

Due to the widespread use of mint oils as flavoring material and green alternative in the food industry, the aim of this study was to develop a sensitive, selective, accurate, absolute and especially a fast high-throughput toolbox, which allows the extraction of the key odorants of mint on a small scale by using only one single mint leaf or a few μL of mint oil, and the quantitation over a concentration range of up to 6 orders of magnitude. This allows one the opportunity to analyze hundreds of samples in a reasonable period of time, compared to the classical approach using SAFE extraction and GC–MS, in order to characterize the flavor profile of the large variety of the different *Mentha* species and to compare the flavor profile of the plant tissue with the corresponding mint oils.

2. Materials and methods

2.1. Chemical and samples

The following compounds were obtained commercially: 3-nitrophenylhydrazine hydrochloride, *N*-(3-(dimethylamino)-propyl)-*N*'-ethylcarbodiimide hydrochloride, glycidyltrimethylammonium chloride, scandium (III) triflate, ammonium acetate solution ~ 5 M in H_2O, (+)–pulegone, 1-octen-3-one, 3–methylbutanal, 2-methylbutanal (+)–menthone, (–)-menthone, citral, β–citronellal, β–pinene, α-pinene, (+)-menthofuran, β–caryophyllene, linalool, limonene, menthalactone, jasmone, neomenthol, (–)–menthol, (+)-menthol, 1-hexanol, 2–phenylethanol, eugenol, *p*-cresol, 1-octen-3-ol, heptanol, 1-penten-3-ol, 4–vinyl–2–methoxyphenol, β-citronellol, (+)-isopulegol, pinocarveol, (–)-isopulegol, dihydrocarveol, 3-methyl-1-buten-1-ol, (*E,Z*)–2,6-nonadien-1-ol, (*Z*)–3–hexen-1-ol, thymol, octanol, 1-pentanol, 1-menthen-9-ol, terpinolene, hexanal-d_{12} (Sigma-Aldrich, Steinheim, Germany), sodium hydroxide 1 M, pyridine (Merck, Darmstadt, Germany), phenylacetaldehyde, nonanal, *p*-cresol-d_8 (Abcr, Karlsruhe, Germany), (+)–isomenthone, sabinene, α–camphene, α-humulene, carvacrol (Extrasynthèse, Genay, France), 1,8–cineole, myrcene, β–damascenone, (*E,Z*)-2,6-nonadienal, (*R,S*)–carvone, hexanal, octanal, α–ionone, β–ionone, geraniol, 3–methyl-1-butanol (Givaudan, Vernier, Switzerland), miglyol (Caesar & Loretz GmbH, Hilden, Germany), 1-hexen-3-one (Alfa Aesar, Kandel, Germany). The isotope labelled standards α-pinene-d_3, (+)–menthofuran-$^{13}C_2$, caryophyllene-$^{13}Cd_2$, terpinolene-d_6, limonene-$^{13}Cd_2$, pulegone–d_6-d_8, vanillin-d_3, menthone-d_6-d_8, β-ionone-d_3, linalool-d_5, 1-octanol-d_5 and β–damascenone-d_4 were purchased from aromaLAB AG (Planegg, Germany). Solvents used for LC–MS/MS analysis were of LC–MS grade (Honeywell, Seelze, Germany). Methanol-d_4, acetonitrile–d_3, dimethyl sulfoxide-d_6 used for quantitative ^{1}H nuclear magnetic resonance (qHNMR) were purchased from Sigma-Aldrich (Steinheim, Germany). Water for chromatography was purified using a Milli-Q water advantage A 10 system (Millipore, Molsheim, France). The mint leaves and oils of Black Mitcham (*Mentha* × *piperita* L.), Arvensis (*Mentha arvensis*), Scotch spearmint (*Mentha* × *gracilis* L.) and Native spearmint (*Mentha spicata* L.) were provided by Mars Wrigley (Chicago, IL), UC Davis (Davis, CA) and Callisons (Lacey, WA). The mint plants were grown under field conditions and were of different sizes. Basil leaves were purchased from a local retailer (Munich, Germany).

The isotope labelled standards (*R,S*)-carvone-d_4, 1-hexen-3-one-d_2, nonanal-d_4, octanal-d_4, 1-octen-3-one-d_4, 3-methylbutanal-d_2, thymol-d_7, (*E,Z*)-2,6-nonadienal-d_2, citral–$^{13}C_2$, 1-octen-3-ol-d_4, 1-hexanol-d_4, 2–phenylethanol–d_5, citral–$^{13}C_2$, 3-methyl-1-butanol-d_2, 4–vinyl–2–methoxyphenol-d_3, β-citronellol-d_2, (*Z*)-3-hexenol-d_5 and geraniol–d_2 were synthesized and provided by Leibniz Institute for Food Systems Biology at the Technical University of Munich.

2.2. Sample preparation of mint leaves and mint oils

A defined volume (20 μL, each) of the three internal standard mixtures (concentration of the internal standard mixture (acetonitrile/water, 70:30, v/v) : IS 1: 1–hexen–3–one-d_2 (95.4 mg/L), pulegone-d_6-d_8 (509.1 mg/L), vanillin-d_3 (18.2 mg/L), nonanal-d_4 (12.3 mg/L), octanal-d_4 (8.2 mg/L), 1–octen–3–one-d_4 (4.00 mg/L), 3–methylbutanal-d_2 (93.7 mg/L), hexanal–d_{12} (5.81 mg/L), (*R,S*)-carvone–d_4 (164.7 mg/L), menthone-d_6-d_8 (231.5 mg/L), (*E,Z*)–2,6–nonadienal-d_2 (4.62 mg/L), β–ionone-d_3 (72.2 mg/L), citral–$^{13}C_2$ (7.02 mg/L), citronellal-d_2 (7.7 mg/L) IS 2: 1–hexanol-d_4 (72.0 mg/L), 2–phenylethanol–d_5 (14.34 mg/L), 3-methyl-1-butanol-d_2 (217.4 mg/L), *p*–cresol-d_8 (588.0 mg/L), 4-methyl–2-methoxyphenol-d_3 (8.34 mg/L), geraniol-d_2 (10.35 mg/L), β-citronellol-d_2 (80.0 mg/L), (*Z*)-3-hexen-1-ol-d_5 (14.55 mg/L), thymol-d_7 (10.4 mg/L), 1-octanol-d_5 (71.43 mg/L), linalool-d_5 (114,0.3 mg/L), 1–octen–3–ol–d_4 (180.0 mg/L) IS 3*:* α-pinene-d_3 (328.6 mg/L), (+)–menthofuran-$^{13}C_2$ (900.0 mg/L), caryophyllene–$^{13}Cd_2$ (714.3 mg/L), terpinolene–d_6 (25.6 mg/L), linalool–d_5 (71.43 mg/L), limonene-$^{13}Cd_2$ (142.8 mg/L), *β*–damascenone–d_4 (50.0 mg/L), (*R,S*)–carvone-d_4 (164.6 mg/L)) were added to fresh mint leaves (0.3 g) and filled up with acetonitrile to 1 mL. After extractive grinding (9000 rpm for 3 × 30 s and 30 s breaks) using the bead -beater (Precellys evolution homogenizer; Bertin Technologies, Montigny Le Bretonneux, France) with extraction

tubes (2 mL, ceramic balls: ck mix 1.4/2.8 mm) and equilibration (1 h), the suspension was centrifuged (10 min, 10000 rpm) using an Eppendorf Centrifuge Minispin (Eppendorf, Hamburg, Germany). The supernatant was membrane filtered (Minisart RC 15, 0.45 μm; Sartorius AG, Göttingen, Germany) before derivatization and analysis.

