Identification and Quantification of Cork Off-flavor Compounds in Natural Cork Stoppers by Multidimensional Gas Chromatographic Methods

Dissertation

zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
– Dr. rer. nat. –

vorgelegt von

Petra Slabizki

geboren in Landstuhl

Fakultät für Chemie
der
Universität Duisburg-Essen

2016

Die vorliegende Arbeit wurde im Zeitraum von Dezember 2011 bis März 2015 unter der Betreuung von PD Dr. habil. Hans-Georg Schmarr am Kompetenzzentrum Weinforschung des Dienstleistungszentrums Ländlicher Raum Rheinpfalz in Neustadt (Weinstraße) durchgeführt.

Tag der Disputation: 15.04.2016

Gutachter: PD Dr. habil. Hans-Georg Schmarr
Prof. Dr. Torsten C. Schmidt
Vorsitzender: Prof. Dr. Rainer Meckenstock

Abstract

The typical cork taint primarily caused by 2,4,6-trichloroanisole (TCA) in cork stoppers is considered today to be less important since its major origin, the utilization of hypochlorite as bleaching agent, during the manufacturing process is avoided and rigorous quality management is applied. Still, TCA and other haloanisoles in wine can originate from a contamination in cellars due to the usage of wood preservatives or flame retardants. Therefore, it is still important to monitor these compounds in the cork and wine industry. Particularly, the trace level analysis of such potent aroma compounds in the complex wine matrix is often hindered due to co-elutions using one-dimensional gas chromatographic (GC) analysis. Thus, a robust analytical method based on headspace solid phase microextraction (HS-SPME), heart-cut multidimensional gas chromatography (H/C MDGC) and halogen-sensitive electron capture detection (ECD) was established for routine application in wine and cork soaks that allowed a reliable quantification in the complex wine matrix below the compounds' odor thresholds at sub-ng/l level that may be crucial in customer conflict situations.

With regard to the atypical cork taint, a clear correlation of tainted wines with this off-flavor was hitherto difficult as this sensory alteration lacked any substantial information. In a study comprising H/C MDGC-olfactometry the responsible off-flavor compounds were identified by analyzing natural cork stoppers with off-odors deviant from the typical cork taint. Here, the identification of trace level aroma compounds benefitted from the additional application of heart-cut and comprehensive multidimensional GC in combination with mass spectrometric detection (H/C MDGC-MS-MS, GC×GC-MS). Basically, well-known off-flavor substances like geosmin, 2-methylisoborneol, and 3-isopropyl-2-methoxypyrazine were detected as well as chlorinated substances. Besides TCA, another potent aroma compound, 3,5-dimethyl-2-methoxypyrazine (MDMP), was present in each sub-group of the off-odorous cork stoppers, obviously playing an important role concerning the atypical cork taint. The unequivocal identification of MDMP was critical since a constitutional isomer, originally associated with another off-flavor in wine, showed similar mass spectrometric data and gas chromatographic behavior on common stationary phases. The GC separation of the isomers that was essential for the unambiguous assignment could be finally achieved on a cyclodextrin-based stationary phase. Targeted trace level analyses of the most important cork off-flavor compounds was achieved in cork soaks and wines below their odor thresholds using an analytical approach based on HS-SPME-H/C MDGC with tandem mass spectrometric detection (MS-MS). Analysis of individual off-odorous cork stoppers revealed elevated concentrations of the targeted compounds correlating with the corresponding sensory description of the stopper. The migration of off-flavor compounds (especially alkyl methoxypyrazines) from cork stoppers into wine and an associated sensory alteration of the

wine could be observed in wines sealed with affected cork stoppers after an appropriate storage period. In particular, MDMP became apparent to be important for the atypical cork taint and should thus be monitored in routine quality control. However, the contribution of MDMP to the characteristic sensory alteration of wine related with the atypical cork taint e.g. reduced fruitiness has to be investigated in more detail. Furthermore, its origin has to be fully elucidated to be able to apply preventive procedures in the cork production process.

Kurzfassung

Der typische Korkgeschmack, der hauptsächlich durch die Verbindung 2,4,6-Trichloranisol (TCA) in Korken verursacht wird, spielt heute nur noch eine untergeordnete Rolle, da die Hauptursache - die Bleichung der Korken mit Hypochlorit - bei deren Herstellung nicht mehr angewendet wird und strenge Qualitätskontrollen durchgeführt werden. Allerdings können TCA und andere Haloanisole aufgrund der Verwendung von Holzschutzmitteln oder Flammschutzmitteln auch im Kellerumfeld gebildet werden, so dass diese Verbindungen regelmäßig in der Kork- und Weinindustrie kontrolliert werden müssen. Die Spurenanalytik solcher potenter Aromastoffe erweist sich in der komplexen Weinmatrix oft als schwierig, da bei der Anwendung von nur eindimensionaler Gaschromatographie (GC) oft Co-elutionen beobachtet werden. Zur Routineanalytik von Wein- und Korkproben wurde daher eine robuste Methode etabliert, in der die Dampfraum-Festphasenmikroextraktion (HS-SPME), *heart-cutting* multidimensionale GC (H/C MDGC) und der halogenempfindliche Elektroneneinfangdetektor (ECD) eingesetzt wurden. Dadurch konnte in der komplexen Weinmatrix eine verlässliche Quantifizierung im Konzentrationsbereich unter den Geruchsschwellenwerten (sub-ng/l) erreicht werden, das gerade in kritischen Fällen von Verbraucherbeschwerden entscheidend sein kann.

Beim Auftreten des untypischen Korkgeschmacks war es bisher schwierig die sensorische Veränderung eines Weines diesem Fehlaroma zuzuordnen, da das Wissen über die verantwortlichen Substanzen fehlte. Diese Verbindungen wurden mittels H/C MDGC in Kombination mit olfaktometrischer Detektion identifiziert, indem Naturkorken mit einem Fehlaroma, das sich vom typischen Korkgeschmack unterscheidet, untersucht wurden. Hierbei wurde die Identifizierung von Aromastoffen im Spurenbereich durch die Anwendung von multidimensionalen GC Methoden mit massenspektrometrischer Detektion (H/C MDGC-MS-MS, GC×GC-MS) begünstigt. Prinzipiell wurden bereits bekannte Fehlaromen wie Geosmin, 2-Methylisoborneol und 3-Isopropyl-2-methoxypyrazin sowie chlorierte Verbindungen nachgewiesen. Jedoch wurde neben TCA ein anderer potenter Aromastoff, das 3,5-Dimethyl-2-methoxypyrazin (MDMP), in allen Untergruppen der sensorisch beeinträchtigten Korken nachgewiesen und scheint daher eine wichtige Rolle im Zusammenhang mit dem untypischen Korkgeschmack zu spielen. Die eindeutige Identifizierung von MDMP war zunächst kritisch, da ein Konstitutionsisomer, das ursprünglich mit einem anderen Weinfehlaroma in Verbindung gebracht wurde, ein ähnliches Massenspektrum und ähnliches gaschromatographisches Verhalten auf üblichen stationären Phasen aufwies. Die eindeutige Zuordnung war nur möglich aufgrund der gaschromatographischen Trennung der beiden Isomere, welche schließlich mittels einer stationären Phase auf Basis eines Cylclodextrin-Derivats erreicht wurde. Die Quantifizierung der wichtigsten Korkfehlaromen in Korkextrakten und Weinen unter deren Geruchsschwelle

im unteren ng/l-Bereich wurde erreicht durch den Einsatz von HS-SPME-H/C MDGC mit Tandemmassenspektrometrie (MS-MS) zur Detektion. Bei der Untersuchung von einzelnen sensorisch auffälligen Korken wurden erhöhte Konzentrationen der untersuchten Verbindungen beobachtet, die mit den entsprechenden sensorischen Beschreibungen der Korken korrelierten. Durch das Verschließen von Weinen mit sensorisch auffälligen Korken wurde nach einer entsprechenden Lagerungszeit die Migration von Fehlaromen, vor allem von Alkylmethoxypyrazinen, aus den Korken in den Wein sowie eine sensorische Beeinflussung des Weines beobachtet. Besonders MDMP ist offensichtlich von großer Bedeutung für den untypischen Korkgeschmack und sollte deshalb in die routinemäßige Qualitätskontrolle mit aufgenommen werden. Allerdings ist die Bedeutung von MDMP in der charakteristischen Wahrnehmung des untypischen Korkgeschmacks (z.B. die reduzierte Frucht des Weines) in ergänzenden sensorischen Studien zu untersuchen. Weiterhin sollten die Ursachen von MDMP auf bzw. in Korken geklärt werden, um präventive Maßnahmen im Produktionsprozess einleiten zu können.

Table of contents

7 Appendix

1 General introduction

1.1 Natural cork stoppers

1.1.1 Botanical origin

Cork is the reproducible bark of the cork oak *Quercus suber* L. that grows in the western Mediterranean region (Portugal, Spain, France, Northern Africa). In plant anatomy, cork is a tissue named phellem that is a part of the periderm in the bark system that surrounds the stem, branches and roots as a protective tissue [1,2]. A schematic illustration of a cross section of the tree stem is shown in Figure 1-1. The inner part of the cross section is the wood (Xylem). The outer part is the bark that consists of the phloem (produced by the cambium) and the periderm. The latter is produced by a secondary meristem, the cork cambium or phellogen. The part of the periderm named phellem represents the cork layer and consists of dead cells filled with air that are regularly arranged without intercellular voids. Periodic variations in cell size and density result from the physiological rhythm of the tree that lead to the formation of growth rings. Furthermore, the periderm is radially streaked by lenticular cells that are dark colored and loosely arranged with large intercellular spaces forming channels for gas exchange (lenticels) [2].

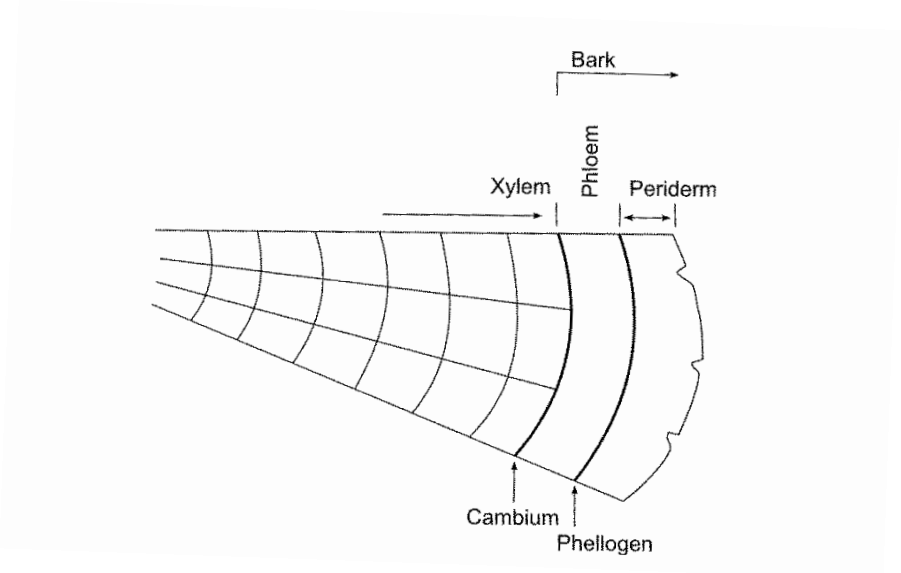


Figure 1-1 Schematic illustration of a cross-section of a cork oak tree stem (reprinted from [2] with permission from Elsevier, Copyright 2007)

The removal of the cork layer results in the formation of a new phellogen and produces a new periderm (traumatic periderm). This process can be repeated as often as necessary during the tree's lifetime. The cork bark is harvested in intervals of ten years. The first extraction is done when the tree is about 25-30 years old and has a minimum diameter of 70 cm. However, the first two harvestings are not used for the cork stopper production but for other applications [1,2].

1.1.2 Chemical composition

The chemical components of cork are primarily suberin (39 %), lignin (22 %) and polysaccharides (18 %). The rest consists of ash (<1 %) and extractives (15 %) [3].

Suberin is the main structural component in the cork cells and is an aliphatic polymer that consists mainly of α,ω-diacids and ω-hydroxyacids. Other monomers are monoacids, 1-alkanols and glycerol. The long-chain monomers are linear and range between 16 and 26 carbons. The aliphatic part is esterified with a polyaromatic part that consists of polymerized ferulic acid and is probably involved in the linkage to lignin [2,4,5].

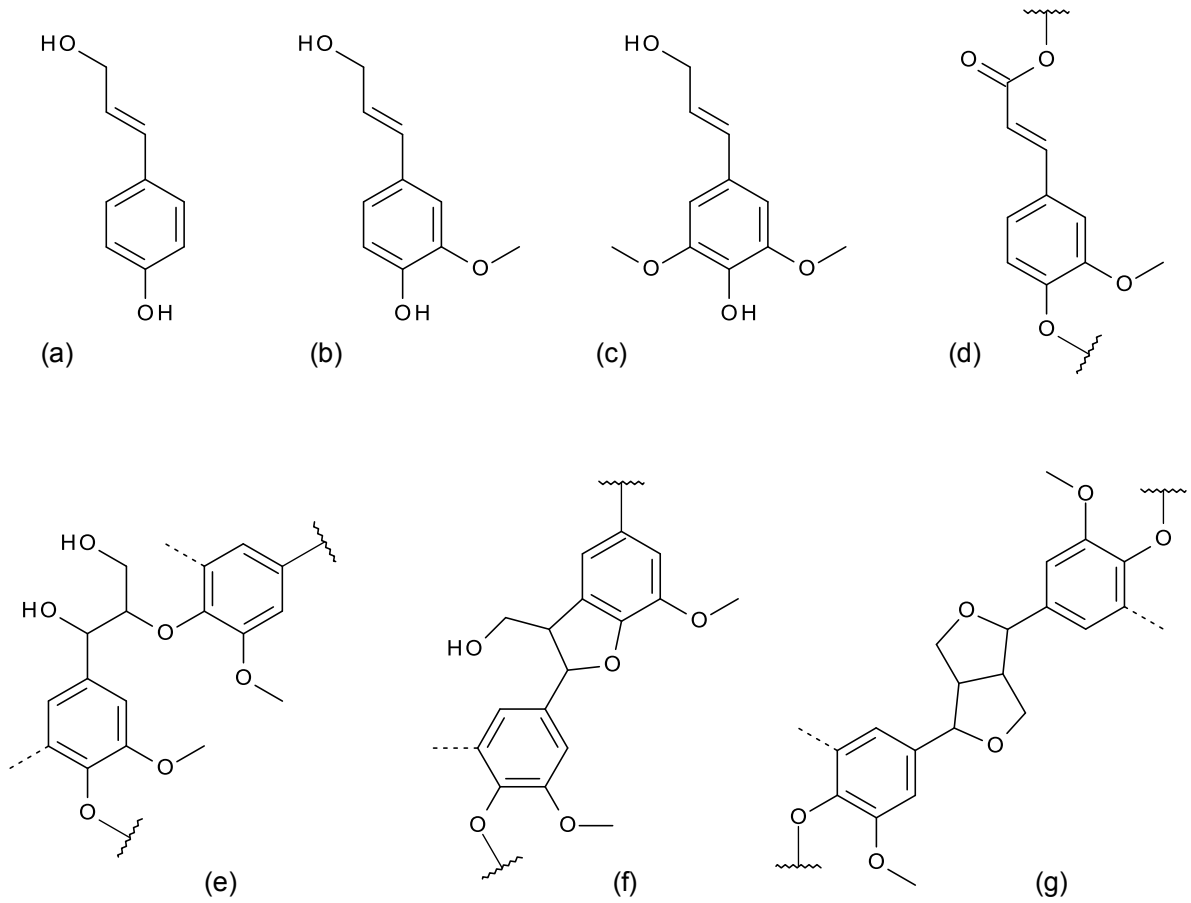


Figure 1-2 Monomer precursors of lignin (*p*-coumaryl alcohol (a), coniferyl alcohol (b), sinapyl alcohol (c)) and main structures in cork lignin (d)-(g) [5]

The second most important structural component of cork cells is lignin that is responsible for the mechanical stability of the cell walls. The three-dimensional macromolecule (7000 - 8000 Da) is formed by the polymerization of phenylpropane monomers that differ in their methoxyl substitution (*p*-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol, Figure 1-2). The structures in lignin are named *p*-hydroxyphenyl, guaiacyl, and syringyl units, respectively, and depending on the moieties different types of lignin are distinguished. Cork lignin contains about 95 % guaiacyl units and minor amounts of syringyl and p-hydroxyphenyl

units and thus is called a G-type lignin. The main structures in cork lignin are presented in Figure 1-2 [2,6,5].