Prior to the quantitation the mint oils were diluted with acetonitrile for analysis of carbonyl compounds (1:100) and for the alcohols (1:250). For the analysis of terpenes by means of atmospheric pressure chemical ionization (APCI), the mint oils were diluted with methanol (1:7500).

2.3. UHPLC–MS/MS analysis

The samples were separated by means of an ExionLC (Sciex, Darmstadt, Germany), consisting of two LC pump systems ExionLC AD Pump, an ExionLC degasser, an ExionLC AD autosampler, an ExionLC AC column oven, and an ExionLC controller. The LC was connected to a QTRAP 6500+ mass spectrometer (Sciex, Darmstadt, Germany) controlled by the Analyst software (version 1.6.3; Sciex, Darmstadt, Germany). Detection was achieved using an electrospray (ESI) and APCI source. Data interpretation was performed using MultiQuant software (version 3.0.2; Sciex, Darmstadt, Germany; Peak model: Signal Finder).

2.4. Quantitation of aldehydes and ketones after 3-NPH derivatization

Following a modified method reported for the analysis of short- and branched-chain fatty acids (Han et al., 2015) and for aroma compounds (Hofstetter et al., 2019), the membrane-filtered extracts (40 μL) or diluted oils (40 μL, 1:100) were mixed with a solution of 3-nitrophenylhydrazine (3–NPH, 20 μL, 200 mmol/L) in acetonitrile/water (50:50; v/v), and *N*–(3–(dimethylamino)propyl)-*N'*-ethylcarbodiimide solution (EDC, 20 μL, 120 mmol/L) in acetonitrile/water (50:50; v/v) containing pyridine (6%). For the quantitation of mint oils IS 1 (20 μL, 1:10; v/v) was added to a safe-lock tube (1.5 mL). After heating in a thermo-shaker (40 °C, 600 rpm, 30 min; Thermo-mixer C, Eppendorf, Hamburg, Germany), the solution of the extracts was made up with acetonitrile/water (70:30; v/v) up to 250 μL and for oils up to 1 mL and then an aliquot (1 μL) was injected into the UHPLC–MS/MS system.

IS Calibration Curve A. A stock solution for the quantitation of 1-hexen-3-one (426.6 μmol/L), (+)-pulegone (2454 μmol/L), (+)-isomenthone (2041.2 μmol/L), vanillin (141.0 μmol/L, leaf: 1410 μmol/L), nonanal (549.6 μmol/L), octanal (989.8 μmol/L), 1–octen-3-one (60.0 μmol/L), isovalerate (28.9 μmol/L, leaf: 43.4 μmol/L), 3–methylbutanal (275.0 μmol/L), 2–methylbutanal (165.0 μmol/L), hexanal (409.8 μmol/L), (*R,S*)-carvone (138740 μmol/L), (+)-menthone (46263.7 μmol/L, leaf: 57829.6 μmol/L), (–)-menthone (43515.6 μmol/L, leaf 54394.45 μmol/L), (*E,Z*)–2,6–nonadienal (87.5 μmol/L), α-ionone (234.2 μmol/L), β-ionone (131.2 μmol/L), phenylacetaldehyde (513.4 μmol/L, leaf 51.34 μmol/L), citral (177.0 μmol/L), β–citronellal (18.04 μmol/L) were prepared in acetonitrile (1 mL) and the exact concentrations of each reference compound was verified by means of qHNMR in acetonitrile-d_3 (Frank et al., 2014). This stock solution was then sequentially diluted 1 + 1. To each dilution IS 1 (20 μL), in accordance 1 + 9 diluted for the oils, were added before derivatization.

2.4.1. Recovery experiments and repeatability of the carbonyl compounds

For the recovery experiments of mint oils, the analytes were spiked into an analyte-free mixture of medium chain triglycerides (MCT oil) using the concentration ranges of the IS calibration curve A each as triplicates. After addition of IS 1 (20 μL, 1:10) the samples were prepared using the instructions above.

For the recovery experiments in mint leaves, IS 1 (20 μL) and the analytes were spiked to ground mint material (0.3 g) using the concentration ranges of the IS calibration curve A as triplicates. For the characteristic compounds menthone, (*R,S*)–carvone, (+)–menthofuran, menthalactone freshly ground basil leaves were spiked with the concentration ranges of the IS calibration curve A as described above (each as biological triplicates). For the determination of the limits of detection (LoD) and the limit of quantitation (LoQ) the standard solution was further diluted. For the LoD the signal-to-noise was set to a ratio of 3, and for the LoQ to a ratio of 9.

2.4.2. LC–MS/MS analysis of 3–NPH derivatized carbonyl compounds

The 3–NPH derivatized samples were separated on a C18 column (100 × 2.1 mm, 1.7 μm; Kinetex, Phenomenex, Aschaffenburg, Germany). The following gradient of formic acid (0.1%) in water (solvent A) and formic acid (0.1%) in acetonitrile (solvent B) at a flow rate of 0.4 mL/min was used for the separation: 0–1 min, 30% B; 5.5 min, 85% B; 6 min, 85% B; 7 min, 100% B; 8 min, 100% B; 9–10 min, 30% B. The method was operated in the negative multiple reaction monitoring (MRM) mode (low mass, ion spray voltage: –4500 V) using the following instrument settings: curtain gas (35 psi), temperature (450 °C), gas 1 (55 psi), gas 2 (65 psi), collision activated dissociation (–2 V), and entrance potential (–10 V).

2.5. Quantitation of alcohols after GTMA derivatization

The membrane-filtered extracts (40 μL) or diluted oils (40 μL, 1:250) were mixed with glycidyltrimethylammonium chloride (GTMA, 40 μL, 1:2 diluted with water), sodium hydroxide (NaOH, 15 μL, 1 M) and scandium(III) triflate (15 μL, 2 mM). For the quantitation of alcohols in mint oils, IS 2 (20 μL, 1:10 diluted with acetonitrile) was added prior to derivatization in a 1.5-mL safe-lock tube. After heating in a thermo-shaker (55 °C, 600 rpm, 2 h; Thermo-mixer C, Eppendorf, Hamburg, Germany), the solution of mint oils was made up with acetonitrile/water (70:30, v/v) to 1 mL and for mint leaves extracts to 500 μL and then an aliquot (1 μL) was injected into the UHPLC–MS/MS system.