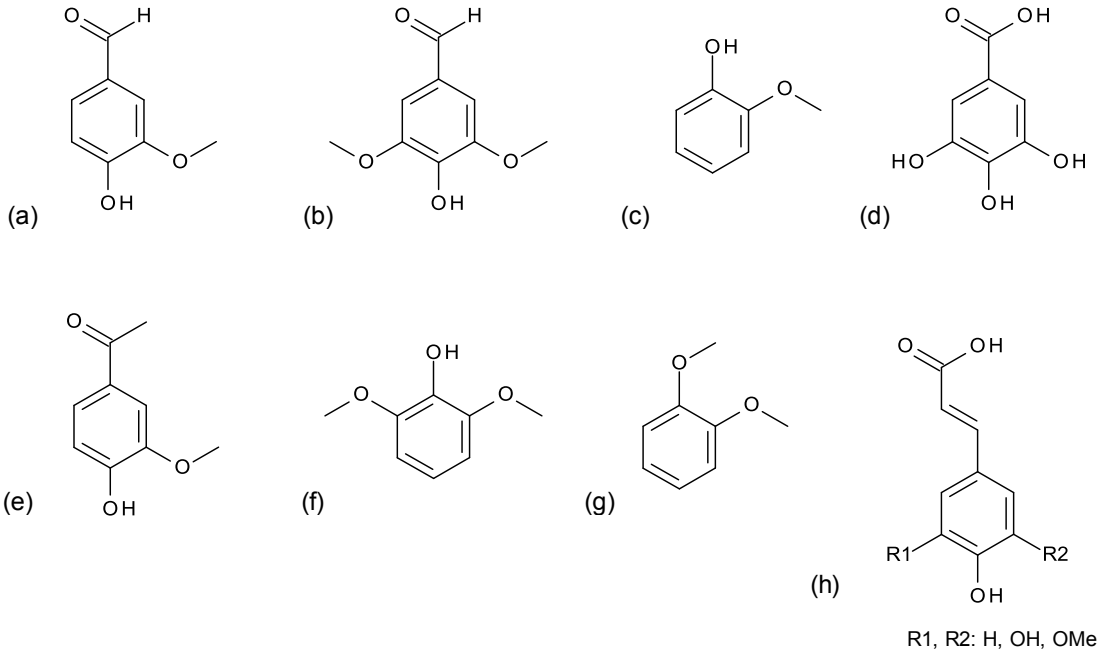


Figure 1-3 Chemical structures of some phenolic compounds present in cork: vanillin (a), syringaldehyde (b), guaiacol (c), gallic acid (d), acetovanillone (e), syringol (f), veratrol (g), hydroxycinnamic acid derivatives (h) [5]

Extractives are low or medium molecular weight compounds that may be extracted with an appropriate solvent without affecting the cellular structure. Based on the solvent used the extractives include alkanes, alcohols, waxes, terpenoids, fatty acids, glycerides, sterols, and phenols. The phenolic extractives comprise polyphenols (tannins) and simple phenolic compounds like benzoic and cinnamic acid derivatives, vanillin, syringaldehyde, acetovanillone, veratrol, syringol, guaiacol and structurally related compounds (Figure 1-3) [2,7,8].

1.1.3 Cork properties

In general, cork is valued due to its low density (0.12-0.25 g/cm^3), low permeability to liquids and gases, compressibility, elasticity, low tendency to rot, high friction, recyclability, and tolerance for temperature and humidity changes [1,2,9]. These properties result from the cellular structure of cork and its chemical composition.

Cork cells are hollow with the solid fraction concentrated in the cell walls that explains the low density of cork. The hydrophobicity and low permeability to liquids derives from the tight cellular structure without intercellular communication and suberin as the major chemical component in the cell wall. Due to the ability of the cell walls to buckle without fracture and

the presence of the lenticels, cork is able to deform under compression and to recover once the mechanical stress is relieved [2].

Especially, the low permeability to liquids, the high friction, and the elasticity of cork are properties that led to its usage as sealant for bottles. Although these beneficial properties of cork were known long before, the widely use of cork as stoppers in wine bottles started first in the 17th century [2]. Due to its unique properties cork is used in a wide range of applications but its use as cork stoppers is probably the most known.

1.1.4 Production process

After being harvested the raw cork planks are first stored under ambient conditions in the field or in the mill yard for a few weeks up to one year. Once refuse planks have been removed, the cork planks are boiled in water for one hour. As an effect, the cork planks increase in volume by approximately 15 % and they are flattened in order to facilitate the following cutting process. In recent years the water boiling process has undergone important modernization. In the past the stacked cork planks were immersed in large tanks that were basically made of a hole in the ground coated with concrete (Figure 1-4). These tanks were difficult to clean and several batches were boiled using the same water with the result that the water obtained a dark brown color and began to foam. Clean water was sometimes added to compensate for losses due to evaporation and absorption into cork. The water was fully replaced only every 4-5 days. With respect to cork taint this was actually problematic since compounds like 2,4,6-trichloroanisole (TCA) or its precursors accumulated in the water and were spread between the cork batches.

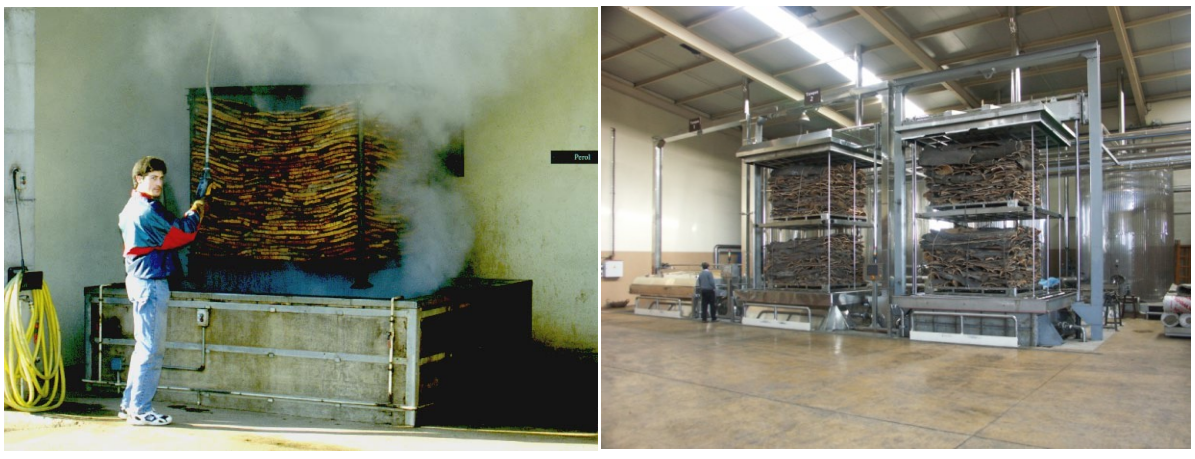


Figure 1-4 Traditional (left) and modern (right) water boiling process of cork planks (photo: Rudolf Ohlinger GmbH)

In the modern boiling process the cork planks are stacked on stainless steel pallets and the boiling occurs in a closed stainless steel autoclave (Figure 1-4). The water re-circulates

during the boiling process and is renewed after each operation. Furthermore, the water used has to be free of chlorine to avoid the possible production of chlorinated compounds. Particularly in Portugal, tap water is usually chlorine-treated, thus the cork mills usually use their own sources of water. After boiling, the planks are left to dry for some days until a moisture content of about 14-18% is reached. The drying period should be kept as short as possible to avoid microbial growth. In the past the boiled cork planks were stored in closed environments for some weeks and they were often allowed to get moldy, sometimes even intentionally (Figure 1-5). Since it is known that microbial growth benefits the formation of cork taint, especially TCA, this is today preferably avoided [2,10].

Figure 1-5 Mildewed cork planks due to inadequate drying conditions after boiling (photo: Rudolf Ohlinger GmbH)

After boiling and drying, the cork planks are cut into smaller parts for better handling and are sorted according to thickness and quality. Defective parts are removed, e.g. parts with holes due to insects or yellow stained or moldy parts. Then, the cork planks are cut horizontally into parallel strips with a width of the approximate length of the dedicated cork stoppers (38, 45, 49 mm). The cylindrical stoppers of a specific diameter (most common 24 mm) are punched from these strips either in a fully automated or a semi-automated system. The latter is done especially with more inhomogeneous material where the punching movement is automatic but the positioning is done manually. In a rectification step the length and thickness of the stopper are adjusted by abrasion [2,11].

The raw cork stoppers are washed in water to clean them and remove dust or loosened material. The water used has to be periodically monitored regarding contamination with TCA and related compounds. In addition, the cork stoppers undergo a bleaching process usually using hydrogen peroxide solution with sodium hydroxide followed by neutralization with a

citric acid solution. This process is done for the purpose of disinfection, but mostly for cosmetic reasons as the stoppers get a lighter color. Depending on the pH value, the grade of bleaching can range from natural washed to very light colored cork stoppers. The traditional chlorine-based bleaching agents like calcium hypochlorite are no longer used due to the formation of chlorinated compounds responsible for TCA-based cork taint.

After the washing process, the cork stoppers are dried at 40-60 °C to reach a final moisture content of 5-8 % where the risk of microbial growth is minimized. The nearly finished cork stoppers are classified by their external appearance (mostly amount and size of lenticels) in an automated process using optical control mechanisms (cameras). The premium quality classes are Flower, Extra and Superior, the lower quality classes vary from 1st to 6th grade and rejects. A final check of the classification is done manually by visual inspection [2,9,10,11,12]. Finally before bottling, the cork stoppers are printed (e.g. with a client logo and batch number) and coated with a lubricant (e.g. silicone) to facilitate the extraction out of the bottle.

1.1.5 Specific production steps

The cork industry has pursued several strategies to prevent and cure cork taint related to TCA. With the knowledge about the formation of TCA (see chapter 1.2.1), the cork stopper production was modified at various points. Microbial activity can be avoided by altering the drying conditions after boiling or the general storage conditions (e.g. lesser contact with soil), or by extensive pre-selection of cork material. Furthermore, the formation of chlorinated compounds is largely prevented by abandoning hypochlorite as bleaching agent and the application of chlorine-free water [2].

In cases of a natural TCA contamination special curative treatments were developed in the cork industry to free cork stoppers from TCA:

- The ROSA® technology, developed by the cork company Amorim, is based on a water steam distillation process for decontamination primarily of cork granules for technical cork stoppers but also of natural cork stoppers. Tests on the effectiveness of this process showed a reduction of 69-80 % [13,14].
- The cork company Oeneo developed an extraction technique using supercritical carbon dioxide for cork granules in the production of agglomerated cork stoppers. They call this technique “Diamant®” and the stoppers are called DIAM closures. On their website they guarantee releasable TCA ≤ 0.3 ng/l [14,15].
- The “Delfin” method uses microwaves to warm the cork stopper and evaporate malodorous volatiles. In addition, microorganisms should be destroyed [16]. This

method showed good results in reducing TCA in the laboratory; however, it did not prove its effectiveness in practice.

- An enzymatic treatment of cork stoppers with Suberase reduces the free phenols by polymerization and hence the precursors for malodorous compounds. The treatment involves washing the stoppers using an aqueous ethanolic solution (15 %vol., pH 5) thus phenols are additionally extracted [17,18].

Further treatment technologies like radiation [19,20] or ozonation [21,22] were tested in order to sterilize cork material and degrade TCA.

With respect to the prevention of a taint in corked wine, it should be mentioned that there are also alternatives to natural cork stoppers for sealing wine bottles. Particularly, the blame of cork closures for cork taint favored the usage of alternative closures, e.g. synthetic stoppers, glass stoppers and screw caps. Today, such alternative closures gain increasingly market shares, particularly in the basic wine quality sector. However, premium wines and sparkling wines are classically sealed with cork stoppers. Although, there has been an intense change in the wine business towards alternative closures, cork stoppers are still widely used and expected by many customers, particularly in the traditional wine-producing countries.

1.2 Typical cork taint

1.2.1 2,4,6-Trichloroanisole (TCA)

The typical cork taint is probably the most well known off-flavor in wine and has caused large financial losses to the wine industry and, especially, to the cork industry. In the 80's the very potent aroma compound TCA was identified as the major cause of cork taint [23,24]. TCA is described with the attributes musty, moldy, and leather-like and only few ng/l of TCA are sufficient to make a wine unpalatable. Depending on wine style and experience of the panelists the odor threshold of TCA varies between 2 and 5 ng/l in white wine and between 3 and 15 ng/l in red wine [24-29].

There has been several hypotheses concerning the origin of TCA in cork. One cause of TCA formation in cork is the usage of hypochlorite as bleaching agent in the production process of cork stoppers. Phenol (and other phenolic compounds) occurring in natural corkwood can be chlorinated under bleaching conditions with the result that 2,4,6-trichlorophenol (TCP) is produced, often co-occurring with less chlorinated compounds like dichlorophenols (Figure 1-6) [24,30,31,32]. Microorganisms from cork then convert trichlorophenol into the corresponding anisole as was shown for various bacteria and mold species [33-36]. In general, chlorination of lignin and further microbial degradation is also a well-known problem particularly found in the wood processing industry during the pulp bleaching process [37-39].

Today in the cork industry, hypochlorite is avoided as much as possible and mostly substituted with hydrogen peroxide to prevent this formation pathway that it should not be longer of any significance. However, it was shown that TCA can be also synthesized biochemically, e.g. via the shikimic acid pathway, by some fungi species in absence of hypochlorite [40,41].

Another discussed origin of TCA are environmental residues of chlorophenols that were used as biocides until a few years ago, e.g. pentachlorophenol (PCP) or TCP. By now such compounds are mostly prohibited in many countries but residues can still be found in water and soil. Commercial preparations of PCP contained 2,3,4,6-tetrachlorophenol and 2,4,6-trichlorophenol as impurities. As a detoxification step the three chlorophenols still ubiquitous existent can be microbially degraded by *O*-methylation into the corresponding anisoles (Figure 1-6) [42,43]. Additionally, PCP can undergo microbial or photochemical dechlorination resulting in tri- and tetrachlorophenols (TeCP) [42,44,45]. Although representatives of the cork industry affirm that no PCP was used in the cork forests, the detection of highly chlorinated phenolic compounds in cork may indicate the opposite [7,43,46,47,48]. Another biocide of interest in TCA formation is Prochloraz. It contains a 2,4,6-trichlorophenyl group that - if cleaved - could produce TCP [49]. Actually, the application of this fungicide to fennel leaded to the formation of TCA and affected the quality of fennel essential oil [50,51].

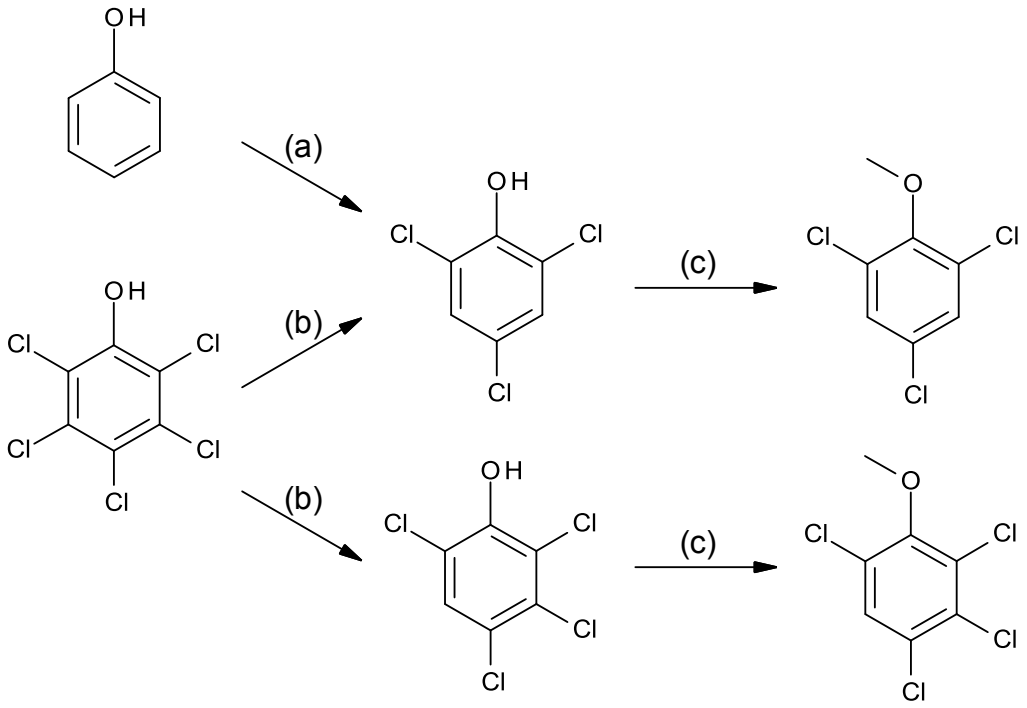


Figure 1-6 TCA and TeCA formation pathways; (a) chlorination (b) dechlorination or byproducts of PCP (c) microbial *O*-methylation

The formation of TCA from chlorophenols that were e.g. used as wood preservatives can also occur elsewhere and then contaminate the cork stoppers by aerial migration during transport or storage. In literature, there was an incidence described with shipping floors that had been treated with a wood preservative, probably containing TCP [51]. The microflora growing on the ship conduced to the formation of TCA and the transported cork stoppers that were stored in cardboard cartons were then contaminated. Similarly, dried fruit was contaminated with TeCA and pentachloroanisole (PCA) during shipping due to the usage of PCP [52]. In addition, a contamination of food can occur if fiberboard is made of recycled wastepaper containing chlorophenolic compounds that are converted by fungi e.g. from the packaged fruit [53,54].

In summary, TCA is still seen as the most important or even sole cause for cork taint in the cork industry. For quality control, TCA is usually monitored in cork soaks (approximately 10 %vol. aqueous ethanol solution) using headspace solid phase microextraction (HS-SPME) and gas chromatographic analysis with mass spectrometric (GC-MS) or electron capture detection (GC-ECD).

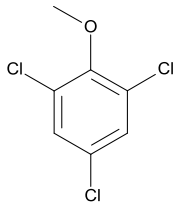
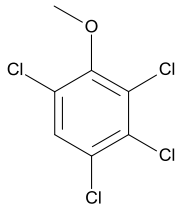
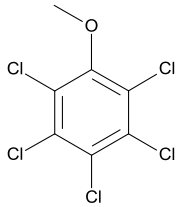
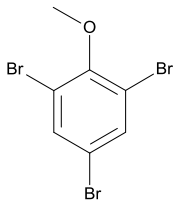
The total TCA amount in cork stoppers appeared to correlate little with the concentration of TCA in wine [55]. The apparent explanation is the location of TCA mainly on the surface or in the outer parts of the cork stoppers [56,57]. Hervé et al. studied the TCA soak kinetics and established the term “releasable TCA” [55]. It is defined as the concentration of TCA (in ng/l) reached at the equilibrium in the soak of one or a group of cork stoppers and it proved to be a good predictor for TCA extracted in bottled wine. The equilibrium in the soak between extracted and reabsorbed TCA was shown to be reached after 24 hours. The affinity of cork for TCA is very high as only a small portion of 0.05-2 % of the total TCA is extracted during soaking [55,58,59]. In this context the following observations were made. Repeated soakings of the same cork stopper revealed nearly constant TCA concentrations and similar TCA concentrations were observed after using different soak volumes, i.e. the extracted amount of TCA is proportional to the soak volume [55].