IS Calibration Curve B. Stock solutions of neomenthol (2356.7 μmol/L), (–)–menthol (76871.8 μmol/L), (+)-menthol (15280.4 μmol/L), 1-hexanol (786.6 μmol/L), 2-phenylethanol (643.05 μmol/L), 3-methyl-1-butanol (592.4 μmol/L), eugenol (2087.2 μmol/L), *p*-cresol (1064.0 μmol/L), 1-octen-3-ol (5556.0 μmol/L), 1-heptanol (190.1 μmol/L), 1–penten-3-ol (724.1 μmol/L), 4-vinyl-2-methoxyphenol (72.8 μmol/L), linalool (793.0 μmol/L, leaf: 634.4 μmol), geraniol (275.0 μmol/L), β–citronellol (577.0 μmol/L), (–)-isopulegol (1895 μmol/L), (+)-isopulegol (733.6 μmol/L), pinocarveol (2335 μmol/L), dihydrocarveol (1004.9 μmol/L), 3–methyl-1-buten-1-ol (1098.0 μmol/L), (*E,Z*)–2,6–nonadien-1-ol (569.9 μmol/L), (*Z*)–3–hexen-1-ol (961.4 μmol/L), thymol (1807.7 μmol/L), carvacrol (1808 μmol/L), 1-octanol (687.5 μmol/L), 1-pentanol (832.4 μmol/L), 1-menthen-9-ol (458.4 μmol/L) were prepared in 1 mL DMSO-d_3 and the exact concentration of each reference compound was verified by means of qHNMR (Frank et al., 2014) This stock solution was then sequentially diluted 1 + 1. To each dilution the same amount of IS 2 (20 μL) was added.

2.6. Recovery experiments and repeatability of GTMA derivatized alcohols

For the recovery experiments, the analytes were spiked into an analyte-free mixture of medium chain triglycerides (1:250) using the concentration ranges of the IS calibration curve B each as triplicates. After addition of IS 2 (20 μL, 1:10) the samples were prepared using the instructions above.

For the recovery experiments in mint leaves, IS 2 (20 μL) and the analytes were spiked to ground mint material (0.3 g) using the concentration ranges of the IS calibration curve B as triplicates. For the characteristic compound menthol fresh ground basil leaves were spiked with the concentration ranges of the IS calibration curve B as described above as triplicates. For the determination of the LoD and the LoQ the standard solution was further diluted. For the LoD the signal-to-noise was set to a ratio of 3, and for the LoQ to a ratio of 9.

2.6.1. LC–MS/MS analysis of GTMA-derivatized alcohols

The samples were separated by a pentafluorophenyl column (100 × 2.1 mm, 1.7 μm; Kinetex, Phenomenex, Aschaffenburg, Germany). A gradient of formic acid (0.1%) in water (solvent A) and formic acid (0.1%) in acetonitrile (solvent B) at a flow of 0.4 mL/min was used for the separation: 0–1.5 min, 5% B; 5 min, 32% B; 6 min, 32% B; 7 min, 65% B; 8–9 min, 100% B; 11–12 min, 5% B. Analysis was in positive multiple reaction monitoring (MRM, low mass) mode (ion spray voltage: 4500 V) using the following instrument settings: curtain gas (35 psi), temperature (450 °C), gas 1 (55 psi), gas 2 (65 psi), collision activated dissociation (2 V), and entrance potential (10 V).

2.7. Quantitation of terpenes by means of APCI

The membrane filtered extract was diluted 1:25 with acetonitrile and the oil was diluted 1:7500 with MeOH and each solution was spiked with 20 μL of IS 3 (1:10 diluted with acetonitrile). An aliquot (1 μL) was directly injected into the UHPLC–MS/MS system.

IS Calibration Curve C. Stock solution of β-pinene (951.2 μmol/L, leaf: 1160 μmol/l), α-pinene (2048.4 μmol/L, leaf: 2205.9 μmol/L), (+)-menthofuran (677.8 μmol/L, leaf: 726.2 μmol/L), β-caryophyllene (607.1 mmol/L leaf: 155.7 μmol/L), myrcene (717.13 μmol/L, leaf: 2151.5 μmol/L), *1,8*-cineole (1837.4 μmol/L, leaf: 2041.6 μmol/L), terpinolene (42.94 μmol/L, leaf: 107.37 μmol/L), linalool (80.66 μmol/L, leaf: 1008.3 μmol/L), limonene (405.6 μmol/L, leaf: 5290 μmol/l), β–damascenone (18.8 μmol/L, leaf: 94.2 μmol/L), sabinene (204.5 μmol/L, leaf: 613.6 μmol/L), α–camphene (104.8 μmol/L, leaf: 108.4 μmol/L), α–humulene (69.2 μmol/L leaf: 415.2 μmol/L), (*R,S*)–carvone (961.0 μmol/L, leaf: 9610.0 μmol/L), menthalactone (24.6 μmol/L, leaf: 122.9 μmol/L), jasmone (105.9 μmol/L) were prepared in methanol (1 mL) and the exact concentration of each reference compound was verified by means of qHNMR solved in methanol-d_4(Frank et al., 2014). This stock solution was then sequentially diluted 1 + 1. To each dilution the same amount of IS 3 (20 μL) was added.

2.7.1. Recovery experiments and repeatability of terpenes

For the recovery experiments, the analytes were spiked into an analyte-free mixture of medium chain triglycerides (1:7500) using the concentration ranges of the IS calibration curve C each as triplicates. After addition of IS 3 (20 μL, 1:10) the samples were prepared using the instructions above.

For the recovery experiments in mint leaves, IS 3 (20 μL) and the analytes were spiked to ground mint material (0.3 g) using the concentration ranges of the IS calibration curve C as triplicates. For the determination of the LoD and the LoQ the standard solution was further diluted. For the LoD the signal-to-noise was set to a ratio of 3, and for the LoQ to a ratio of 9.

2.7.2. LC–MS/MS analysis of terpenes

The samples were separated on a C18 column (150 × 2.1 mm, 1.7 μm; Kinetex Phenomenex, Aschaffenburg, Germany). A gradient of formic acid (0.1%) and NH_4Ac (2 mM) in water (solvent A) and formic acid (0.1%) and NH_4Ac (2 mM) in methanol (solvent B) at a flow of 0.4 mL/min was used for separation: 0–1 min 30% B; 2.5 min, 50% B; 6 min, 75% B; 7 min, 80% B; 10.5 min, 90% B; 12–13 min, 100% B; 14–15 min, 30%. Detection was operated in the positive MRM APCI mode (ion spray voltage: 5500 V) using the following instrument settings: curtain gas (35 psi), temperature (500 °C), gas 1 (55 psi), gas 2 (0 psi), collision activated dissociation (2 V), and entrance potential (10 V).

2.8. Quantitative 1H nuclear magnetic resonance spectroscopy (qNMR)

Quantitative proton NMR-spectroscopy (qHNMR) was recorded on a 400 MHz Avance III spectrometer (Bruker, Rheinstetten, Germany) equipped with a Broadband Observe BBFO plus Probe (Bruker, Rheinstetten, Germany). Methanol-d_4, acetonitrile-d_3, dimethyl sulfoxide-d_6 (600 μL) were used as solvents and chemical shifts are reported in parts per million relative to the methanol-d_4 solvent signal. Data processing was performed using Topspin NMR software (version 3.2; Bruker, Rheinstetten, Germany). Quantitative NMR spectroscopy (qHNMR) was performed as reported earlier through calibration of the spectrometer by applying the ERETIC 2 tool using the PULCON methodology (Frank et al., 2014).