The good absorptive properties of cork can also be helpful if the wine is already tainted before bottling (see chapter 1.2.2) [59]. Due to the extraction of TCA out of the wine, the concentration in the wine can be reduced until it drops below the odor threshold and the wine is palatable again.

1.2.2 Cellar-derived cork taint

Bottle closures like cork stoppers are not always responsible for musty off-flavors in wine. Such off-flavors can arise even if wine bottles are sealed with screw caps [60]. In these cases the whole lot is often affected and a bottle-to-bottle variation cannot be observed. A

Table 1-1 Typical and cellar-derived cork off-flavor compounds (odor thresholds are in white wine unless otherwise indicated)

Compound	CAS no.	Structural formula	MW (g/mol)	Odor description	Odor threshold
2,4,6-trichloroanisole (TCA)	87-40-1		211.47	musty, moldy, dank cellar, wet cardboard	2-5 ng/l [25-29]
2,3,4,6-tetrachloroanisole (TeCA)	938-22-7		245.92	musty, moldy	10-25 ng/l [26,66]
pentachloroanisole (PCA)	1825-21-4		280.36	musty	3.2-4 µg/l (water) [26,67]
2,4,6-tribromoanisole (TBA)	50-31-7		344.83	musty	4 ng/l [63]

reason is the former usage of PCP and TCP as wood preservatives, also known under the trade names Raco, Xylamon® (PCP) or Dowicide® 25, Phenaclor (TCP). Treated wood or wood-based materials can be located in the wine cellar surrounding, e.g. wooden pallets, wooden barrels, wall coverings, wooden crates, cardboard packaging, and doors. Due to microbial activity PCP, its by-product TeCP, and TCP are converted into the volatile anisoles (PCA, TeCA, TCA). Structural and odorous information about these substances are described in Table 1-1. Materials such as rubber seals, plastic stoppers, filter layers, and fining agents e.g. bentonite can be stored in the contaminated air and represent good sorbents for these compounds. Consequently, wines are indirectly contaminated via the contact with these materials [49,61].

After the prohibition of PCP in Europe in the late 1980s, 2,4,6-tribromophenol (TBP; or other bromophenolic derivatives) has been introduced as an alternative fungicide. Additionally, TBP is widely used as flame retardant in wood, plastics, and paints. Similar to TCA,

microorganisms are able to methylate TBP into the corresponding 2,4,6-tribromoanisole (TBA), which has a similar musty odor like TCA and a very similar odor threshold (Table 1-1) [60,61,62,63].

Another problem occurring in German wine cellars is the usage of cleaning products and sanitizers containing chlorine that are used for floors, pallets, or barrels even though experts explicitly recommend to avoid such products in the surroundings of wine production [64,65]. This is because microorganisms occurring in wine cellars, e.g. *Penicillium* sp., are able to synthesize under suitable conditions phenols via the shikimic acid pathway and utilize the chlorine to produce TCA [40].

1.3 Atypical cork taint

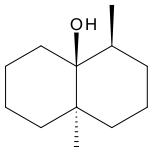
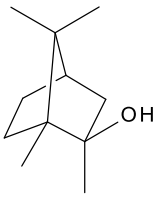
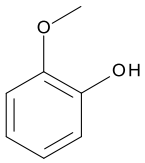
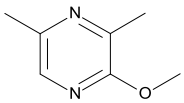
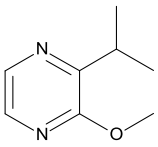
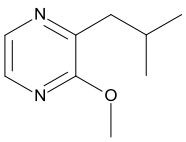
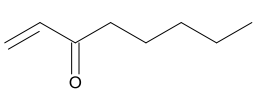
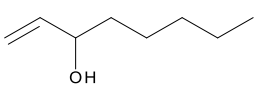
Over the years, the importance of the typical cork off-flavor decreased due to the reduction of microbiological growth on cork during the production process, the avoidance of hypochlorite as bleaching agent and rigorous quality management in the production of natural cork stoppers. Still, some experts in the wine industry report a sensory alteration of wines caused by cork stoppers different from the typical cork taint. It is described with a reduced fruitiness and moldy or musty notes. However, this *atypical* cork taint has not been fully characterized so far. The most important compounds discussed in previous studies [25,32,49,68-70] associated with atypical cork off-flavors are described in Table 1-2 and in the following sections.

1.3.1 Geosmin

The bicyclic alcohol geosmin (GSM; 2,6-dimethylbicyclo[4.4.0]decan-1-ol or 4,8a-dimethyl-1,2,3,4,5,6,7,8-octahydronaphthalen-4a-ol) is the characteristic aroma compound in table beets [75] and its smell is reminiscent of wet garden soil. GSM is also a well-known earthy off-odor substance in water supplies, fish and other marine foods, mostly in combination with 2-methylisoborneol [76-78]. It probably originates from microorganisms like actinomycetes [79] or blue-green algae (cyanobacteria) [80]. The biosynthesis of GSM occurs via the terpenoid synthesis pathway. Starting with isopentenyl pyrophosphate, a bicyclic sesquiterpene is synthesized that is further degraded to GSM [81-83].

GSM is considered to contribute to earthy, musty off-flavors in wine that are associated with cork stoppers [25]. Here, microorganisms isolated from cork were able to produce GSM among other compounds responsible for cork taint [70]. However, GSM was also detected in wine and must made from rotten grapes assuming that the microorganisms on the grapes are responsible for earthy odors in wine [71,84,85]. *Penicillium* spp. and *Streptomyces* spp.

Table 1-2 Atypical cork off-flavor compounds discussed in literature (odor thresholds are in white wine unless otherwise indicated)

Compound	CAS no.	Structural formula	MW (g/mol)	Odor description	Odor threshold
Geosmin (GSM)	23333-91-7		182.31	earthy, musty, muddy,	25 ng/l [25]; 60-65 ng/l (red wine) [71]
2-Methylisoborneol (MIB)	2371-42-8		168.28	earthy, musty, muddy, in higher concentrations camphoraceous	30 ng/l [25]
Guaiacol	90-05-1		124.14	smoky, phenolic, medicinal	20 µg/l [72]
3,5-Dimethyl-2-methoxypyrazine (MDMP)	92508-08-2		138.17	wet cardboard, musty, moldy, dusty, earthy, nutty, in higher concentrations coffee, chocolate	2 ng/l [68]
3-Isopropyl-2-methoxypyrazine (IPMP)	25773-40-4		152.19	green, vegetative, pea, potato-like	1-2 ng/l [73,74]
3-Isobutyl-2-methoxypyrazine (IBMP)	24683-00-9		166.22	green, vegetative, bell pepper	1 ng/l [73]
1-Octen-3-one	4312-99-6		126.20	mushroom, metallic	20 ng/l [25]
1-Octen-3-ol	3391-86-4		128.21	mushroom, metallic	20 µg/l [25]

isolated from rotten grapes were able to produce GSM [71,85] whereas *Botrytis cinerea* was suggested to induce the GSM production of other microorganisms [86,87]. Furthermore, Darriet et al. identified the more odoriferous (-)-GSM as the major enantiomer of GSM in wine and microbial cultures [71].

Still, the role of GSM in wine was questioned as it was rapidly converted into the odorless argosmin (4a,8-dimethyl-2,3,4,5,6,7-hexahydro-1H-naphthalene) under acidic conditions in model systems [25,79,88]. On the other hand Darriet et al. found high concentrations of GSM in red and rosé wines. They stated that GSM was relatively stable in acidic wines [84].

1.3.2 Methylisoborneol

In nature 2-Methylisoborneol (MIB; 1,2,7,7-tetramethylbicyclo[2.2.1]heptan-2-ol) often occurs in combination with GSM. Together they are responsible for most earthy off-flavors in water supplies and marine food, possibly originating from microorganisms [77,78,89,90]. Additionally, MIB is reported to be responsible for a musty off-odor in coffee that is probably of microbial origin if coffee beans are exposed to contact with soil [91].

Similar to GSM, MIB is a metabolite of soil bacteria (actinomycetes) [89,92] and blue-green algae (cyanobacteria) [93]. Basically, MIB is a methylated monoterpene and is formed via the terpenoid biosynthesis pathway [94].

In association with cork taint, MIB was detected in affected wines and their corresponding cork stoppers [25] as well as in cork samples inoculated with microorganisms [70] or infested by molds [95]. In literature, it is further hypothesized, that the occurrence of MIB in wine is not only due to tainted cork stoppers but could also originate from microorganisms on grapes. La Guerche et al. identified some fungi isolated from rotten grapes that were able to produce MIB together with GSM and C8 compounds [85].

1.3.3 3,5-Dimethyl-2-methoxypyrazine (MDMP)

3,5-Dimethyl-2-methoxypyrazine (MDMP) is an extremely potent aroma compound with an odor threshold of 1 pg/l in air [96]. It is described with the unpleasant sensory attributes “wet cardboard”, “musty”, “moldy”, “dusty”, “earthy”, and “potato”. In higher concentrations it is also described as “nutty”, “herbaceous”, “chocolate”, or “coffee” [68,97].

One of the first descriptions of MDMP as musty smelling off-flavor compound was in machine cutting fluid emulsions [98]. Mottram et al. isolated an aerobic, gram-negative bacterium responsible for this malodor, but could not fully characterize it. They suggested that MDMP and the bacterial species involved might have been responsible for common occurrences for such off-odors in the environment, including the food industry [98]. However, MDMP was

thereafter not often reported in literature as cause of off-odors, maybe, due to its low odor threshold and therewith the difficulty to analyze this compound [68]. In recent years, it was described as aroma compound in some food products, like raw hazelnuts [99,100], raw arabica coffee beans [96], or cooked brown rice [101]. However, in these cases it was not associated with an off-odor.

In cork stoppers MDMP was identified for the first time by Simpson et al. causing a “fungal must” taint in wine. They determined an odor threshold in a white wine matrix with about 2 ng/l [68]. Later, it was also found as a malodorous compound in water supplies [102]. The incidence of MDMP as off-flavor compound in cork was confirmed by Chatonnet et al. who also described this compound in oak chips and further investigated its microbiological origin [97]. They isolated the bacterium *Rhizobium excellensis* that is able to produce a high amount of MDMP and that is widespread in soil. Consequently, cork material could be contaminated when stored on or near soil or at another stage during the cork stopper production process [97]. Furthermore, Prat et al. detected MDMP in cork samples that were inoculated with microorganisms isolated from cork [70]. It has also been shown that MDMP is a volatile metabolite released by the myxobacterium *Chondromyces crocatus* [103,104].

A hypothetical pathway for biosynthesis of MDMP proposes amino acids as starting material. It is thought that an amidation of alanine followed by a condensation with methylglyoxal forms a hydroxypyrazine that is subsequently methylated [98,103]. The hypothesis about amino acids as nitrogen source has been supported by the detection of high MDMP concentrations after culturing *R. excellensis* in medium supplemented with alanine and leucine [63].

In literature, MDMP is seen as the most important substance affecting cork stoppers and thus wine next to TCA [68,97]. The migration kinetics of MDMP from cork into wine has not yet been studied in detail as it has been with TCA. There are indications about a low affinity of natural cork stoppers for 3-isobutyl-2-methoxypyrazine (IBMP) in contrast to their high affinity for TCA [105], thus suggesting also a low affinity for related alkyl methoxypyrazines like MDMP. Consequently, the equilibrium of MDMP would be more likely on the side of the wine than on the side of the cork with the result that in comparison to TCA less amounts of MDMP on the cork stopper would probably be sufficient to spoil a wine.

Concerning off-flavors in wine, a structural isomer of MDMP, namely 2,5-dimethyl-3-methoxypyrazine (DMMP), has been described in relation with the so-called “ladybug taint”, an off-odor problem associated with beetles harvested together with the grapes [106]. Some authors described DMMP - together with other 3-alkyl-2-methoxypyrazines - in *Harmonia axyridis* (multicolored Asian lady beetle) and in *Coccinella septempunctata* (seven-spotted lady beetle) responsible for the ladybug taint in wine [107,108].

1.3.4 3-Isopropyl-2-methoxypyrazine and 3-isobutyl-2-methoxypyrazine

The alkyl methoxypyrazines, 3-isopropyl-2-methoxypyrazine (IPMP) and IBMP, with a vegetative, green odor are flavor relevant compounds with very low odor thresholds in many vegetables [109] and also in *V. vinifera* varieties with IBMP as the major methoxypyrazine [110] (odor threshold in white wine about 1 ng/l [73]). However, they can also contribute to earthy off-flavors in water [111], fish [112] and other foodstuffs [113]. IPMP is also known to be the major component contributing to a green, earthy, potato-like off-flavor in wine, the ladybug taint [106]. Lady beetles like *Harmonia axyridis* and *Coccinella septempunctata* contain olfactorily potent alkyl methoxypyrazines like IBMP, DMMP, 3-*sec*-butyl-2-methoxypyrazine besides the major pyrazine IPMP [107,114]. These compounds are particularly present in their haemolymph, a reflex bleed released in stress situations. *H. axyridis* (multicolored Asian lady beetle) was first introduced as a biological control agent in Europe and North America where it spread rapidly and is now more considered as a pest e.g. in vineyards [115]. If these beetles are harvested and processed together with the grapes, the off-flavor described above may occur in wine.

IPMP and other alkyl methoxypyrazines may also be produced by microorganisms as shown with *Pseudomonas* spp. [112,116,117], *Chondromyces crocatus* [103,104], and *Serratia* and *Cedecea* strains [118]. They are most likely synthesized from amino acids as well as in plants as in bacteria [116,119,120].

So far little work has been published about the contamination of cork stoppers with IPMP or IBMP. In an assessment of cork taint in natural cork stoppers over nine years a “methoxypyrazine” taint was described among others. This term was chosen due to its similarity to the aroma found in Sauvignon Blanc wines and it was supposed to be derived from IPMP [57]. A possible contamination of cork stoppers with IPMP and its migration in wine was hypothesized in a study conducted by Allen et al. [121] in which individual IPMP concentrations were found for different bottles of the same wine. Capone et al. studied the extraction of IBMP from cork stoppers during wine storage and found a low affinity of natural cork stoppers for IBMP compared to TCA [105].

1.3.5 Guaiacol

2-Methoxyphenol or guaiacol in cork was identified early by Lefèbvre et al. [122] and Amon et al. [25]. Simpson et al. demonstrated the migration of guaiacol from contaminated cork stoppers into the wine as they found individual guaiacol concentrations in different bottles of the same wine. The wines tainted with guaiacol were described with a phenolic, medicinal off-flavor [72]. It was shown that guaiacol is the primary off-flavor compound in defective corkwood with a yellow discoloration.

Guaiacol is a degradation product of lignin and can be of microbial origin. Microorganisms isolated from cork samples, like *Streptomyces* spp., were able to convert vanillin and vanillic acid into guaiacol [122,123]. The defect originating in discolored corkwood is well-known in cork producing companies and faulty sections of corkwood are removed early in the production process. Besides, guaiacol in wine could also originate from oak barrels used during barrel aging [124].

Amon et al. detected guaiacol in concentrations below its odor threshold in cork tainted wines [25] and Prat et al. found guaiacol concentrations in tainted cork samples not significantly different from the control [70]. Both concluded that guaiacol does not contribute significantly to cork taint. However, they proposed the possibility that guaiacol could act in combination with other compounds of similar aroma or through synergisms.

1.3.6 1-Octen-3-one and 1-octen-3-ol

1-Octen-3-one and its corresponding alcohol are characteristic flavor compounds in edible mushrooms and they are common metabolites of molds, e.g., *Aspergillus* and *Penicillium* species [125,126]. The formation of 1-octen-3-ol and other C8 compounds derives from enzymatic oxidation of unsaturated fatty acids, e.g. linoleic acid [127]. Both compounds were detected in affected as well as in unaffected cork samples [25,7,128]; Octen-3-on was also found in synthetic stoppers [128]. Nevertheless, they were considered a contributor to cork taint by Sefton and Simpson [69]. They usually occur together with other taint compounds thus it is possible that they play an important role in synergisms.

Octen-3-one seems to be generally present in wine in concentrations in the range of its odor threshold as Culleré et al. found concentrations of 7-61 ng/l in “normal”, non-spoiled wines [129]. They further stated that a modification of wine aroma occurs at a minimum concentration of about 120 ng/l. Furthermore, octen-3-one and octen-3-ol are produced, among GSM, MIB and other C8 compounds, by several fungi found on grapes and thus contributing to earthy, mushroom, mossy odors in wine [85]. Especially, in grapes contaminated with powdery mildew, octen-3-one was one of the most potent flavor compounds [130]. However, according to these authors it was reduced to a less odorous compound during alcoholic fermentation.

1.3.7 Other compounds

In the early work of Buser et al. about compounds causing cork taint in wine, other chlorinated compounds besides TCA were identified in cork, e.g. dichloroanisoles [24]. Additionally, Simpson detected a chlorinated *o*-cresol (2,4-dichloro-6-methylanisole) as a

moldy, musty compound in wine [32] and Kugler and Rapp identified various chloroguaiacols and 6-chlorovanillin by GC-MS in corkwood [7]. These compounds probably derived from the chlorination of lignin during the cork bleaching process using hypochlorite as bleaching agent, then followed by a microbial degradation. Today, hypochlorite is no longer used in the production process of cork stoppers, hence, such chlorinated compounds are not seen to contribute significantly to cork taint as stated by Sefton and Simpson [69].

Furthermore, a range of sesquiterpenes were detected in GC-O experiments of mold cultures isolated from cork possibly contributing to moldy, earthy, musty attributes of these cultures [131,132]. However, they were identified only partially and their contribution to cork taint was not further investigated in this study.

Further compounds possibly causing mushroom-like off-odors in wine and cork are the unsaturated C8 compounds like (*Z*)-1,5-octadien-3-ol and (*Z*)-1,5-octadien-3-one that were tentatively identified by Simpson [32]. Similar to 1-octen-3-one, they are formed by lipidperoxidation. However, a lower chemical stability of these compounds was assumed and Sefton and Simpson hypothesized that they may be not present at high enough concentrations to contribute to cork taint [69].

1.4 Gas chromatography-olfactometry

In flavor analysis the human nose is used as a detector after gas chromatographic separation (gas chromatography-olfactometry, GC-O) in order to determine the odor-active compounds among the many volatiles occurring in food. In cases of odor-active compounds that often occur at low concentrations, the human nose is sometimes even more sensitive for these compounds than the “chemical” detectors. The odorous elution zones derived from GC-O involve a range of retention indices and may be generated by a number of individual flavor compounds. Therefore, the terms “aroma” or “odor event” are used for odorous “peaks” in GC-O analysis. A higher resolution of odor events can be achieved by applying H/C MDGC-O (Figure 1-7) [133,134]. Compound identification is based on comparison of the odor description and the linear retention indices on at least two stationary phases of different polarities with authentic reference substances. The identification should be additionally supported by mass spectrometric data or other spectroscopic data [135].

Due to the low concentrations of flavor compounds in food samples, pre-concentration steps are often necessary prior to injection. Furthermore, the stability of odor compounds has to be considered during sample preparation in order to obtain a representative extract of volatiles [136,137]. Common isolation techniques are static and dynamic headspace extraction or distillation methods like simultaneous distillation-extraction (SDE) [138] or solvent assisted flavor evaporation (SAFE) [139].

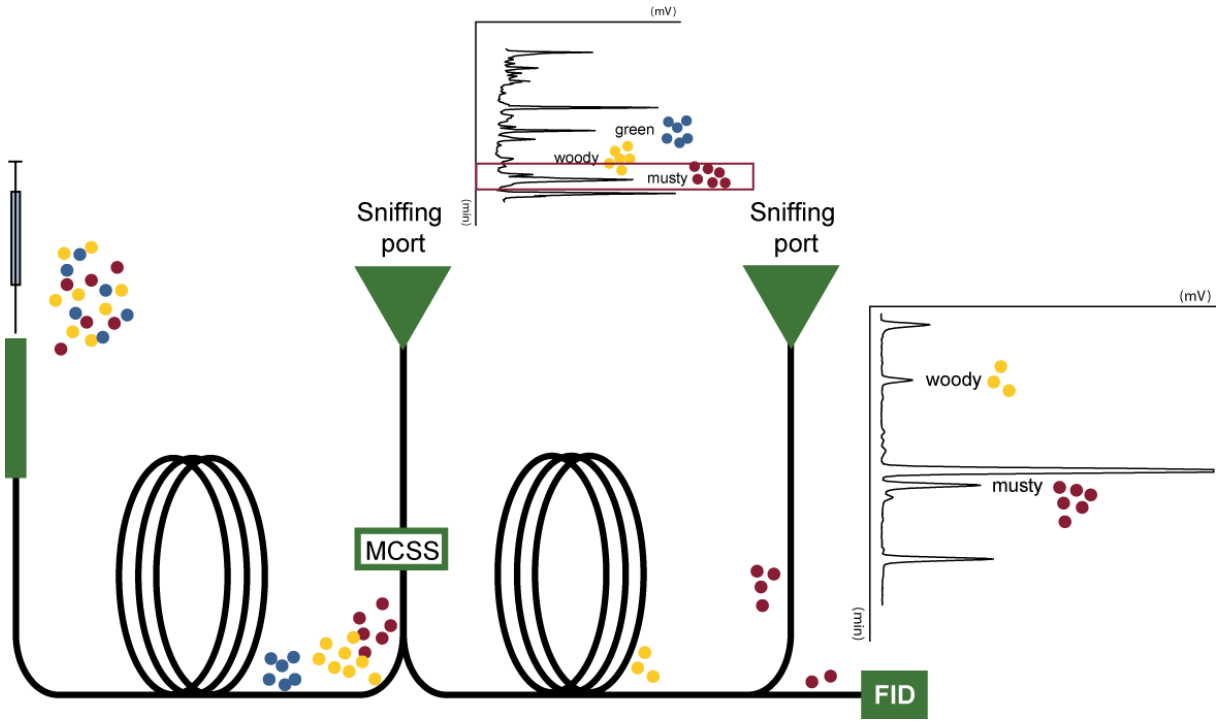


Figure 1-7 Schematic illustration of a H/C MDGC-O system (MCSS: moving capillary stream switching, FID: flame ionization detector)

Several methods were developed to collect and process GC-O data for the estimation of the sensory contribution and the flavor relevance of single odor-active compounds and are summarized in reviews [133,137]. The most common techniques are dilution analysis methods based on stepwise dilution to threshold for producing dimensionless values representing the potency of the flavor compound (combined hedonic response measurement, CHARM; aroma extraction dilution analysis, AEDA) [140,141]. Further methods are based on the detection frequency in a group of panelists or on posterior intensity methods to produce estimates of the perceived odor intensity [137,142,143]. Besides, GC-O is applied in qualitative attempts in situations where fully quantitative data is not required, e.g. for sensitive screening purposes of wine off-flavors [144].

In order to evaluate the contribution of a specific odor component to the flavor of a specific food sample, the concept of the odor activity value (OAV) was introduced. The OAV is defined as the ratio of the concentration of an odorant to its odor threshold in a specific food sample [145]. However, OAVs do not correlate linearly with the perceived intensity of an odor component. The correlation of odor intensity and concentration of an odor compound follows the Steven's law: $\Psi = k\Phi^n$, where Ψ is the perceived intensity, k is a constant, Φ is the stimulus level (OAV), and n is the Steven's exponent (0.3 – 0.8) [136,146,147].

1.5 Scope and aim

Although the occurrence of TCA in natural cork stoppers is minimized, it is still important to monitor TCA and other haloanisoles responsible for typical cork taint for quality control in the cork and wine industry, particularly relating to the cellar-derived cork taint. Especially, in customer conflict situations a reliable quantification in the concentration range of the odor thresholds at low ng/l levels is necessary. Whereas cork stoppers are usually monitored as cork soaks (a relatively simple matrix) such conflict situations have to be performed on the much more complex wine matrix. Since an earlier established method based on one-dimensional GC and ECD has shown to be inapplicable in wine at low ng/l level due to severe co-elutions, a method suitable for routine analysis and for reliable quantification at low ng/l level (or even sub-ng/l) in wine has to be developed.

In recent years the atypical cork taint is increasingly becoming an issue for the wine industry. It is characterized in wine by a reduced fruitiness and musty notes that do not resemble the typical cork taint. Customers, however, often ascribe this atypical cork taint to the wine itself and not to the cork stopper. Eventually, this assignment can result in a bad reputation of the winery. As long as this sensory alteration lacks substantial information, a clear correlation of tainted wines to the atypical cork taint is difficult. Therefore, the resulting financial losses for the wine industry due to this off-flavor cannot even be estimated. As the atypical cork off-flavor has not been fully characterized so far, the aim of this work is to identify and characterize the compounds responsible for this off-flavor by multiple gas chromatographic approaches.

Furthermore, an analytical method to quantify the most important compounds in cork and wine samples in a concentration range of their odor thresholds at the lower ng/l level has to be established for quality control applications. Since trace level analysis in wine is often critical due to co-elution problems, the analytical approach should be based on heart-cut multidimensional gas chromatography (H/C MDGC) and specific detection like tandem mass spectrometry (MS-MS). The potential migration of off-flavor compounds from cork stoppers into wine should be studied in real-life samples.

1.6 References

1. APCOR (2011) Technical Guide Cork Stoppers - Culture, Nature, Future. http://www.realcork.org.
2. Pereira H (2011) Cork: Biology, Production and Uses: Biology, Production and Uses. Elsevier Science.

3. Pereira H (1988) Chemical composition and variability of cork from *Quercus suber* L. Wood Science and Technology 22 (3):211-218.

4. Graca J, Pereira H (1997) Cork suberin: a glyceryl based polyester. Holzforschung 51:225-234.

5. Marques AV, Rencoret J, Gutiérrez A, del Rio JC, Pereira H (2015) Ferulates and lignin structural composition in cork. Holzforschung ahead of print, DOI:10.1515/hf-2015-0014

6. Marques AV, Pereira H, Meier D, Faix O (1999) Structural characterization of cork lignin by thioacidolysis and permanganate oxidation. Holzforschung 53:167-174.

7. Kugler D, Rapp A (1997) Bildung und Entwicklung von Inhaltstoffen in Korkborke während des Herstellungsprozesses von Flaschenkorken. Deutsche Lebensmittel-Rundschau 93 (6):174-177.

8. Mazzoleni V, Caldentey P, Silva A (1998) Phenolic compounds in cork used for production of wine stoppers as affected by storage and boiling of cork slabs. American Journal of Enology and Viticulture 49 (1):6-10.

9. Amon JM, Simpson RF (1986) Wine corks: a review of the incidence of cork related problems and the means for their avoidance. Australian Grapegrower & Winemaker 268:63-80.

10. Fischer C, Rudolf Ohlinger GmbH & Co. KG, personal communication.

11. C.E. Liège-Confédération Européene du Liège (2006) International Code of Cork Stopper Manufacturing Practices (Version 5).

12. Mesquita AC, Cork Supply Portugal, personal communication.

13. Cabral M (2006) Cork winning the war against TCA. Australian & New Zealand Wine Industry Journal 21 (5):50-53.

14. Coque JJR, Alvarez-Rodriguez ML, Goswami M, Feltrer-Martinez R (2006) Causes and origins of wine contamination by haloanisoles (chloroanisoles and bromoanisoles). edited by ASECOR, Spain.

15. http://www.diam-closures.com/Diam-Cork-Sensorial-neutrality-Consistency-Choice. Accessed 9.9.2015

16. Jäger JP (2000) Delfin - ein neues Verfahren für die Produktion von Korken. Schweizerische Zeitschrift für Obst- und Weinbau 136 (17):412-415.

17. Garrigues B (1999) Élimination des composés phénoliques présents dans les bouchons de liège par une phénoloxydase: Suberase. Revue des Oenologues 26 (93):20-22.

18. Sponholz WR (2000) Suberase: Eine biotechnologische Möglichkeit Korken zu reinigen. Schweizerische Zeitschrift für Obst- und Weinbau, Wädenswil 136 (24):621-625.

19. Mazzoleni V, Molteni R, Fumi MD, Musci M (2000) Effect of accelerated electron beam irradiation on cork used for stopper production. Industrie delle Bevande 29 (167):247-257.

20. Pereira C, Gil L, Carrico L (2007) Reduction of the 2,4,6-trichloroanisole content in cork stoppers using gamma radiation. Radiation Physics and Chemistry 76 (4):729-732.

21. Vlachos P, Kampioti A, Kornaros M, Lyberatos G (2007) Development and evaluation of alternative processes for sterilization and deodorization of cork barks and natural cork stoppers. European Food Research and Technology 225 (5-6):653-663.

22. Qi F, Xu B, Zhao L, Chen Z, Zhang L, Sun D, Ma J (2012) Comparison of efficiency and mechanism of catalytic ozonation of 2,4,6-trichloroanisole by iron and manganese modified bauxite. Applied Catalysis, B: Environmental 121-122:171-181.

23. Tanner H, Zanier C, Buser HR (1981) 2,4,6-Trichloroanisole: A dominant component of cork flavour. Schweizerische Zeitschrift für Obst- und Weinbau 117:97-103.

24. Buser HR, Zanier C, Tanner H (1982) Identification of 2,4,6-trichloroanisole as a potent compound causing cork taint in wine. Journal of Agricultural and Food Chemistry 30 (2):359-362.

25. Amon JM, Vandepeer JM, Simpson RF (1989) Compounds responsible for cork taint in wine. The Australian and New Zealand Wine Industry Journal 4 (1):62-69.

26. Fischer C (1997) Neue und kostengünstige Analyse. Dem Korkton auf der Spur. Das Deutsche Weinmagazin 16 (17):30-33.

27. Rudy H, Scholten G (2005) Neue Analytik schützt moderne Rieslingweine Der Deutsche Weinbau 3:26-27.

28. Prescott J, Norris L, Kunst M, Kim S (2005) Estimating a “consumer rejection threshold” for cork taint in white wine. Food Quality and Preference 16 (4):345-349.

29. Mazzoleni V, Maggi L (2007) Effect of wine style on the perception of 2,4,6-trichloroanisole, a compound related to cork taint in wine. Food Research International 40 (6):694-699.

30. Burtschell RH, Rosen AA, Middleton FM, Ettinger MB (1959) Chlorine derivatives of phenol causing taste and odour. Journal - American Water Works Association 51:205-214.

31. Tindale CR, Whitfield FB (1989) Production of chlorophenols by the reaction of fiberboard and timber components with chlorine-based cleaning agents. Chemistry & Industry (London, United Kingdom) 24:835-836.

32. Simpson RF (1990) Cork Taint in Wine: A Review of the Causes. Australian and New Zealand Wine Industry Journal 5:286-296.

33. Jäger J, Diekmann J, Lorenz D, Jakob L (1996) Cork-borne bacteria and yeasts as potential producers of off-flavors in wine. Australian Journal of Grape and Wine Research 2 (1):35-41.

34. Alvarez-Rodriguez ML, Lopez-Ocana L, Lopez-Coronado JM, Rodriguez E, Martinez MJ, Larriba G, Coque J-JR (2002) Cork taint of wines: role of the filamentous fungi isolated from cork in the formation of 2,4,6-trichloroanisole by *O*-methylation of 2,4,6-trichlorophenol. Applied and Environmental Microbiology 68 (12):5860-5869.

35. Coque JJR, Alvarez-Rodriguez ML, Larriba G (2003) Characterization of an inducible chlorophenol *O*-methyltransferase from *Trichoderma longibrachiatum* involved in the formation of chloroanisoles and determination of its role in cork taint of wines. Applied and Environmental Microbiology 69 (9):5089-5095.

36. Maggi L, Mazzoleni V, Fumil MD, Copete ML, Salinas MR (2007) Capability of fungi isolated from cork and of Botrytis cinerea to transform 2,4,6-trichlorophenol to 2,4,6-trichloroanisole. Enologos 9 (48):38-41.

37. Knuutinen J (1982) Analysis of chlorianted guaiacols in spent bleach liquor from a pulp mill. Journal of Chromatography, A 248 (2):289-295.

38. Eriksson KE, Kolar MC, Ljungquist P, Kringstad KP (1985) Studies on microbial and chemical conversions of chlorolignins. Environmental Science and Technology 19 (12):1219-1224.

39. Brownlee BG, MacInnis GA, Noton LR (1993) Chlorinated anisoles and veratroles in a Canadian river receiving bleached kraft pulp mill effluent. Identification, distribution, and olfactory evaluation. Environmental Science and Technology 27 (12):2450-2455.

40. Maujean A, Millery P, Lemaresquier H (1985) Explications biochimiques et metaboliques de la confusion entre gout de moisi. Revue Francaise d'Oenologie 99:55-62.

41. Neidleman SL, Geigert J (1986) Biohalogenation: principles, basic roles, and applications. E. Horwood.

42. Crosby DG, Beynon KI, Greve PA, Korte F, Still GG, Vonk JW (1981) Environmental chemistry of pentachlorophenol. Pure and Applied Chemistry 53:1051-1080.

43. Sponholz WR, Muno H (1994) Der Korkton - ein mikrobiologisches Problem? Wein-Wissenschaft, Wiesbaden 49 (1):17-22.

44. Weiss UM, Scheunert I, Klein W, Korte F (1982) Fate of pentachlorophenol-^{14}C in soil under controlled conditions. Journal of Agricultural and Food Chemistry 30 (6):1191-1194.

45. Haggblom M, Apajalahti J, Salkinoja-Salonen M (1986) Metabolism of chloroguaiacols by *Rhodococcus chlorophenolicus*. Applied Microbiology and Biotechnology 24 (5):397-404.

46. Rigaud J, Issanchou S, Sarris J, Langlois D (1984) Effect of volatiles from cork on cork taint of wine. Sciences des Aliments, Paris 4 (1):81-93.

47. Simpson RF, Sefton MA (2007) Origin and fate of 2,4,6-trichloroanisole in cork bark and wine corks. Australian Journal of Grape and Wine Research 13 (2):106-116.

48. Alvarez-Rodriguez ML, Recio E, Coque JJR (2009) The analysis of natural cork stoppers in transversal sections as an effective tool to determine the origin of the taint by 2,4,6-trichloroanisole. European Food Research and Technology 230 (1):135-143.

49. Chatonnet P, Guimberteau G, Dubourdieu D, Boidron J (1994) Nature et origine des odeurs de “moisi” dans les caves. Incidences sur la contamination des vins. Journal International des Sciences de la Vigne et du Vin 28:131-151.

50. Bricout J, Brunerle P, Desmarest P (1990) Influence of fungicide on the quality of fennel essential oil. In: Bhattacharya NS, Sethi KL (eds) Proceedings of the 11th International Congress of Essential Oils, Fragrances & Flavours (New Delhi, India, 1989).

51. Lee TH, Simpson RF (1993) Microbiology and Chemistry of Cork Taint in Wine. In: Fleet GH (ed) Wine Microbiology and Biotechnology. Taylor & Francis, New York, pp 353-372.

52. Tindale CR (1987) Shipping container floors: a potential source of chloroanisole contamination in packaged dried fruit. Chemistry & Industry (London, United Kingdom) 13:458-459.