2.9. Statistical analysis

The quantitative data was visualized as a heatmap using the visualization platform R (version 4.0.3) and the pheatmap package (version 1.0.12) (Kolde, 2019; Team, 2018). Clusters were formed according to Ward's minimum variance method, while for cluster analysis Euclidean distances were applied as distance measurement (Ward Jr, 1963). LoD and LoQ values and ratios of LoQ/thresholds were visualized using ggplot (3.3.3) package (Wickham et al., 2016).

The chemical similarity network was obtained from the pairwise similarity matrix calculated using Pubchem Fingerprints (Kim et al., 2016) and the Tanimoto similarity coefficient. After conversion to a dissimilarity matrix, visualization was performed using Gephi (version Gephi 0.9.3-SNAPSHOT 202004172136) (Bastian et al., 2009) and the following parameters: edge weight cutoff 0.7, ForceAtlas 2 layout, tolerance 1.0, approximation 1.2, scaling 300, edge weight influence 1.0, gravity 1.0, and the stronger gravity, dissuade hubs, and prevent overlap options. The connection indicates the significant correlation between the nodes. The nodes and connections of the same color were affiliated with the same compound class. The Tanimoto coefficient ranges from 0 when the fingerprints have no bits in common, to 1 when the fingerprints are identical.

3. Results and discussion

To meet the new requirements of a high-throughput analysis, a new method for extraction and quantitation of the key aroma compounds in single mint leaves without any time and laborious consuming steps was developed by using a high-throughput extraction approach. In the past, many research groups worked on the aroma profiles of the different mint species and the majority of the key aroma compounds are already identified. In total, 59 aroma actives were selected to be important and contributing to the specific aroma or unpleasant off-flavors. To visualize the chemical similarities and differences between these 59 compounds, a network structure was constructed (Fig. 1). According to their compound classes the aroma compounds were sorted and color-coded. As displayed in Fig. 1, a large number of different structures and functional groups are characterizing the overall aroma of mint. In addition to ketones and aldehydes such as menthone (**13**), (*R,S*)–carvone (**12**) and hexanal (**11**), alkenyl, aliphatic and phenolic alcohols are moreover contributing to the aroma of mint. Another compound class of major importance for mint is represented by terpenes, like monoterpenes (e.g., **46**, **53**, **55**), sesquiterpenes (**49**, **57**) and bicyclic terpenes (**48**, **58**). They are defined by a carbon skeleton derived from isopentenyl pyrophosphate (IPP) and formed in the terpene biosynthesis pathway (Ruzicka, 1953).

However, for analytics, it is not always the chemical similarity of the compounds that is important to ensure separation and detection. Derivatizable functional groups are of greater relevance to adjust analyte properties of such highly volatile compounds to perform UHPLC–MS/MS analysis and to enable liquid chromatographic separation, improvement of detection and to enhance sensitivity as well as accuracy of the method. By subdivision of the 59 selected analytes according to their functional groups, there are mainly carbonyl compounds, alcohols and terpenes, for which an extraction, liquid chromatographic separation and detection approach has to be developed by means of UHPLC–MS/MS, in order to characterize the aroma profiles in different mint species.

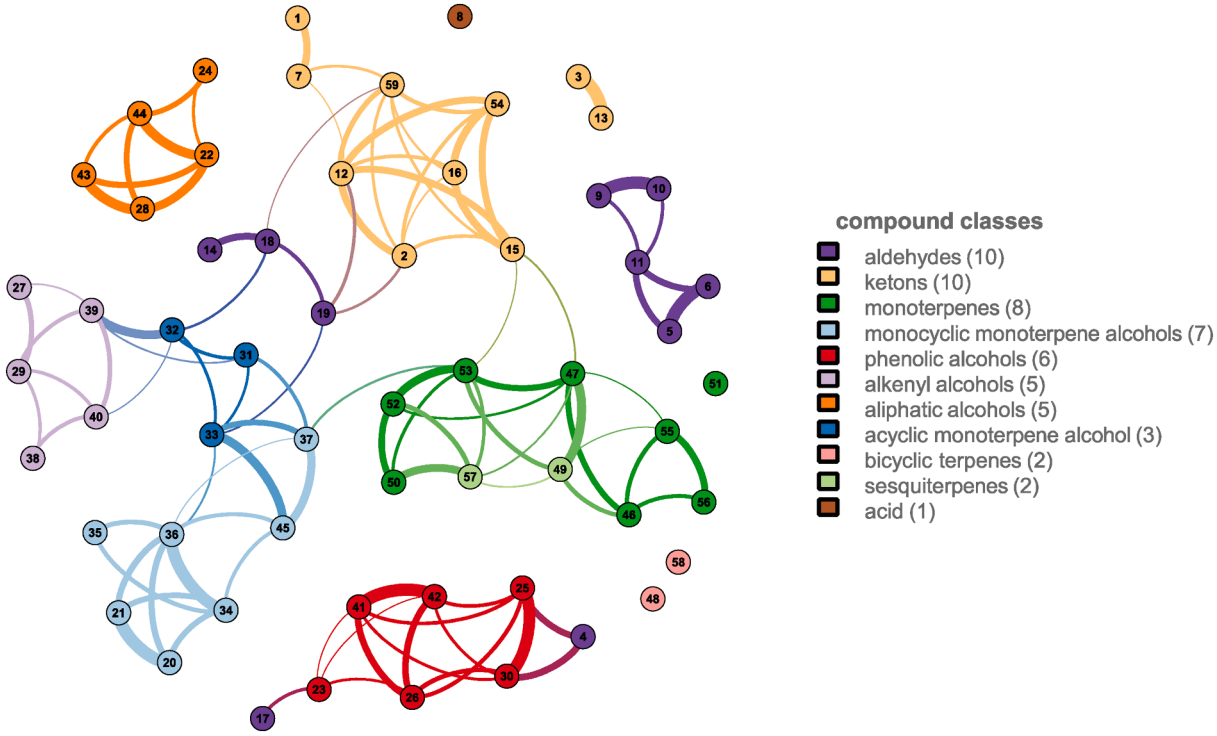

Fig. 1. Chemical similarity network. Network describing the chemical similarity of the compounds of the analyte classes. Thickness of connecting lines represents the size of the correlation coefficient between the chemical similarity of the structures.

3.1. Sample Workup, method development and Validation experiments

3.1.1. Sample workup

The traditional and very time consuming and labor-intensive aroma approach usually starts with a separation of the volatile fraction from the nonvolatile compounds, e.g., by SAFE extraction, SDE or steam distillation of a few grams of plant material to extract a sufficient quantity of aroma compounds for further GC-analysis. While the most common solvents for SAFE extraction are diethyl ether, pentane, dichloromethane (Engel et al., 1999), solvents like, e.g., methanol, acetonitrile, isopropanol and water meet the requirements of an extraction by means of a bead-beater-homogenizer and subsequent UHPLC separation. In order to extract non-polar terpenes as well as polar alcohols and to avoid potential reactions of derivatization reagents with hydroxy groups of solvents such as methanol, mint leaves were preferably extracted with acetonitrile.

This new extraction approach for aroma compounds by means of a bead-beater homogenizer requires a minimum of 0.3 g mint material – roughly the equivalent of one mature mint leaf – and a solvent volume of just 1.0 mL acetonitrile. This proved to be enough material to identify and quantify the main aroma compounds in the huge variety of *Mentha* species.