53. Whitfield FB, Ly Nguyen TH, Shaw KJ, Last JH, Tindale CR, Stanley G (1985) Contamination of dried fruit by 2,4,6-trichloroanisole and 2,3,4,6-tetrachloroanisole adsorbed from packaging materials. Chemistry & Industry (London, United Kingdom) 19:661-663.

54. Whitfield FB, Nguyen THL, Last JH (1991) Effect of relative humidity and chlorophenol content on the fungal conversion of chlorophenols to chloroanisoles in fiberboard

cartons containing dried fruit. Journal of the Science of Food and Agriculture 54 (4):595-604.

55. Hervé E, Price S, Burns G, Weber P Chemical analysis of TCA as a quality control tool for natural corks. In: American Society of Enology and Viticulture, Annual Meeting, Reno NV, 1999. available at www.corkqc.com/currentresearch/CorkTaint/ETS%20CQC-SPME.pdf.

56. Howland PR, Pollnitz AP, Liacopoulos D, McLean HJ, Sefton MA (1997) The location of 2,4,6,-trichloroanisole in a batch of contaminated wine corks. Australian Journal of Grape and Wine Research 3 (3):141-145.

57. Simpson R, Capone D, Duncan B, Sefton M (2005) Incidence and nature of “fungal must” taint in wine corks. Australian and New Zealand Wine Industry Journal 20:26-31.

58. Pollnitz AP, Pardon KH, Liacopoulos D, Skouroumounis GK, Sefton MA (1996) The analysis of 2,4,6-trichloroanisole and other chloroanisoles in tainted wines and corks. Australian Journal of Grape and Wine Research 2 (3):184-190.

59. Capone DL, Skouroumounis GK, Barker DA, McLean HJ, Pollnitz AP, Sefton MA (1999) Absorption of chloroanisoles from wine by corks and by other materials. Australian Journal of Grape and Wine Research 5 (3):91-98.

60. Rudy H, Scholten G (2006) Korkgeschmack ohne Kork? Der Deutsche Weinbau 15:24-27.

61. Schäfer V, Jung R (2010) Nicht immer ist es der Korken... Der Deutsche Weinbau 22:14-18.

62. Whitfield FB, Hill JL, Shaw KJ (1997) 2,4,6-Tribromoanisole: a Potential Cause of Mustiness in Packaged Food. Journal of Agricultural and Food Chemistry 45 (3):889-893.

63. Chatonnet P, Bonnet S, Boutou S, Labadie M-D (2004) Identification and Responsibility of 2,4,6-Tribromoanisole in Musty, Corked Odors in Wine. Journal of Agricultural and Food Chemistry 52 (5):1255-1262.

64. Rudy H, Slabizki P (2013) Mufftöne in Wein. Fortbildungstagung für Lehr- und Beratungskräfte in Weinbau, Oenologie und Marktwirtschaft des Landes Rheinland-Pfalz, Neustadt an der Weinstraße.

65. Schäfer V (2013) Untersuchungen zum Auftreten, der Herkunft, Behandlung und Vermeidung sensorisch wirksamer, dumpf-muffiger Fehltöne im Wein, die durch Trauben, Weinbereitung, Schönung, Weinbehandlung und Abfüllung verursacht werden können. Dissertation, Justus Liebig-University, Gießen

66. Chatonnet P, Labadie D (1995) Contamination des locaux vinicoles et altération des vins - Contrôle de la qualité de l'atmosphère des chais. Revue des Oenologues 74:19-21.

67. Curtis RF, Land DG, Griffiths NM, Gee M, Robinson D, Peel JL, Dennis C, Gee JM (1972) 2,3,4,6-Tetrachloroanisole Association with Musty Taint in Chickens and Microbiological Formation. Nature 235 (5335):223-224.

68. Simpson RF, Capone DL, Sefton MA (2004) Isolation and Identification of 2-Methoxy-3,5-dimethylpyrazine, a Potent Musty Compound from Wine Corks. Journal of Agricultural and Food Chemistry 52 (17):5425-5430.

69. Sefton MA, Simpson RF (2005) Compounds causing cork taint and the factors affecting their transfer from natural cork closures to wine – a review. Australian Journal of Grape and Wine Research 11 (2):226-240.

70. Prat C, Trias R, Cullere L, Escudero A, Antico E, Baneras L (2009) Off-Odor Compounds Produced in Cork by Isolated Bacteria and Fungi: A Gas Chromatography-Mass Spectrometry and Gas Chromatography-Olfactometry Study. Journal of Agricultural and Food Chemistry 57 (16):7473-7479.

71. Darriet P, Lamy S, La Guerche S, Pons M, Dubourdieu D, Blancard D, Steliopoulos P, Mosandl A (2001) Stereodifferentiation of geosmin in wine. European Food Research and Technology 213 (2):122-125.

72. Simpson RF, Amon JM, Daw AJ (1986) Off-flavor in wine caused by guaiacol. Food Technology in Australia 38 (1):31-33.

73. Allen MS, Lacey MJ, Harris RLN, Brown WV (1991) Contribution of Methoxypyrazines to Sauvignon blanc Wine Aroma. American Journal of Enology and Viticulture 42 (2):109-112.

74. Pickering GJ, Karthik A, Inglis D, Sears M, Ker K (2007) Determination of ortho- and retronasal detection thresholds for 2-isopropyl-3-methoxypyrazine in wine. Journal of Food Science 72 (7):468-472.

75. Acree TE, Lee CY, Butts RM, Barnard J (1976) Geosmin, the earthy component of table beet odor. Journal of Agricultural and Food Chemistry 24 (2):430-431.

76. Lovell RT, Broce D (1985) Cause of musty flavor in pond-cultured penaeid shrimp. Aquaculture 50 (1-2):169-174.

77. Martin JF, McCoy CP, Tucker CS, Bennett LW (1988) 2-Methylisoborneol implicated as a cause of off-flavor in channel catfish, *Ictalurus punctatus* (Rafinesque), from

commercial culture ponds in Mississippi. Aquaculture and Fisheries Management 19 (2):151-157.

78. Watson SB, Brownlee B, Satchwill T, Hargesheimer EE (2000) Quantitative analysis of trace levels of geosmin and MIB in source and drinking water using headspace SPME. Water Research 34 (10):2818-2828.

79. Gerber NN, Lechevalier HA (1965) Geosmin, a earthy-smelling substance isolated from actinomycetes. Applied Microbiology 13 (6):935-938.

80. Safferman RS, Rosen AA, Mashni CI, Morris ME (1967) Earthy-smelling substance from a blue-green algae. Environmental Science & Technology 1 (5):429-430.

81. Dickschat JS, Bode HB, Mahmud T, Muller R, Schulz S (2005) A novel type of geosmin biosynthesis in myxobacteria. Journal of Organic Chemistry 70 (13):5174-5182.

82. Jiang J, He X, Cane David E (2006) Geosmin biosynthesis. Streptomyces coelicolor germacradienol/germacrene D synthase converts farnesyl diphosphate to geosmin. Journal of the American Chemical Society 128 (25):8128-8129.

83. Nawrath T, Dickschat JS, Mueller R, Jiang J, Cane DE, Schulz S (2008) Identification of (8S,9S,10S)-8,10-dimethyl-1-octalin, a key intermediate in the biosynthesis of geosmin in bacteria. Journal of the American Chemical Society 130 (2):430-431.

84. Darriet P, Pons M, Lamy S, Dubourdieu D (2000) Identification and Quantification of Geosmin, an Earthy Odorant Contaminating Wines. Journal of Agricultural and Food Chemistry 48 (10):4835-4838.

85. La Guerche S, Dauphin B, Pons M, Blancard D, Darriet P (2006) Characterization of some mushroom and earthy off-odors microbially induced by the development of rot on grapes. Journal of Agricultural and Food Chemistry 54 (24):9193-9200.

86. La Guerche S, Chamont S, Blancard D, Dubourdieu D, Darriet P (2005) Origin of (-)-geosmin on grapes: on the complementary action of two fungi, *Botrytis cinerea* and *Penicillium expansum*. Antonie Van Leeuwenhoek 88 (2):131-139.

87. Morales-Valle H, Silva LC, Paterson RRM, Venancio A, Lima N (2011) Effects of the origins of *Botrytis cinerea* on earthy aromas from grape broth media further inoculated with *Penicillium expansum*. Food Microbiology 28 (5):1048-1053.

88. Hsieh W-H, Hung W-N, Wang G-S, Hsieh S-T, Lin T-F (2012) Effect of pH on the analysis of 2-MIB and geosmin in Water. Water, Air, and Soil Pollution 223 (2):715-721.

89. Medsker LL, Jenkins D, Thomas JF, Koch C (1969) Odorous compounds in natural waters. 2-exo-Hydroxy-2-methylbornane, the major odorous compound produced by several actinomycetes. Environmental Science & Technology 3 (5):476-477.

90. Farmer LJ, McConnell JM, Hagan TDJ, Harper DB (1995) Flavour and off-flavour in wild and farmed Atlantic salmon from locations around Northern Ireland. Water Science and Technology 31 (11):259-264.

91. Blank I, Grosch W (2002) On the Role of (−)-2-Methylisoborneol for the Aroma of Robusta Coffee. Journal of Agricultural and Food Chemistry 50 (16):4653-4656.

92. Gerber N (1977) Three highly odorous metabolites from an actinomycete: 2-Isopropyl-3-methoxy-pyrazine, methylisoborneol, and geosmin. Journal of Chemical Ecology 3 (4):475-482.

93. Tsuchiya Y, Matsumoto A, Okamoto T (1981) Identification of Volatile Metabolites produced by Blue-green Algae, *Oscillatoria splendida*, *O. amoena*, *O. Geminata* and *Aphanizomenon sp.* Yakugaku Zasshi 101 (9):852-856.

94. Dickschat JS, Nawrath T, Thiel V, Kunze B, Müller R, Schulz S (2007) Biosynthesis of the Off-Flavor 2-Methylisoborneol by the Myxobacterium *Nannocystis exedens*. Angewandte Chemie International Edition 46 (43):8287-8290.

95. Rocha S, Delgadillo I, Ferrer Correia AJ (1996) GC-MS study of volatiles of normal and microbiologically attacked cork from *Quercus suber* L. Journal of Agricultural and Food Chemistry 44:865-876.

96. Czerny M, Grosch W (2000) Potent odorants of raw arabica coffee. Their changes during roasting. Journal of Agricultural and Food Chemistry 48 (3):868-872.

97. Chatonnet P, Fleury A, Boutou S (2010) Origin and incidence of 2-methoxy-3,5-dimethylpyrazine, a compound with a “fungal” and “corky” aroma found in cork stoppers and oak chips in contact with wines. Journal of Agricultural and Food Chemistry 58 (23):12481-12490.

98. Mottram DS, Patterson RLS, Warrilow E (1984) 2,6-Dimethyl-3-methoxypyrazine: a microbiologically-produced compound with an obnoxious musty odor. Chemistry & Industry (London, United Kingdom) (12):448-449.

99. Burdack-Freitag A, Schieberle P (2010) Changes in the key odorants of italian hazelnuts (*Coryllus avellana* L. Var. Tonda Romana) induced by roasting. Journal of Agricultural and Food Chemistry 58 (10):6351-6359.

100. Burdack-Freitag A, Schieberle P (2012) Characterization of the key odorants in raw italian hazelnuts (*Corylus avellana* L. var. Tonda Romana) and roasted hazelnut paste

by means of molecular sensory science. Journal of Agricultural and Food Chemistry 60 (20):5057-5064.

101. Jezussek M, Juliano BO, Schieberle P (2002) Comparison of key aroma compounds in cooked brown rice varieties based on aroma extract dilution analyses. Journal of Agricultural and Food Chemistry 50 (5):1101-1105.

102. Ventura F, Quintana J, Gomez M, Velo-Cid M (2010) Identification of alkyl-methoxypyrazines as the malodorous compounds in water supplies from northwest spain. Bulletin of Environmental Contamination and Toxicology 85 (2):160-164.

103. Dickschat JS, Reichenbach H, Wagner-Doebler I, Schulz S (2005) Novel pyrazines from the myxobacterium Chondromyces crocatus and marine bacteria. European Journal of Organic Chemistry (19):4141-4153.

104. Schulz S, Fuhlendorff J, Reichenbach H (2004) Identification and synthesis of volatiles released by the myxobacterium *Chondromyces crocatus*. Tetrahedron 60 (17):3863-3872.

105. Capone D, Sefton M, Pretorius I, Høj P (2003) Flavour scalping by wine bottle closures—the winemaking continues post vineyard and winery. The Australian and New Zealand Wine Industry Journal 18 (5):16-20.

106. Pickering G, Lin J, Riesen R, Reynolds A, Brindle I, Soleas G (2004) Influence of *Harmonia axyridis* on the sensory properties of white and red wine. American Journal of Enology and Viticulture 55 (2):153-159.

107. Cai L, Koziel JA, O'Neal ME (2007) Determination of characteristic odorants from *Harmonia axyridis* beetles using in vivo solid-phase microextraction and multidimensional gas chromatography-mass spectrometry-olfactometry. Journal of Chromatography A 1147 (1):66-78.

108. Botezatu AI, Kotseridis Y, Inglis D, Pickering GJ (2013) Occurrence and contribution of alkyl methoxypyrazines in wine tainted by *Harmonia axyridis* and *Coccinella septempunctata*. Journal of the Science of Food and Agriculture 93 (4):803-810.

109. Murray KE, Whitfield FB (1975) Occurrence of 3-alkyl-2-methoxypyrazines in raw vegetables. Journal of the Science of Food and Agriculture 26 (7):973-986.

110. Lacey MJ, Allen MS, Harris RLN, Brown WV (1991) Methoxypyrazines in Sauvignon blanc grapes and wines. American Journal of Enology and Viticulture 42 (2):103-108.

111. Sung Y-H, Li T-Y, Huang S-D (2005) Analysis of earthy and musty odors in water samples by solid-phase microextraction coupled with gas chromatography/ion trap mass spectrometry. Talanta 65 (2):518-524.

112. Miller A, 3rd, Scanlan RA, Lee JS, Libbey LM, Morgan ME (1973) Volatile compounds produced in sterile fish muscle (*Sebastes melanops*) by *Pseudomonas perolens*. Applied Microbiology 25 (2):257-261.

113. Maga JA (1987) Musty/earthy aromas. Food Reviews International 3 (3):269-283.

114. Al Abassi S, Birkett MA, Pettersson J, Pickett JA, Woodcock CM (1998) Ladybird beetle odour identified and found to be responsible for attraction between adults. Cellular and Molecular Life Sciences CMLS 54 (8):876-879.

115. Roy HE, Wajnberg E (2008) From Biological Control to Invasion: the Ladybird *Harmonia axyridis* as a Model Species. Springer Netherlands.

116. Gallois A, Kergomard A, Adda J (1988) Study of the biosynthesis of 3-isopropyl-2-methoxypyrazine produced by *Pseudomonas taetrolens*. Food Chemistry 28 (4):299-309.

117. Cheng TB, Reineccius GA, Bjorklund JA, Leete E (1991) Biosynthesis of 2-methoxy-3-isopropylpyrazine in *Pseudomonas perolens*. Journal of Agricultural and Food Chemistry 39 (5):1009-1012.

118. Gallois A, Grimont PA (1985) Pyrazines responsible for the potatolike odor produced by some *serratia* and *cedecea* strains. Applied and Environmental Microbiology 50 (4):1048-1051.

119. Murray KE, Shipton J, Whitfield FB (1970) 2-Methoxypryazines and the flavor of green peas (Pisum sativum). Chemistry & Industry (London, United Kingdom) (27):897-898.

120. Schulz S, Dickschat JS (2007) Bacterial volatiles: the smell of small organisms. Natural Product Reports 24 (4):814-842.

121. Allen MS, Lacey MJ, Boyd SJ (1995) Methoxypyrazines in Red Wines: Occurrence of 2-Methoxy-3-(1-methylethyl)pyrazine. Journal of Agricultural and Food Chemistry 43 (3):769-772.

122. Lefebvre A, Riboulet J-M, Boidron J-N, Ribereau-Gayon P (1983) The incidence of micro-organisms on cork and their effect on the olfactive alterations of wine. Sciences des Aliments, Paris 3 (2):265-278.

123. Alvarez-Rodriguez ML, Belloch C, Villa M, Uruburu F, Larriba G, Coque J-JR (2003) Degradation of vanillic acid and production of guaiacol by microorganisms isolated from cork samples. FEMS Microbiology Letters 220 (1):49-55.

124. Chatonnet P, Boidron JN, Pons M (1990) Maturation of red wines in oak barrels: Evolution of some volatile compounds and their aromatic impact. Sciences des Aliments, Paris 10 (3):565-587.

125. Kaminski E, Libbey LM, Stawicki S, Wasowicz E (1972) Identification of the predominant volatile compounds produced by *Aspergillus flavus*. Applied Microbiology 24 (5):721-726.

126. Kaminski E, Stawicki S, Wasowicz E (1974) Volatile Flavor Compounds Produced by Molds of Aspergillus, Penicillium, and *Fungi imperfecti*. Applied Microbiology 27 (6):1001-1004.

127. Wurzenberger M, Grosch W (1984) The formation of 1-octen-3-ol from the 10-hydroperoxide isomer of linoleic acid by a hydroperoxide lyase in mushrooms (*Psalliota bispora*). Biochimica et Biophysica Acta, Lipids and Lipid Metabolism 794 (1):25-30.

128. Culleré L, Cacho J, Ferreira V (2009) Comparative study of the aromatic profile of different kinds of wine cork stoppers. Food Chemistry 112 (2):381-387.

129. Culleré L, Cacho J, Ferreira V (2006) Validation of an analytical method for the solid phase extraction, in cartridge derivatization and subsequent gas chromatographic–ion trap tandem mass spectrometric determination of 1-octen-3-one in wines at ng L^{-1} level. Analytica Chimica Acta 563 (1–2):51-57.

130. Darriet P, Pons M, Henry R, Dumont O, Findeling V, Cartolaro P, Calonnec A, Dubourdieu D (2002) Impact odorants contributing to the fungus type aroma from grape berries contaminated by powdery mildew (*Uncinula necator*); incidence of enzymatic activities of the yeast *Saccharomyces cerevisiae*. Journal of Agricultural and Food Chemistry 50 (11):3277-3282.

131. Heimann W, Rapp A, Völter I, Knipser W (1983) Beitrag zur Entstehung des Korktons in Wein. Deutsche Lebensmittel-Rundschau 79 (4):103-107.

132. Caldentey P, Fumi MD, Mazzoleni V, Careri M (1998) Volatile compounds produced by microorganisms isolated from cork. Flavour and Fragrance Journal 13 (3):185-188.

133. Delahunty CM, Eyres G, Dufour J-P (2006) Gas chromatography-olfactometry. Journal of Separation Science 29 (14):2107-2125.

134. Marriott PJ, Chin S-T, Maikhunthod B, Schmarr H-G, Bieri S (2012) Multidimensional gas chromatography. Trends in Analytical Chemistry 34:1-21.

135. Molyneux RJ, Schieberle P (2007) Compound identification: A Journal of Agricultural and Food Chemistry perspective. Journal of Agricultural and Food Chemistry 55 (12):4625-4629.

136. Mayol AR, Acree TE (2001) Advances in Gas Chromatography-Olfactometry. In: Leland JV, Schieberle P, Buettner A, Acree TE (eds) Gas Chromatography-Olfactometry, vol 782. ACS Symposium Series, vol 782. American Chemical Society, pp 1-10.

137. van Ruth SM (2001) Methods for gas chromatography-olfactometry: a review. Biomolecular Engineering 17 (1389-0344 (Print)):121-128.

138. Flath RA, Forrey RR (1977) Volatile components of papaya (*Carica papaya* L., Solo variety). Journal of Agricultural and Food Chemistry 25 (1):103-109.

139. Engel W, Bahr W, Schieberle P (1999) Solvent assisted flavour evaporation – a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. European Food Research and Technology 209 (3-4):237-241.

140. Acree TE, Barnard J, Cunningham DG (1984) A procedure for the sensory analysis of gas chromatographic effluents. Food Chemistry 14 (4):273-286.

141. Ullrich F, Grosch W (1987) Identification of the most intense volatile flavor compounds formed during autoxidation of linoleic acid. Zeitschrift für Lebensmittel-Untersuchung und -Forschung 184:277-282.

142. Linssen JPH, Janssens JLGM, Roozen JP, Posthumus MA (1993) Combined gas chromatography and sniffing port analysis of volatile compounds of mineral water packed in polyethylene laminated packages. Food Chemistry 46 (4):367-371.

143. Ferreira V, Pet'ka J, Aznar M, Cacho J (2003) Quantitative gas chromatography–olfactometry. Analytical characteristics of a panel of judges using a simple quantitative scale as gas chromatography detector. Journal of Chromatography, A 1002 (1–2):169-178.

144. De La Fuente A, Lopez R, Cacho J, Ferreira V (2014) Chapter 79 - Evaluation of Gas Chromatography-Olfactometry for Screening Purposes of Wine Off-Flavors. In: Lopez VF (ed) Flavour Science. Academic Press, San Diego, pp 423-428.

145. Rothe M, Thomas B (1963) Aromastoffe des Brotes - Versuch einer Auswertung chemischer Geschmacksanalysen mit Hilfe des Schwellenwertes. Zeitschrift für Lebensmittel-Untersuchung und -Forschung 119:302-310.

146. Stevens SS (1961) To Honor Fechner and Repeal His Law. Science 133 (3446):80-86.

147. Acree TE (1997) GC/Olfactometry GC With a Sense of Smell. Analytical Chemistry 69 (5):170A-175A.

2 Analysis of corky off-flavor compounds at ultra trace level with multidimensional gas chromatography-electron capture detection

Adapted with permission from P. Slabizki, H.-G. Schmarr, Analysis of corky off-flavour compounds at ultra trace level with multidimensional gas chromatography-electron capture detection, *Journal of chromatography A*, **2013**, 1271, 181-184, Copyright (2012) Elsevier.

2.1 Abstract

A robust method for routine quality control of corky off-flavor compounds in wine and cork soak matrices has been established. Based on an automated headspace solid phase microextraction (HS-SPME), the method needs only marginal sample preparation and achieves low (sub-ng/l) trace level detection limits (LODs) for the most relevant off-flavor compounds, such as 2,4,6-trichloroanisole (TCA), 2,3,4,6-tetrachloroanisole (TeCA) and 2,4,6-tribromoanisole (TBA). Particularly for wine matrix, reliable trace level quantification had only been achieved after applying heart-cutting multidimensional gas chromatography (MDGC). Using a halogen-sensitive electron capture detector (ECD) and quantification with a stable isotope dilution assay (SIDA), LODs of 0.1 ng/l for TCA, TeCA and TBA could be obtained. Since a SIDA based quantification method is used with a non-mass spectrometric detector, the necessary chromatographic resolution of internal standard and target analyte peaks resulted from the use of highly deuterated [$^2H_5$]-isotopologues.

2.2 Introduction

Musty cork taint is one of the most known off-flavors in wine, with 2,4,6-trichloroanisole (TCA) as the primary responsible compound [1,2]. However, other haloanisoles such as 2,3,4,6- tetrachloroanisole (TeCA) and 2,4,6-tribromoanisole (TBA) are also important for quality control in the cork and wine industry [3-5]. Known sources are from wood preservatives used in packaging or in the cellar surroundings [6,7]. Their sensory thresholds have been reported to be at the lower ng/l level in wine. Depending on the wine style, off-flavor can be detected at some 2–5 ng/l for TCA; e.g. in flavor-accentuated white wines with

a particularly fruity character [3,7-9]. Therefore, aroma-relevant haloanisoles have to be monitored in a quality-control situation for the cork and wine industry at low levels, or in customer conflict situations, even at sub-ng/l levels. The cork industry usually monitors cork soaks (approximately 10% by volume ethanol solutions) for quality control, thus being relatively simple in matrix composition. In such situations, the applied analytical methods are often based on one-dimensional gas chromatographic analysis (^{1}D-GC) with mass spectrometric (MS) or electron capture detection (ECD) as standard procedures [10-18]. However, customer conflicts originate from rejected wines, due to “corkiness” detected during tasting. In such conflict situations, chemical analysis has to be performed on the much more complex and analytically demanding wine matrix.

In the control laboratory of the Dienstleistungszentrum Ländlicher Raum Rheinpfalz, an earlier-established method based on headspace solid phase microextraction (HS-SPME) and ^{1}D-GC-ECD analysis [10] failed to produce reliable data in certain trace-level (low ng/l) situations with wine matrices due to severe co-elution problems. Such problems could be overcome by applying a method based on (off-line) solid phase extraction (SPE) and multidimensional GC (MDGC)–MS [19]. Detection limits of sub-ng/l levels could be achieved, but at the cost of relatively extensive sample preparation and clean-up procedures, also incorporating a large volume on-column injection. The procedure proved to be time consuming and somewhat demanding for the operator, thus not suitable for a routine application. For high-throughput analyses, a more practical and automated sample preparation had to be targeted. Based on previous methods using automated HS-SPME as extraction technique [10,20-23], the original ^{1}D-GC-ECD system should be modified by increasing the chromatographic separation efficiency with a second separation dimension (^{2}D) and by heart-cutting the haloanisole fractions to this second dimension column. Reliable quantification for the HS-SPME-MDGC-ECD analytical method then should be assured by using highly deuterated isotopologues as internal standards, thus quantifying via a stable isotope dilution assay (SIDA) approach, which had been introduced by Rittenberg and Foster [24] and has found wide application, particularly in trace-level flavor analyses since then [25]. In recent years, quantitative analyses of haloanisoles in wine have often been based on SIDA methods published in literature, but, usually with MS or MS-MS detection [14,19,20,26-28].

2.3 Methods and materials

2.3.1 Chemicals and reagents

2,3,4,6-Tetrachloroanisole (CAS no. 938-22-7) was from LGC Promochem (Wesel, Germany), 2,4,6-trichloroanisole (CAS no. 87-40-1) and 2,4,6-tribromoanisole (CAS no. 607-

99-8) were from Sigma–Aldrich (Steinheim, Germany), ethanol (absolute) was from VWR (Darmstadt, Germany), and sodium chloride and ethanol (denaturated with 1% methyl ethylketone) were from Roth (Karlsruhe, Germany). The deuterated reference substances [$^2H_5$]-2,4,6-trichloroanisole (TCA-d_5; CAS no. 352439-08-8) and [$^2H_5$]-2,4,6-tribromoanisole (TBA-d_5; CAS no. 1219795-33-1) were synthesized in-house as described earlier [19]. Commercial chemicals were usually of analytical grade.

2.3.2 HS-SPME conditions

HS-SPME extraction was done on 5 ml sample volumes, using 10 ml headspace vials with silicone/polytetrafluoroethylene (PTFE) septa and metallic screw caps. Sample preparation involved addition of 1 g of sodium chloride (previously conditioned at 180 °C), a glass-coated magnetic stir bar, and the internal standards TCA-d_5 and TBA-d_5 in a concentration of 2 ng/l each in an ethanolic solution (10 µl of 1 pg/µl). SPME utilized a 1 cm fiber coated with 100 µm of polydimethylsiloxane (PDMS; Supelco, Steinheim, Germany). Automation was done with a CombiPal autosampler (CTC, Zwingen, Switzerland), comprising a single magnet mixer (Chromtech, Idstein, Germany) for agitation and incubation. Since no fiber conditioning station was available, the SPME fiber was conditioned for 10 min in the GC injector at 250 °C, prior to starting an analytical sequence. Extraction conditions involved a 1 min pre-incubation at 35 °C, and extraction for 20 min at 35 °C and 250 rpm agitation speed. Desorption of the fiber was done in the GC injector at 250 °C utilizing a 2 min splitless time and a liner dedicated for SPME application (Supelco). Instrument control was with the Cycle Composer Software version 1.5.2 (CTC).

2.3.3 Gas chromatographic conditions

Heart-cutting MDGC was based on the Deans' switch principle [29], using the capillary flow technology from Agilent (Waldbronn, Germany). The MDGC system consisted of two HP 6890 Series GCs (Agilent) equipped with a flame ionization detector (FID) for ^{1}D and an ECD for ^{2}D detection.

The two GC instruments were connected via a heated transferline (kept at 230 °C). The ^{1}D separation column was a fused silica capillary (30 m × 0.25 mm i.d.) with a film thickness of 0.25 µm of DB-XLB (J&W; Agilent), the ^{2}D separation column was a fused silica capillary (15 m × 0.25 mm i.d.) coated with 0.25 µm TG-1301MS (ThermoFisher Scientific, Dreieich, Germany). The restrictor column between the Deans' switch and FID consisted of a 2 m phenylmethyl deactivated fused silica capillary (0.15 mm i.d.). The ^{1}D and ^{2}D analytical columns were connected via a phenylmethyl deactivated fused silica capillary (1.2 m × 0.25 mm i.d.). Deactivated capillaries were from Agilent. The carrier gas used was

hydrogen in constant pressure mode at 129 kPa (^{1}D inlet pressure). Mid-point pressure for the Deans' switch was applied at 80 kPa via an auxiliary electronic pressure regulator (EPC).

^{1}D oven temperature was programmed from 50 °C (2 min isothermal) with 20 °/min to 120 °C (0.5 min hold), and then with 5 °/min to 250 °C (5 min hold). ^{2}D oven temperature was programmed from 50 °C (20 min isothermal) with 25 °/min to 85 °C (0.5 min hold), then with 2 °/min to 140 °C and finally with 40 °/min to 250 °C (5 min hold). FID and ECD were each kept at 250 °C, using nitrogen as make-up gas in both cases. A scheme of the HS-SPME-MDGC-ECD system is given in Figure 2-1.

Instrument control and data processing was with GC ChemStation software, rev. B. 04.03 (Agilent). Gas flows and restrictor parameters were optimized using Deans Switch Calculator Software Version A.01.01 (Agilent).

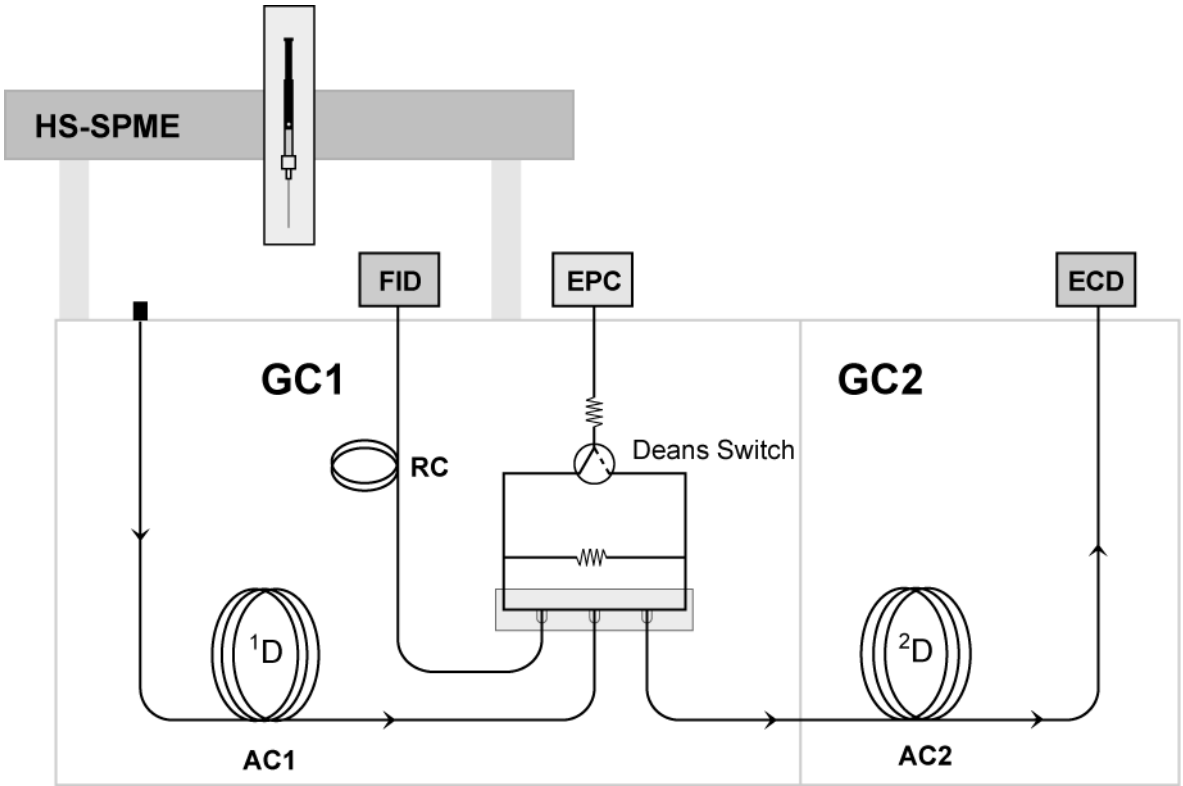


Figure 2-1 Scheme of the automated HS-SPME-MDGC-ECD system. Heart-cuts are performed with a Deans' Switch, transferring from ^{1}D analytical column (AC1; DB-XLB) to the ^{2}D analytical column (AC2; TG-1301MS); restrictor capillary (RC); electronic pressure controller (EPC)

2.3.4 Method calibration

Calibration was carried out in ethanol/water solutions (10 % by volume) spiked with ethanolic standard solutions of the haloanisoles and the internal standards. The concentration ranges were 0.1-6.1 ng/l (TCA), 0.1-8.2 ng/l (TeCA) and 0.1-6.3 ng/l (TBA). Quantification was done for TCA and TBA with their deuterated isotopologues, TeCA was quantified via TCA-d_5 as

standard. Detection limits (LODs) and quantification limits (LOQs) were calculated with DINTEST, vers. 2005 DE software (Georg Schmitt, Inst. F. Rechtsmedizin, Universitätsklinikum Heidelberg, Germany; www.analytiksoft.de) according to DIN 32645.

2.4 Results and discussion

In the trial to increase separation efficiency, thus switching from a previous ^{1}D to a ^{2}D HS-SPME-GC-ECD set-up, several column combinations were considered with respect to selectivity, bleed, and robustness. As a GC setup with two independent ovens was used, film thickness in the second dimension was not critical and could be kept in standard dimensions (here 0.25 µm), without the need for additional cryo-trapping instrumentation. This could simply be achieved by setting the ^{2}D initial oven temperature low enough to prevent band broadening of the early eluting TCA, whilst waiting for the last cut with the higher boiling TBA. Considering the performance and stability of the ECD, a low column bleed was a further postulate for the targeted routine instrumentation.