Lysing kits with a volume of just 2 mL and mixed ceramic beads were perfectly suited for this application. Compared to the traditional workup, this approach significantly required less sample material, is much faster and therefore enables a high-throughput workup of hundreds of samples in a comparable short period. Mint oils did not require any further sample workup and could directly be used after a dilution step for derivatization (alcohols, ketones and aldehydes) or direct (terpenes) UHPLC–MS/MS analysis.

Using SIDA with (^{13}C, ^{2}H)–labeled molecules as internal standards, workup losses are considered based on the molar ratio. Due to limited commercial availability of some stable isotope labeled standards, a compound isotopically similar in structure was used for quantification purposes: e.g. 3-methylbutanal-d_2 was used to quantitate 2–methylbutanal (**10**) and isovalerate (**8**) and 1-octanol-d_5 was used as standard for 1-pentanol (**44**) and 1-heptanol (**28**) (Table S1–S3 in Supplementary Material).

3.1.2. Derivatization of aroma compounds by 3–NPH and GTMA

To make the volatile carbonyl compounds **1**–**19** more suitable for UHPLC–MS/MS analysis, a derivatization step by 3–NPH, already reported for aroma compounds in apple juice, was required (Hofstetter et al., 2019). After optimization of the derivatization parameters, a highly sensitive MS detection over a large concentration range could be achieved for compounds **1**–**19** (Fig. 2 A). Using the derivatization reagent glycidyltrimethylammonium chloride (GTMA) active hydrogens of the hydroxy groups of the alcohols were modified and chemical properties of the alcohols **20**–**45** were adjusted to ensure an UPHLC–MS/MS analysis (Fig. 2 B). For detection and quantification of the alcohols in mint oils or extracts, a derivatization using GTMA for 2 h at 55 °C was fully adequate to generate chemical modifications of primary alcohols such as **40** as well as secondary alcohols like **20** and **21** or tertiary alcohols like **31** and **37**. This reaction was catalyzed by the Lewis acid scandium(III) triflate, which has the ability to activate electron-rich functional groups such as imides or carbonyls and, in contrast to other catalysts, shows a high stability in aqueous solutions (Kobayashi, 1999). With this approach, in total 35 out of the 59 aroma compounds were modified by 3–NPH or GTMA respectively and it enables the conversation of volatile aroma-active into non-volatile compounds, which then could be easily separated by liquid chromatography and detected by mass spectrometry.

3.1.3. Optimization of UHPLC–MS parameters

In comparison to aldehydes, ketones or alcohols, terpenes are non-polar compounds with no functional group for derivatization. Therefore, atmospheric pressure chemical ionization (APCI) in positive mode was deemed the ionization method of choice for the determination of compounds **45**–**59** (Fig. 2 C), whereas 3–NPH derivatized carbonyl compounds (**1**–**19**) and GTMA-derivatized alcohols (**20**–**45**) were

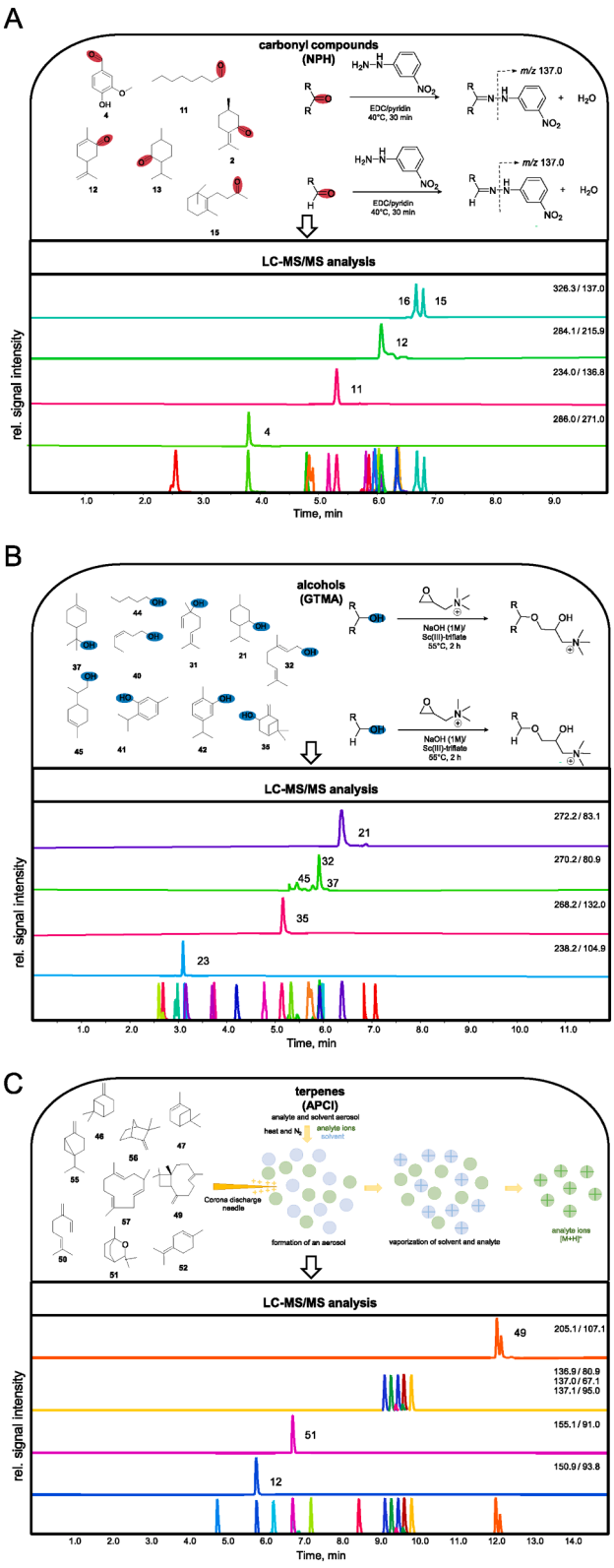

Fig. 2. High-throughput toolbox for comprehensive flavor compound mapping in mint. A Derivatization of carbonyl compounds with 3–NPH and the catalyst EDC and mass transition of 4, 11, 12, 15 and 16. B Derivatization of alcohols with glycidyltrimethylammonium chloride (GTMA) using scandium(III) triflate as the catalyst in a basic milieu and mass transition of 23, 35, 45, 32, 37 and 21. C Direct UHPLC–MS/MS analysis of terpenes by means of atmospheric pressure chemical ionization (APCI) and mass transitions of 12, 51, 55, 56, 50, 47, 53, 52, 46 and 49. The signal intensity of each mass transition is normalized.

efficiently ionized with an electrospray ionization (ESI) source in negative mode (3-NPH) and in positive mode (GTMA). Due to their chemical structure, linalool (**31**) and (*R*,*S*)–carvone (**12**) could be analyzed after the respective derivatization step by ESI as well as without any further modification of the functional groups using APCI. Due to higher sensitivity, for quantification ESI mode was preferred for these two compounds.

To facilitate appropriate detection *via* MS/MS, ionization parameters were optimized by continuous infusion of solutions of the reference compounds **1**–**59** and the stable isotope labeled standards into the MS/MS system to optimize the ion intensities by means of software-assisted ramping of the ion source and ion path potentials in order to analyze the 59 compounds by using the multiple reaction monitoring (MRM) mode (Table S1-S2 in Supplementary Material). For quantitation, the most intensive mass transitions of the individual compounds were used, and the second mass transition was selected for peak qualification of the target analytes. To prevent a saturating of compounds **12** and **13** due to comparative high concentrations in the samples, non-optimized declustering potential values for MRM transitions were used.

By comparison of the retention time and MS/MS transitions 19 carbonyl compounds including the isomers α– and β–ionone (**15**, **16**) as well as menthone (**13**) and (+)–isomenthone (**3**), were separated after 3–NPH derivatization on a C18 column.

In contrast to the separation of the carbonyl compounds and terpenes on a standard C18 column, a pentafluorophenyl phase was suitable for the separation and analysis of the by GTMA modified alcohols **20**–**45**. The mechanism of interaction and separation is based on a neutral/hydrophobic retention of the carbon skeleton of linker and aromatic ring. A π–π interaction of the electron deficit carbon ring with the π–electrons of the analytes, e.g., carvacrol (**42**), dipole moments caused by the high electronegativity of the fluorine groups aiding in polar compound retention and at least a hydrogen bonding based on an interaction of the electron greedy fluorine with the polar functional group of the alcohols (Euerby et al., 2003; Pellati & Benvenuti, 2008; Reta et al., 1999; Schindler et al., 2011).

Since the monoterpenes **46**, **47**, **50**, **52**, **53**, **55** and **56** are formed of isoprene (C5) units biosynthetically, the chemical structures are very similar and form identical pseudomolecular ions of $[M + H]^+ \rightarrow m/z$ 137.0 and respond to the same mass transition m/z 137.0 → 80.9, m/z 137.0 → 95.0 and m/z 137.0 → 67.1, appropriate chromatographic separation was essential to enable accurate results (Fig. 2 C). For every terpene, the most intensive mass transition was used for quantification and chromatographic separation was achieved using a C18-reversed column. Due to the similar structures, identical mass transitions and insufficient chromatographic separation of the seven monoterpenes, a spectral overlap for these compounds could not be prevented. Through the co-elution of terpinolene (**52**) and limonene (**53**) as well as β–pinene (**46**) and camphene (**56**) a unified quantitation of these compounds was performed.

To summarize, by comparison of retention time and MS/MS transitions, 19 carbonyl compounds could be analyzed in 10 min (ESI^-), 26 alcohols in a 12 min run (ESI^+) and 16 terpenes within 15 min using atmospheric pressure chemical ionization ($APCI^+$).

As mentioned previously for highly precise and accurate results, the quantitation was performed by stable isotope dilution analysis (SIDA) using internal standards added to mint leaves or to the mint oils prior to sample workup and measurement. For analytes without corresponding stable isotope labeled standard, structural similar standards were selected. For quantitative analysis calibration standards were prepared with a fixed concentration of IS 1–3 and serial dilution of the calibration standards were analyzed as replicates. The linear regressions were calculated from the area ratios *versus* their concentration ratios. An excellent linearity and precision were achieved for all 59 compounds through accurate quantitative determination of the stock solutions using the qHNMR technique (Frank et al., 2014) (Table S4–S6 in Supplementary Material).

3.1.4. Method validation experiments

For the recovery experiments, additional amounts at three concentrations levels covering the whole calibration range were added to ground mint leaves. Due to the naturally occurring high concentrations of menthone (**13**), menthol (**21**) and (+)–menthofuran (**48**) in ground mint leaves, fresh basil leaves as an alternative matrix were spiked with additional amounts of these three compounds to calculate the recovery rates.

For the mint oils, the recovery rates were calculated for the 59 analytes by spiking an analyte free mixture of medium chain triglycerides with **1**–**59** in concentrations covering the entire concentration range. After extraction of the mint leaves, dilution of the mint oils and derivatization by means of 3–NPH and GTMA, each solution was analyzed by SIDA–UHPLC–MS/MS analysis and recovery rates were calculated (Table S7–S9 in Supplementary Material). The ranges of the recovery rates of all three analyte classes in mint oils were between 80.9% for 1,8–cineole (**51**) and 121.7% for α–terpineol (**37**) and in mint leaves between 78.5% for *p*–cresol (**26**) and 133.7% for β–caryophyllene (**49**), excluding (+)–menthofuran (**48**) with a recovery rate of only 37%, explained by the tendency of its autoxidation (Woodward & Eastman, 1950).

Also, the limits of detection (LoD) and limits of quantitation (LoQ), shown in Fig. 3 A–B were calculated for **1**–**59**. For the carbonyl compounds the LoDs ranged from 0.0002 μmol/L (**8**), over 0.018 μmol/L (**19**) to 8.468 μmol/L (**12**). The LoQs of the carbonyl compounds were in a range of 0.0005 μmol/L (**8**) – 16.936 μmol/L (**12**). LoDs of the derivatized alcohols were higher compared to the carbonyl compounds and between 0.003 μmol/L (**23**) and 0.671 μmol/L (**43**), while LoQ values were determined in a range of 0.006 μmol/L (**23**) to 1.343 μmol/L (**43**). For the secondary alcohols higher LoD and LoQ values were evaluated, such as 2.812 μmol/L (LoD) and 5.625 μmol/L (LoQ) for menthol (**21**) and 4.603 μmol/L (LoD) and 9.206 μmol/L (LoQ) for neomenthol (**20**), due to a lower detector response. Besides that, the LoD and LoQs of the 16 terpenes detected directly by means of APCI were in the same range compared to the other two compound classes, ranging from 0.004 μmol/L (LoD) and 0.017 μmol/L (LoQ) for jasmone (**59**) over 0.051 μmol/L (LoD) and 0.101 μmol/L (LoQ) for β–caryophyllene (**49**) to 1.401 μmol/L (LoD) and 2.801 μmol/L (LoQ) for myrcene (**50**). Therefore, 26 out of the 59 compounds, e.g., hexanal (**11**), 2–phenylethanol (**23**) and α–humulene (**57**), could be quantified by means of UHPLC–MS/MS below their odor thresholds in water (Fig. 3 C). Eleven compounds, like α–ionone (**15**) and jasmone (**59**), were in the same range of the odor thresholds and of the remaining compounds such as menthone (**13**), β–citronellol (**33**) and limonene (**53**), the LoQ values were higher than the odor thresholds. Nevertheless, all 59 aroma compounds could be analyzed by means of UHPLC–MS/MS without any enrichment of the extracts. On the contrary, the mint oils required a dilution step to ensure accurate results.

These data clearly show that the extraction of aroma compounds by means of a bead-beater homogenizer in combination with the three developed UHPLC–MS/MS methods is a reliable toolbox for a rapid and accurate quantification of 59 aroma compounds in mint leaves as well as in mint oils of different *Mentha* species.