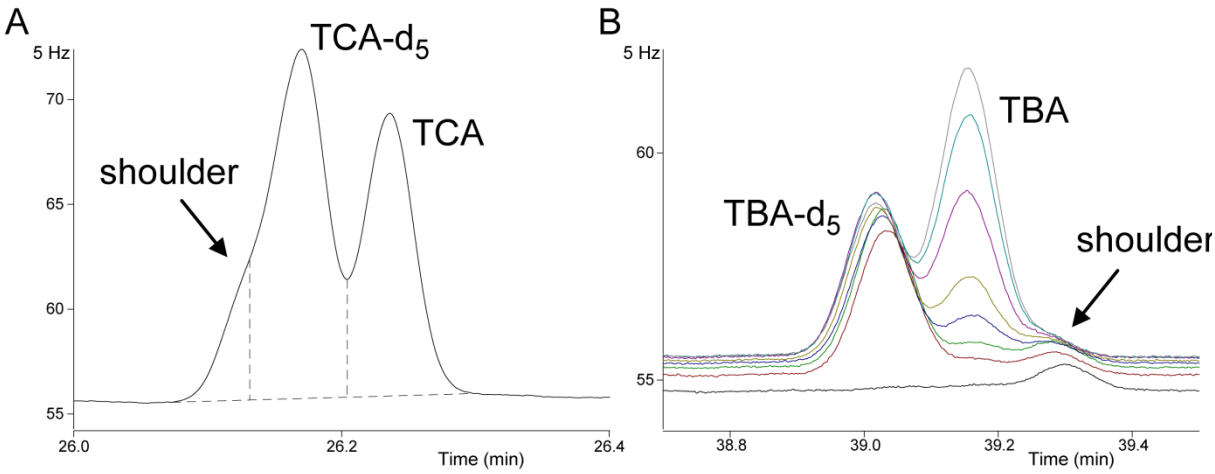


Figure 2-2 HS-SPME-MDGC-ECD ^{2}D chromatograms of (A) TCA-d_5/TCA and (B) TBA-d_5/TBA standards. The indicated shoulder visible at the peak of TCA-d5 indicates co-elution, problematic for quantification on a 35 % diphenylpolysiloxane stationary phase column. Only minor co-elution (shoulder) of a system background compound (lowest trace; chromatogram B) with TBA on TG-1301MS. Integration of TBA is not hampered for investigated calibration ranges (overlayed traces)

A good combination was found in heart-cutting from a DB-XLB column. Separation in ^{2}D was then performed on a more polar column to provide different retention behavior, further allowing separation of potential co-eluting compounds. Here, a good compromise with a cyano-based column (6 % cyanopropylphenylpolysiloxane) was found. With respect to bleed, column stability, and polarity combination, this column combination proved to be appropriate for ECD operation and chromatographic robustness. In earlier tests, combinations were tried using 5 % and 35 % diphenylpolysiloxanes, which also fulfill low-bleed and stability postulates. However, co-elution problems hampered reliable quantification, as e.g. presented

in Figure 2-2A, showing an almost perfect co-elution with the internal standard peak of TCA-d_5. Changing to a 6 % cyanopropylphenylpolysiloxane stationary phase (TG-1301MS) as ^{2}D separation column, this problem could be solved. However, a marginal co-elution of an unknown compound from system background with TBA could be observed, as presented in Figure 2-2B. Luckily, integration was not critical as can be seen for high calibration levels, having increasing peak widths for TBA, eventually merging with the co-eluting compound. Working with a calibration range up to below 10 ng/l, this co-elution situation was tolerable. In principal, such situations clearly show the drawback of a non-MS based detection, as ECD response and retention time are the only means for compound identification.

Another prerequisite for the proposed SIDA approach with a non-MS detector is the chromatographic resolution of target analyte and isotope standard. Here, the cyanopropyl based column showed a less pronounced negative isotope effect than, e.g. a more apolar dimethylpolysiloxane column. Still, the achieved resolution (Rs) was around 0.71 (TCA-d_5/TCA) and 0.75 (TBA-d_5/TBA), calculated from chromatographic raw data according to standard procedures [30]. Although not perfect (Rs ≥ 1.0) [31], integration was only with marginal errors, as reflected in the quality of validation data (Table 2-1).

Table 2-1 Method validation data for HS-SPME-MDGC-ECD analysis. Calibration graphs based on 8 calibration points (n = 3), calculated with equal weighting according to DINTEST, with ranges from 0.1 to <10 ng/l (details in Section 2.3.4)

Analyte	Calibration graph	R^2	LOD (ng/l)	LOQ (ng/l)
TCA	y = 0.407 x - 0.002	0.9994	0.1	0.4
TeCA	y = 0.709 x + 0.047	0.9998	0.1	0.4
TBA	y = 0.572 x - 0.014	0.9994	0.1	0.5

Calibration graphs express good linearity in the targeted concentration ranges with LODs and LOQs below or equal to 0.5 ng/l. This allows the quantification at relevant concentration levels below sensory thresholds. Recoveries and repeatability for method validation were determined after spiking about 3 ng/l of each analyte to the two targeted matrices: (i) three cork soaks, each prepared from 20 corks showing no sensory defects; (ii) three white wines (Riesling, Chardonnay, Müller-Thurgau) without detectable amounts of the targeted haloanisoles. Recoveries (n = 3) obtained were 108 ± 11 % (TCA), 94 ± 6 % (TeCA), and 99 ± 9 % (TBA) in cork soaks, and 113 ± 10 % (TCA), 85 ± 6 % (TeCA), and 101 ± 14 % (TBA) in wine, respectively. Repeatability (n = 3) was 3.4 ± 0.4 ng/l (TCA), 3.8 ± 0.3 ng/l (TeCA), and 3.1 ± 0.3 ng/l (TBA) in cork soaks, and 3.6 ± 0.3 ng/l (TCA), 3.7 ± 0.4 ng/l (TeCA), and 3.2 ± 0.4 ng/l (TBA) in wine, respectively.

A successful application of the proposed method is shown in Figure 2-3. On a wine sample spiked at 3 ng/l for each analyte, the ^{2}D separation still shows a considerable number of ECD active compounds, but well separated from the target analytes.

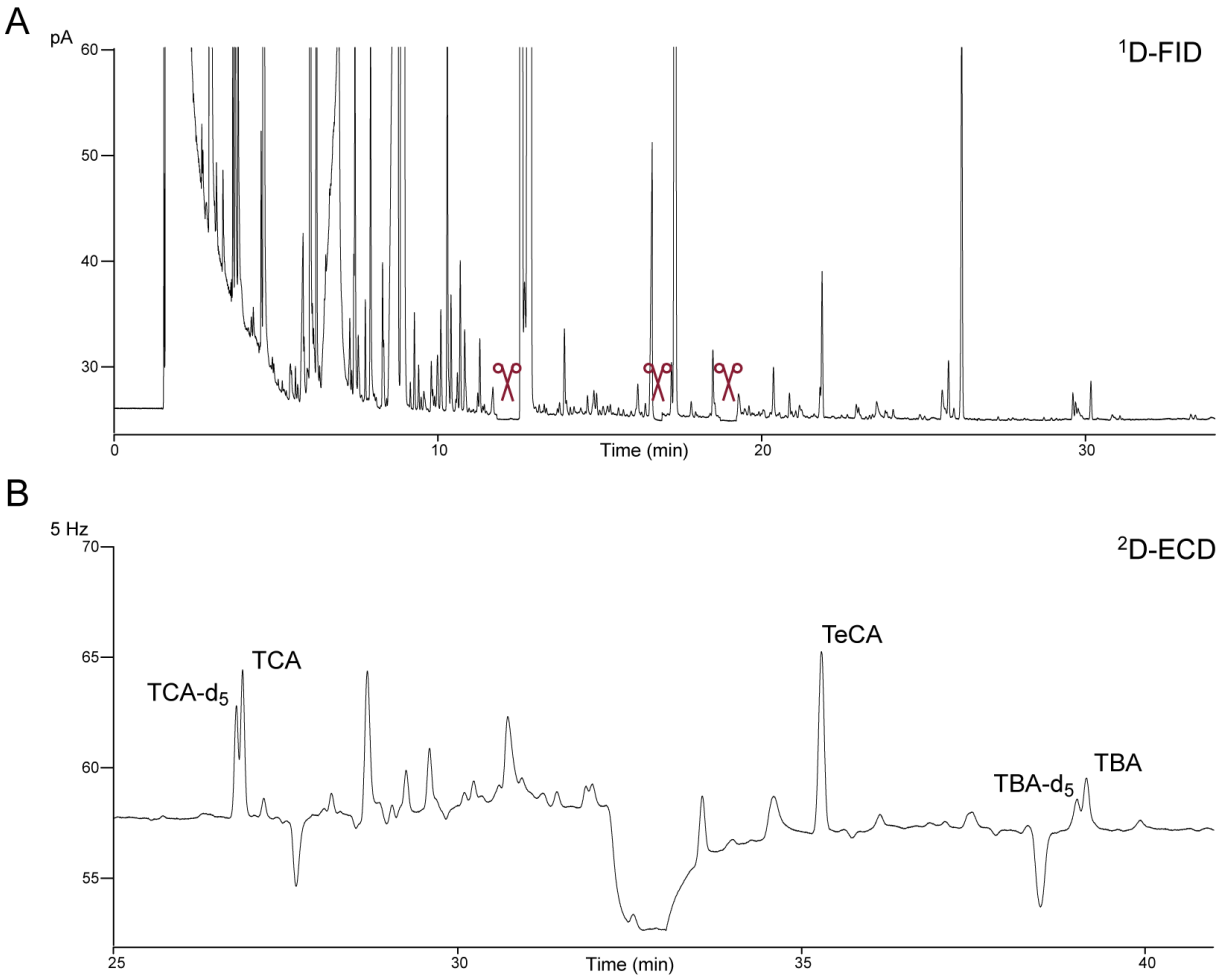


Figure 2-3 (A) ^{1}D-GC pre-separation (FID) of a wine spiked with about 3 ng/l for each analyte. Heart-cut regions indicated with symbols of scissors. (B) ^{2}D-GC separation (ECD) for the cumulative cuts of targeted haloanisoles

2.5 Conclusion

Analysis of trace-level haloanisoles in wine matrix with ^{1}D-GC is critical due to co-elution problems, and reliable quantification clearly benefits from the additional separation achieved with the described MDGC setup. The halogen sensitive ECD allows low limits of detection at sub-ng/l level, which may be crucial in customer conflict situations. The previously described laborious sample preparation with SPE [19] could be successfully substituted with an automated HS-SPME method, utilizing only minor sample preparation steps, such as salt and standard addition. Careful selection of chromatographic parameters allowed reliable quantification via the SIDA approach in a non-MS detection mode. Thus, this method has proven its usefulness in routine application.

2.6 References

1. Tanner H, Zanier C, Buser HR (1981) 2,4,6-Trichloroanisole: A dominant component of cork flavour. Schweizerische Zeitschrift für Obst- und Weinbau 117:97-103.
2. Buser HR, Zanier C, Tanner H (1982) Identification of 2,4,6-trichloroanisole as a potent compound causing cork taint in wine. Journal of Agricultural and Food Chemistry 30 (2):359-362.
3. Fischer C (1997) Neue und kostengünstige Analyse. Dem Korkton auf der Spur. Das Deutsche Weinmagazin 16 (17):30-33.
4. Tanner H, Zanier C (1981) Analytical differentiation of musty taint and cork taste in wines. Schweizerische Zeitschrift für Obst- und Weinbau 117 (26):752-757.
5. Chatonnet P, Bonnet S, Boutou S, Labadie M-D (2004) Identification and Responsibility of 2,4,6-Tribromoanisole in Musty, Corked Odors in Wine. Journal of Agricultural and Food Chemistry 52 (5):1255-1262.
6. Chatonnet P, Guimberteau G, Dubourdieu D, Boidron JN (1994) Nature and origin of musty odors in cellars: contamination of wines. Journal International des Sciences de la Vigne et du Vin 28 (2):131-151.
7. Mazzoleni V, Maggi L (2007) Effect of wine style on the perception of 2,4,6-trichloroanisole, a compound related to cork taint in wine. Food Research International 40 (6):694-699.
8. Rudy H, Scholten G (2005) Neue Analytik schützt moderne Rieslingweine Der Deutsche Weinbau 3:26-27.
9. Prescott J, Norris L, Kunst M, Kim S (2005) Estimating a “consumer rejection threshold” for cork taint in white wine. Food Quality and Preference 16 (4):345-349.
10. Fischer C, Fischer U (1997) Analysis of cork taint in wine and cork material at olfactory subthreshold levels by solid phase microextraction. Journal of Agricultural and Food Chemistry 45 (6):1995-1997.
11. Pollnitz AP, Pardon KH, Liacopoulos D, Skouroumounis GK, Sefton MA (1996) The analysis of 2,4,6-trichloroanisole and other chloroanisoles in tainted wines and corks. Australian Journal of Grape and Wine Research 2 (3):184-190.
12. Sponholz WR, Hoffmann A, David F, Sandra P (2001) Detection of corkiness in wine by analysis of 2,4,6-trichloroanisole with Stir Bar Sorptive Extraction (SBSE) and Thermal Desorption GC/MS. Mitteilungen Klosterneuburg 51 (6):248-253.

13. Lizarraga E, Irigoyen A, Belsue V, Gonzalez-Penas E (2004) Determination of chloroanisole compounds in red wine by headspace solid-phase microextraction and gas chromatography-mass spectrometry. Journal of Chromatography, A 1052 (1-2):145-149.

14. Lopes P, Marques J, Lopes T, Lino J, Coelho J, Alves C, Roseira I, Mendes A, Cabral M (2011) Permeation of d_5-2,4,6-trichloroanisole via vapor phase through different closures into wine bottles. American Journal of Enology and Viticulture 62 (2):245-249.

15. Alzaga R, Ortiz L, Sanchez-Baeza F, Marco MP, Bayona JM (2003) Accurate determination of 2,4,6,-trichloroanisole in wines at low parts per trillion by solid-phase microextraction followed by GC-ECD. Journal of Agricultural and Food Chemistry 51 (12):3509-3514.

16. Ozhan D, Anli RE, Vural N, Bayram M (2009) Determination of chloroanisoles and chlorophenols in cork and wine by using HS-SPME and GC-ECD detection. Journal of the Institute of Brewing 115 (1):71-77.

17. Riu M, Mestres M, Busto O, Guasch J (2007) Comparative study of 2 chromatographic methods for quantifying 2,4,6-trichloroanisole in wines. Journal of Chromatography, A 1138 (1-2):18-25.

18. Weingart G, Schwartz H, Eder R, Sontag G (2010) Determination of geosmin and 2,4,6-trichloroanisole in white and red Austrian wines by headspace SPME-GC/MS and comparison with sensory analysis. European Food Research and Technology 231 (5):771-779.

19. Schmarr H-G, Koschinski S, Sang W, Slabizki P (2012) Trace level analysis of corky off-flavor compounds: Development of a new analytical method based on solid phase extraction and analysis by multidimensional gas chromatography with mass spectrometric detection. Journal of Chromatography, A 1226:96-102.

20. Butzke CE, Evans TJ, Ebeler SE (1998) Detection of cork taint in wine using automated solid-phase microextraction in combination with GC/MS-SIM. ACS Symposium Series 714 (Chemistry of Wine Flavor):208-216.

21. Boutou S, Chatonnet P (2007) Rapid headspace solid-phase microextraction/gas chromatographic/mass spectrometric assay for the quantitative determination of some of the main odorants causing off-flavours in wine. Journal of Chromatography, A 1141 (1):1-9.

22. Martinez-Urunuela A, Gonzalez-Saiz JM, Pizarro C (2004) Optimization of a headspace solid-phase microextraction method for the direct determination of chloroanisoles related to cork taint in red wine. Journal of Chromatography, A 1056 (1-2):49-56.

23. Berthod R, Flaction O, Frey U (2004) Fast determination of Chlorophenols at the ppt level. A new analytical tool for quality control of cork stoppers? Chimia 58 (7-8):560-562.

24. Rittenberg D, Foster GL (1940) A new procedure for quantitative analysis by isotope dilution, with application to the determination of amino acids and fatty acids. Journal of Biological Chemistry 133:733-744.

25. Milo C, Blank I (1998) Quantification of impact odorants in food by isotope dilution assay: strength and limitations. ACS Symposium Series 705 (Flavor Analysis):250-259.

26. Bianco G, Novario G, Zianni R, Cataldi TRI (2009) Comparison of two SPME fibers for the extraction of some off-flavor cork-taint compounds in bottled wines investigated by GC-HRMS. Analytical and Bioanalytical Chemistry 393 (8):2019-2027.

27. Evans TJ, Butzke CE, Ebeler SE (1997) Analysis of 2,4,6-trichloroanisole in wines using solid-phase microextraction coupled to gas chromatography-mass spectrometry. Journal of Chromatography, A 786 (2):293-298.

28. Vestner J, Fritsch S, Rauhut D (2010) Development of a microwave assisted extraction method for the analysis of 2,4,6-trichloroanisole in cork stoppers by SIDA-SBSE-GC-MS. Analytica Chimica Acta 660 (1-2):76-80.

29. Deans D (1968) A new technique for heart cutting in gas chromatography. Chromatographia 1 (1):18-22.

30. Rohrschneider L (1980) Analysen und Messverfahren. Ullmanns Encyclopädie der technischen Chemie, vol 5. Verlag Chemie, Weinheim, Germany.