3.2. Quantitation of the 59 aroma compounds in four different species of the genus Mentha and the corresponding oils.

The motivation for developing this new extraction approach for volatile compounds in combination with modern UHPLC–MS/MS methods was to characterize the aroma composition of each mint species by the analysis of one single mint leaf, in order to avoid the traditional and crucial, as well as time and cost intensive distillation step of the mint oils prior to analysis. It therefore enables a high-throughput analysis of hundreds of samples in a short time and provides a great toolbox for the support of, e.g., breeding programs. Changes in the composition of the aroma compounds caused by the distillation process of the mint oils as compared to the mint leaves will be shown in later studies.

In the following, single mint leaves of the four main and commercially important mint species Black Mitcham (*Mentha* × *piperita* L.), Arvensis (*Mentha arvensis*), Scotch and Native spearmint (*Mentha spicata* L.) and steam distilled mint oils from the same mint leaf material were analyzed as biological quadruplicates. Native spearmint is considered to be a hybrid of *Mentha longifolia* and *Mentha suaveolens*, Scotch spearmint a hybrid of *Mentha arvensis* and *Mentha spicata*, and *Mentha* × *piperita* L. a hybrid of *Mentha aquatica* and *Mentha spicata*. Due to the complexity of this genus and the great readiness for hybridization, each mint variety shows a characteristic qualitative and quantitative composition of aroma compounds and therefore represents a major challenge for analytics.

The results obtained from the analysis with the toolbox are visualized in a heatmap that was combined with hierarchical agglomerative clustering of the compounds and is shown in Fig. 4 A. The cluster analysis, visually displayed as a dendrogram, quantifies the degree of similarity between the aroma compounds by calculating the distance between all possible pairs of molecules. The closer the aroma compounds are to each other in the dendrogram, the similar are the concentrations in the analyzed samples, e.g., menthone (**13**) and (*R*,*S*)-carvone (**12**) with a concentration range of 0.14 g/mL (**13**) to 0.44 g/mL (**12**), and α–ionone (**15**) and citral (**18**) in a range of approximately 20.0 μg/mL in the mint oils.

The traditional aroma analysis by GC–MS allows one to capture large

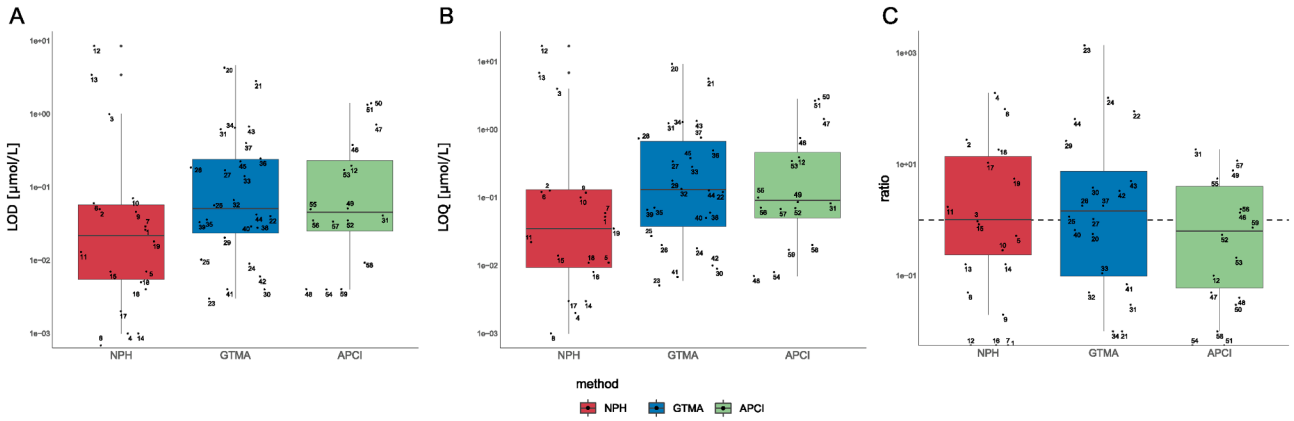

Fig. 3. Validation experiments for the quantitation of aroma compounds in Mint. A. Limit of detection (LoD) values for the three UHPLC–MS/MS methods. B. Limit of quantitation (LoQ) values for the carbonyl compounds, alcohols and terpenes. C. Ratio of odor thresholds to limit of quantitation (LoQ). Source data are provided in Table S7–9 in the Supplementary Material.

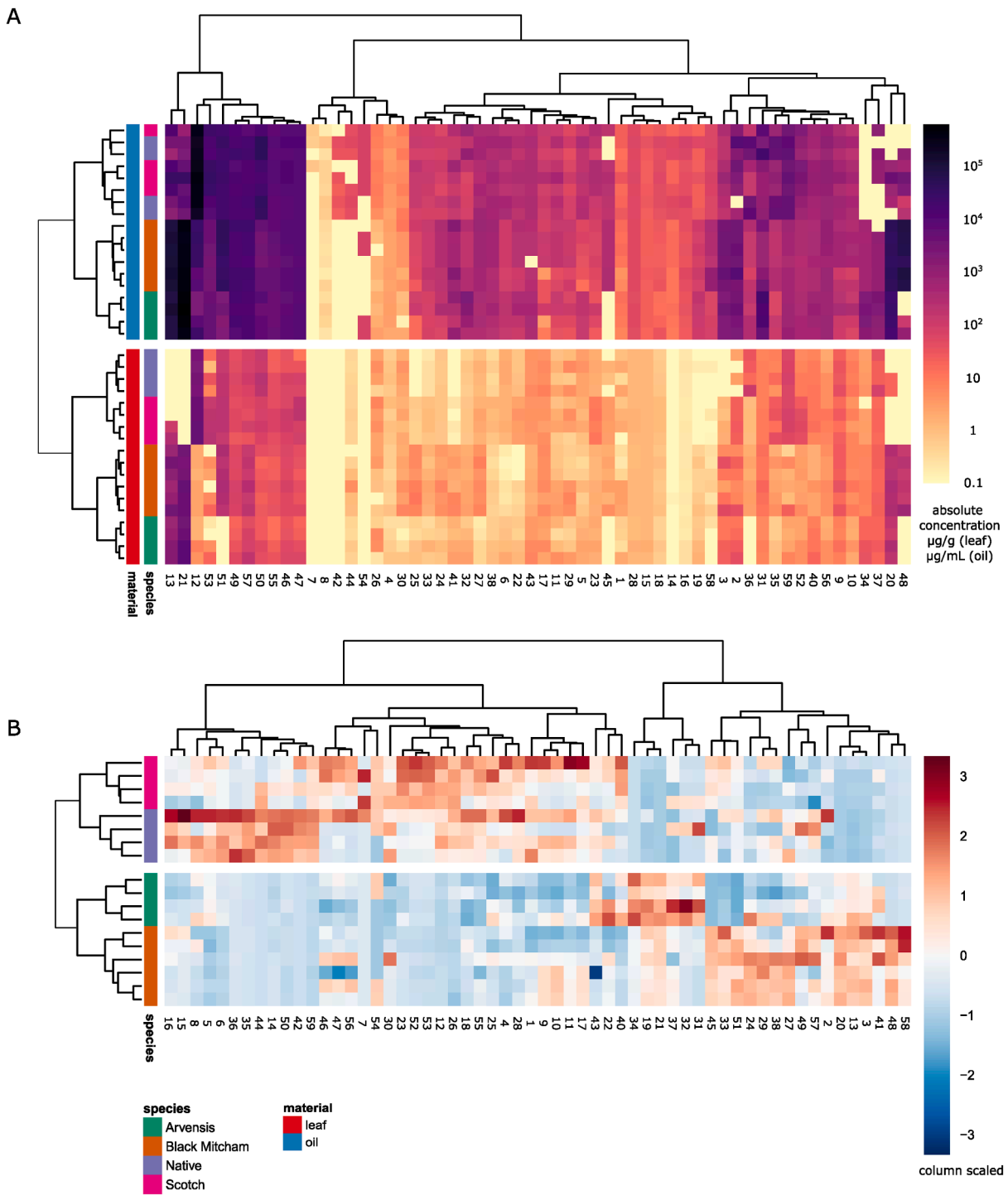

Fig. 4. Quantitation results of the aroma composition of Black Mitcham, Arvensis, Scotch spearmint and native spearmint. A. Comparison of the absolute concentrations of the main aroma compounds in mint leaves and corresponding oils of the four commercially important *Mentha* species plotted as heatmaps. B. Heatmaps displaying the column scaled concentrations of the analyzed 59 aroma compounds in mint oils. Source data are provided in Table S10–11 in the Supplementary Material.

concentration differences only through work-up of different sample volumes or injection several times of different dilutions of the samples. With these new developed methods, analysis with linearity over a large concentration range could be achieved, e.g., for carbonyl compounds from 0.17 µg/mL for isovalerate (**8**) and 218 µg/mL for octanal (**6**) up to 123190 µg/mL for menthone (**13**). The concentration range covered for alcohols was between 0.98 µg/mL for *p*-cresol (**26**) up to 372993 µg/mL for menthol (**21**) and for terpenes from 60 µg/mL for menthalactone (**58**) up to 485692 µg/mL for (*R*,*S*)–carvone (**12**).

In general, the mint oils showed higher amounts of the analytes compared to the mint leaves due to the concentration process of the aroma compounds during the steam distillation of the mint leaves, but quantitative dominance of menthone (**21**) with concentration up to 3621.2 µg/g and menthol (**13**) with amounts up to 5984.6 µg/g could be observed in the mint leaves as well as in the mint oils of the species Black Mitcham and Arvensis. Furthermore, the compound (+)-menthofuran (**48**), formed

enzymatically through oxidative bioconversion of (+)–pulegone (**2**) while flowering, could exclusively be detected in leaves of Black Mitcham up to an amount of 645.5 μg/g (Fuchs et al., 1999; Reitsema, 1958). Compounds that were also present in high concentration in the leaves and oils of Black Mitcham as well as Arvensis were 1,8–cineole (**51**) (205.2–398.8 μg/g (leaf), 1693.3–41303.5 μg/mL (oil)), neomenthol (**20**) (171.5–387.8 μg/g (leaf), 16231.7–48937 μg/mL (oil)), α–humulene (**57**) (46.7–263.2 μg/g (leaf), 11009.7–22089.2 μg/mL (oil)) and α–pinene (**47**) (19.7–59.0 μg/g (leaf), 7895.7–9689.2 μg/mL (oil)).

The related species Native and Scotch Spearmint show a similar aroma compound composition and are characterized by high amounts of (*R,S*)-carvone (**12**) up to 4797.7 μg/g in plant material and up to 443271 μg/mL in mint oils and compounds like limonene (**53**) (128.0–2.0 μg/g (leaf), 9339.7–31573.9 μg/mL (oil)) and myrcene (**50**) (71.6–245.9 μg/g (leaf), 12973.9–47263.1 μg/mL (oil)). Compounds dominating Black Mitcham and Arvensis (**51**, **49**, **57**, **50**, **55**, **46** and **47**), were also present in high amounts in the spearmint species. In comparison to these high concentrations, lower amounts were found for (*E,Z*)–2,6–nonadienal (**14**) (18.3–55.9 μg/mL), hexanal (**11**) (90.3–448.7 μg/mL), linalool (**31**) (636.1–854.1 μg/mL) in these two types of spearmint oils. These concentration range were well in line with literature reports on the key aroma compounds in Scotch and Native Spearmint oil analyzed by means of GC–FID and GC–MS (Kelley & Cadwallader, 2017). In general, it can be observed that terpenes like limonene (**53**),α–/β–pinene (**47**,**46**), sabinene (**55**), α–humulene (**57**), β–caryophyllene (**49**) and myrcene (**50**) occur in very high concentrations independent of the variety.

To illustrate the concentration differences within the samples the quantitative data of the mint oils were centered, scaled and after hierarchical agglomerative clustering of the normalized data depicted as a heatmap in Fig. 4 B. It is noticeable that the native spearmint oils showed the highest concentration of carbonyl compounds such as **5**, **8**, **14**, **15**, and **16** and alcohols such as **35**, **36**, **42** and **44**, while Scotch - spearmint showed high amounts of **12**, **23**, **52**, **53** und **26**. The Black Mitcham oils were characterized by the highest contents of **13**, **20**, **48**, **51** and **58** compared to the other three analyzed mint species, while the highest amounts of **31**, **32**, **37**, **34** and the isomer of menthol neomenthol (**20**) were found in Arvensis oils.

Finally, an important aspect highlighted in these two heatmaps is the congruence of the aroma profiles of the biological quadruple determination of each species, that indicates that the variance of a plant within the species can be negligible and the differences among the species are considerably greater. Therefore, the greater the similarities in the composition of the aroma compounds, the greater the biological relationship of the mint species, demonstrated in this study for Native and Scotch spearmint.

4. Conclusion

A high-throughput toolbox analyzing the most important aroma compounds in mint leaves or oils, avoiding the time-consuming and labor-intensive traditional flavor analysis was developed. After a quick extraction step of one single mint leaf by bead-beater-homogenization, a sufficient amount of aroma compounds was obtained to analyze in total 59 aroma compounds by means of UHPLC–MS/MS. To achieve a liquid chromatographic separation and sensitive detection of such volatile compounds, 19 carbonyl compounds were derivatized by 3–NPH and 26 alcohols by GTMA, while 16 terpenes were directly analyzed by means of APCI. By analytical determination of 59 compounds, this toolbox enabled for the first time an analysis and a comparison of the aroma composition of mint leaves and mint oils of several species over a concentration range of 6 orders of magnitude. As already reported in the literature, it could be shown that the main aroma compounds in Black Mitcham are menthol (**20**), menthone (**13**), (+)-menthofuran (**48**), neomenthol (**20**) and 1,8–cineole (**51**), whereas higher concentration of (*R,S*)-carvone (**12**), limonene (**53**), 1,8–cineole (**51**) and myrcene (**50**) were representative of Scotch and Native spearmint. Application of this toolbox for quantification of the aroma compounds in the four commercial important species is the beginning of the aroma characterization of the genus *Mentha* and enables a mapping of flavor alterations depending on, e.g., growing stages or environmental conditions.

Declaration of Competing Interest

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

Acknowledgments

The work was supported by Mars Wrigley, Chicago, IL, USA. Thanks to Callisons, Lacey, WA and UC Davis, CA for providing the sample material and to the Leibniz Institute for Food Systems Biology at the Technical University of Munich for supplying some of the stable isotope labeled internal standards.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130522.

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