31. Sandra P (1989) Resolution–definition and nomenclature. Journal of High Resolution Chromatography 12 (2):82-86.

2.7 Appendix

Table 2-2 Screening for haloanisoles in wines, cellar atmospheres and various materials from the cellar surroundings from different wineries (A-D) to localize the cause of contamination. Origins of haloanisole contamination in exemplary wineries were: TCA formation due to unknown source of chlorine and microbial activity (winery A), TBA contamination of cardboard and wooden box possibly due to usage of recycled material (winery B), TCA contamination of cork stopper (winery C), TeCA contamination of wood paneling (winery D)

Sample	TCA [ng/l]	TeCA [ng/l]	TBA [ng/l]	Sample information
Winery A				
Pinot Noir, 2009	11.8	<LOD	<LOD	
cork soak	2.8	<LOD	<LOD	soak of corresponding cork stopper of Pinot Noir, 2009
Riesling, 2012	0.8	<LOQ	<LOD	
cork soak	0.6	<LOQ	<LOD	soak of corresponding cork stopper of Riesling, 2012
rubber stopper	1.7	<LOQ	<LOD	[a]
cellarcheck 1	3.3	<LOQ	<LOQ	[b]
cellarcheck 2	1.6	<LOQ	<LOD	cellar with stainless steel barrels [b]
cellarcheck 3	5.0	<LOQ	<LOQ	cellar with wooden barrels [b]
Winery B				
Müller-Thurgau, 2011	<LOD	<LOD	0.7	sealed with screw cap
cardboard	0.5	1.1	94.9	[a]
wooden box	<LOQ	0.9	17.2	[a]
rubber gasket	<LOQ	<LOQ	4.6	[a]
cellarcheck	<LOD	<LOD	1.9	cellar with barrels [b]
Winery C				
Riesling, 2011	2.2	<LOD	<LOD	
cork soak	5.2	<LOD	<LOD	soak of corresponding cork stopper of Riesling, 2011
Winery D				
white wine, 2013	<LOD	0.4	<LOD	
cellarcheck 1	0.4	9.3	<LOD	[b]
cellarcheck 2	<LOD	1.9	<LOQ	[b]
rubber gasket	<LOQ	14.6	<LOD	[a]
rubber stopper	<LOQ	11.8	<LOD	[a]
wooden piece	0.5	215.2	0.6	piece from wood paneling [a]

[a] soaked in 10%vol. aqueous ethanolic solution

[b] aqueous ethanolic solution (10%vol.) as passive sampler for cellar atmosphere

3 Characterization and analysis of structural isomers of dimethyl methoxypyrazines in cork stoppers and ladybugs (*Harmonia axyridis* and *Coccinella septempunctata*)

Adapted with permission from P. Slabizki, C. Legrum, R. Meusinger, H.-G. Schmarr, Characterization and analysis of strucural isomers of dimethyl methoxypyrazines in cork stoppers and ladybugs (*Harmonia axyridis* and *Cooccinella septempunctata*), *Analytical and Bioanalytical Chemistry*, **2014**, 406, 6429-6429, Copyright (2014) Springer-Verlag.

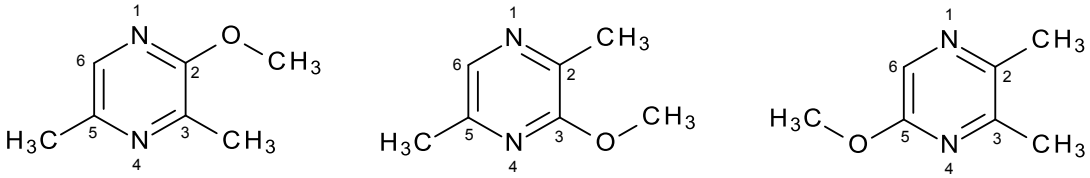
3.1 Abstract

The three constitutional isomers of dimethyl-substituted methoxypyrazines: 3,5-dimethyl-2-methoxypyrazine **1**; 2,5-dimethyl-3-methoxypyrazine **2**; and 2,3-dimethyl-5-methoxypyrazine **3** are potent flavor compounds with similar mass spectrometric, gas chromatographic, and nuclear magnetic resonance spectroscopic behavior. Therefore, unambiguous analytical determination is critical, particularly in complex matrices. The unequivocal identification of **1-3** could be achieved by homo- and heteronuclear NMR correlation experiments. The observed mass fragmentation for **1-3** is proposed and discussed, benefitting from synthesized partially deuterated **1** and **2**. On common polar and apolar stationary phases used in gas chromatography (GC) **1** and **2** show similar behavior whereas **3** can be separated. In the focus on off-flavor analysis with respect to wine aroma, **1** has been described as a “moldy” off-flavor compound in cork and **2** as a constituent in *Harmonia axyridis* contributing to the so-called “ladybug taint,” whereas **3** has not yet been described as a constituent of wine aroma. A successful separation of **1** and **2** could be achieved on octakis-(2,3-di-*O*-pentyl-6-*O*-methyl)-γ-cyclodextrin as stationary phase in GC. Applying heart-cut multidimensional GC analysis with tandem mass spectrometric detection the presence of **1** as a “moldy” off-flavor compound in cork could be confirmed. However, in the case of *Harmonia axyridis*, a previous identification of **2** has to be reconsidered. In the described experiments the constitutional isomer **1** was identified, which was also found in *Coccinella septempunctata*, another species discussed with respect to the “ladybug taint.” The analysis of such structurally related compounds is a demonstrative example for the importance of a chromatographic separation, as mass spectrometric data by itself could not guarantee the unequivocal identification.

3.2 Introduction

Alkyl methoxypyrazines have been identified in many foodstuffs of plant origin as aroma-relevant compounds [1] but can also be generated as Maillard reaction products [2]. Due to their, in many cases, low odor thresholds they contribute significantly to the aroma of such foodstuffs or products made thereof. Well-known representatives are 3-alkyl-2-methoxypyrazines such as 3-isobutyl-2-methoxypyrazine, 3-isopropyl-2-methoxypyrazine, or 3-*sec*-butyl-2-methoxypyrazine which are responsible for characteristic aroma attributes as e.g. in bell peppers [3], peas [4], carrots [5] or some *Vitis vinifera* varieties like Sauvignon blanc [6].

Alkyl methoxypyrazines with two methyl groups have also been identified in a variety of matrices. 3,5-dimethyl-2-methoxypyrazine **1** was found as an obnoxious musty odor compound from the metabolism of Gram-negative bacteria [7] and was also detected in a machine cutting fluid emulsion with an off-odor [8]. Later, **1** was also described in raw hazelnuts [9], raw arabica coffee beans [10], or cooked brown rice [11]. Sensory attributes for **1** are described as “wet cardboard,” “musty,” “moldy,” “dusty,” and “earthy,” and in higher concentrations with attributes such as “chocolate,” “coffee,” and “nutty” [9,10,12-14]. Czerny and Grosch determined an odor threshold for **1** as low as 1 pg/l in air [10].



1 **2** **3**

Figure 3-1 Structural isomers of dimethyl methoxypyrazines: 3,5-dimethyl-2-methoxypyrazine **1**; 2,5-dimethyl-3-methoxypyrazine **2**; and 2,3-dimethyl-5-methoxypyrazine **3**

For dimethyl-substituted methoxypyrazines, three structural isomers **1**–**3** (Figure 3-1) are possible. Changing the position of the methyl groups causes a drastic change in sensory properties of the individual compounds. A dramatic increase in odor intensity is found for compound **1** (1 pg/l in air) in comparison with 2,5-dimethyl-3-methoxypyrazine **2** (56 ng/l air) [10]. Such a structural dependent quantitative (and sometimes also qualitative) change of the sensory properties of substituted pyrazines is often found and has been described in extensive studies, e.g., by Mihara et al. [15,16].

Sensory properties for **2** described in literature are associated with attributes such as “earthy,” “musty,” “roasted peanuts,” or “dead leaves” [10,17,18]. In foodstuffs, **2** has been described in pepper (*Piper nigrum* L.) [19], cheese [20], peanuts [21], sesame paste [22], and in Mentha species [23]. To the best of the author’s knowledge, there has been no odor

threshold described for 2,3-dimethyl-5-methoxypyrazine **3**, yet. Furthermore, only one incidence has been found in literature for the occurrence of **3** in nature. Recently, Poehlmann and Schieberle described it as one of the odor-active compounds in Styrian pumpkin seed oil [24]. The odor quality (determined by gas chromatography–olfactometry; GC-O) was given as “roasty, sweet;” however, no odor threshold had been determined. According to these authors, identification was based on mass spectral data, retention indices, and odor descriptions compared to reference substances. However, no spectral data was given.

In the ongoing research on the occurrence of alkyl methoxypyrazines in wine and related matrices [25,26], the investigation of dimethyl methoxypyrazines became interesting since they had been described as off-odor compounds. Simpson et al. identified compound **1** in cork stoppers causing a “fungal must” taint in wine. They determined the odor threshold for **1** in a white wine matrix with 2.1 ng/l [13]. **1** has also been found as a malodorous compound in water supplies [27] and seems to be generated by bacteria (as well as **2**) [28,29]. This first incidence of **1** as off-flavor compound in cork was later confirmed by Chatonnet et al. who also described **1** in oak chips and further discussed the microbiological origin of **1** [14]. In recent years, the wine industry has been confronted with an off-odor problem associated with beetles that are harvested together with the grapes, leading to the so-called “ladybug taint” in wine [30]. In this respect, **2** has been described together with other 3-alkyl-2-methoxypyrazines as a constituent of *Harmonia axyridis* [17] and also in *Coccinella septempunctata* [18], the beetles that were eventually causing the “ladybug taint.”

Identification of dimethyl methoxypyrazines in earlier work was often done by GC-O (odor description), comparison of retention index and mass spectrometric detection. Since mass spectral and chromatographic data for compounds **1** and **2** are very similar and may cause erroneous results [31], all constitutional isomers of dimethyl methoxypyrazines **1-3** were first characterized. For trace-level analytical studies with respect to wine off-flavor analysis, an analytical method based on heart-cut multidimensional gas chromatography coupled to a triple quadrupole mass spectrometer (H/C MDGC-MS-MS) was then developed.

3.3 Materials and methods

3.3.1 Chemicals

Dichloromethane, methanol, and methyl tert-butyl ether were from Carl Roth (Karlsruhe, Germany); sodium, sodium sulfate, sodium carbonate (anhydrous), and calcium chloride hexahydrate were from Merck (Darmstadt, Germany); [$^2H_1$]-chloroform and sodium hydroxide were from KMF Laborchemie (Lohmar, Germany). 3,5-dimethyl-2-chloropyrazine (CAS no. 38557-72-1) and methyl-deuterated [$^2H_3$]-methanol (CAS no. 1849-29-2) were from ABCR (Karlsruhe, Germany); 2,5-dimethyl-3-chloropyrazine (CAS no. 95-89-6) and

trimethyloxonium tetrafluoroborate were from TCI Europe (Eschborn, Germany); butane-2,3-dione (CAS no. 431-03-8), 3-ethyl-2-methoxypyrazine **4** (CAS no. 25680-58-4) and 3-(1-methylethyl)-2-methoxypyrazine **5** (3-isopropyl-2-methoxypyrazine; CAS no. 25773-40-4) were from Sigma-Aldrich (Steinheim, Germany); glycinamide hydrochloride (CAS no. 1668-10-6) was from Alfa Aesar (Karlsruhe, Germany); and hydrochloric acid was from Riedel-de Haën (Seelze, Germany). The [$^2H_3$]-isotopologue of **5** (**d-5**) was synthesized as described earlier [25,26]. Commercial chemicals were usually of analytical grade and used as such, except butane-2,3-dione which was freshly distilled. The reference substance **1** (CAS no. 92508-08-2) was purchased from Bellen Chemistry Co. Ltd. (Beijing, China).

3.3.2 Syntheses of other reference compounds

(i) 2,5-dimethyl-3-methoxypyrazine **2**

The synthesis of **2** was done according to the procedure described earlier by Czerny and Grosch [10]. 2,5-dimethyl-3-chloropyrazine (1.99 g, 14 mmol) was dissolved in methanol (5 ml) and freshly prepared sodium methoxide solution (~400 mg, ~17 mmol sodium in 7 ml methanol) was added and refluxed until completeness of the reaction (monitored by gas chromatography–mass spectrometry (GC-MS)). After reaching room temperature, distilled water (20 ml) was added and the products were extracted with methyl *tert*-butyl ether (5 × 15 ml). The combined organic phases were dried with sodium sulfate and concentrated using a micro distillation apparatus with a spinning band column. The residual solvent was purged with argon gas using a fused silica capillary. The obtained purity of **2** was 97 % (determined by GC-MS).

(ii) 2,5-dimethyl-3-[$^2H_3$]-methoxypyrazine **d-2**

d-2 was prepared as described for **2** but substituting methanol with [$^2H_3$]-methanol. Purity was 95 % (determined by GCMS).

(iii) 3,5-dimethyl-2-[$^2H_3$]-methoxypyrazine **d-1**

d-1 was prepared as described for **d-2** but using 3,5-dimethyl-2-chloropyrazine as starting material. Purity was 99 % (determined by GC-MS).

(iv) 2,3-dimethyl-5-hydroxypyrazine **6**

Synthesis of **3** followed a common approach for generation of the heterocyclic ring system described earlier [32,33], condensing a vicinal dicarbonyl compound with hydrohalides of the

appropriate amino acid amides. Here, glycinamide hydrochloride (9 mmol) was dissolved in methanol (15 ml) and freshly distilled butane-2,3-dione (9 mmol in 5 ml water) was added. With continuous stirring, 12 M sodium hydroxide solution (2 ml) was added dropwise at a temperature kept at -25 °C (ice-calcium chloride hexahydrate mixture). After 2 h stirring at room temperature, the reaction mixture was allowed to warm to 0 °C and then 12 M hydrochloric acid (2 ml) was added. After multiple extractions with dichloromethane, the organic extract was dried with sodium sulfate, filtered, and the solvent was evaporated, yielding 0.3 g of a yellow amorphous raw product that was characterized by GC-MS with **6** as the major product. qMS (EI+): **42** (100), **124** (78, M+), **95** (60), **96** (37), **81** (22), **54** (18), **41** (14), **32** (10), **52** (9), **43** (8); linear retention index (LRI, based on n-alkanes and determined as described earlier [34]): ZB-Wax 2366, ZB-5 1173 (fronting peak, retention taken at the front of the peak).

(v) 2,3-dimethyl-5-methoxypyrazine **3**

Selective *O*-methylation of **6** was achieved using a Meerwein salt (trimethyloxonium tetrafluoroborate, TMO) [35]. Raw product **6** was mixed with sodium carbonate (0.3 g) in water (6 ml) and TMO (0.3 g) was added in portions under stirring at room temperature. After 12 h of stirring, another portion of 0.2 g of TMO was added and stirred overnight. The dark red reaction mixture was transferred into a GC headspace vial with a silicone septum cap and purged with argon by inserting a fused silica capillary through the septum and into the liquid mixture. The purged volatiles left the vial through another piece of fused silica capillary (5 cm × 0.53 mm i.d. capillary) and were trapped on a LiChrolut® EN solid phase extraction (SPE) cartridge (200 mg/3 ml; Merck) fitted via a press-fit connector and a piece of a polyethylene tube to the capillary. The total purge time was 3 h with a flow of some 25 ml/min. To avoid a potential breakthrough, every 45 min, the cartridge was replaced with a new one. Each of the (four) cartridges was eluted with 1 ml of [$^2H_1$]-chloroform. After pooling and concentrating with a micro distillation apparatus [36], the residual solution was used for further characterization by GC-MS and nuclearmagnetic resonance spectroscopy (NMR).

3.3.3 Gas chromatographic and mass spectrometric analysis

GC-MS was done on three different systems:

(i) A Finnigan Trace GC Ultra (Thermo Fisher Scientific, Dreieich, Germany) was equipped with a programmed temperature vaporizing injector (PTV) and coupled to an ion trap PolarisQ mass spectrometer (Thermo Fischer Scientific). The analytical column used was a 30 m × 0.25 mm i.d. fused silica capillary coated with 0.25 µm of 5 % diphenyl 95 % dimethyl

polysiloxane (ZB-5, Phenomenex, Aschaffenburg, Germany). Helium was used as carrier gas at a constant flow of 1.2 ml/min. Split injection was done at 240 °C (split ratio 1:10). Oven temperature was programmed from 40 °C (2 min isothermal) with 5 °/min to 250 °C (5 min hold). MS detection was performed in positive electron ionization mode (EI) at 70 eV with a scan range of m/z 29-350. Ion source temperature was held at 230 °C and the transfer line was set at 250 °C.

(ii) A 8000 series GC instrument (C. E. Instruments, now Thermo Fisher Scientific) was coupled to a MD 800 quadrupole mass spectrometer (Fisons Instruments, now Thermo Fisher Scientific). Separation was done with a fused silica capillary (30 m × 0.25 mm i.d.) coated with 0.5 µm polyethylene glycol (ZB-WAX, Phenomenex) which was connected to a 2 m × 0.32 mm i.d. phenylmethylsilylated fused silica capillary as a precolumn. The split/splitless injector base was at 210 °C and injection was done at a split ratio of about 1:50. Helium was used as carrier gas in constant pressure mode at 75 kPa. Oven temperature was programmed from 40 °C (5 min isothermal) with 5 °/min to 240 °C (10 min hold). MS acquisition was done in positive EI mode at 70 eV in full scan mode from m/z 29 to 350. Ion source and transfer line were heated at 230 and 200 °C, respectively.

(iii) A Trace GC Ultra was coupled to a triple quadrupole mass spectrometer (Quantum Ultra; both Thermo Fisher Scientific). The GC was upgraded (S+H Analytik GmbH, Mönchengladbach, Germany) with a Deans' switching device (SGE, Victoria, Australia) for H/C MDGC and a cryo-trap made in-house using a dual cryo-jet GC×GC modulator (Thermo Fisher Scientific) and liquid CO_2 as coolant, allowing trapping of material being transferred from ^{1}D into ^{2}D. The ^{1}D analytical column used was a 15 m × 0.25 mm i.d. fused silica capillary coated with 0.25 µm of polyethylene glycol (ZB-WAX, Phenomenex) and a ^{2}D separation column consisting of a 25 m × 0.25 mm i.d. fused silica capillary coated with a derivatized cyclodextrin stationary phase (LIPODEX G®, Macherey-Nagel, Düren, Germany). Helium was used as carrier gas at constant pressure modes. A PTV injector was connected to the ^{1}D separation column with pressure set to 146 kPa. Using the second (split/splitless) injector for auxiliary gas supply, this was set to 121 kPa. Flow diversion was achieved by redirecting the auxiliary gas using a three-port valve (Valco Instruments Co. Inc., Houston, TX, USA). Actuation of the switching (H/C) events was achieved via the event and valve functions of the instrument and programmed in the manufacturer's software. Headspace solid phase microextraction (HS-SPME) was done at the PTV injector in splitless mode (2 min, 270 °C, 1 mm i.d. liner). The oven temperature was programmed from 40 °C (2 min isothermal) at 8 °/min to 137 °C (0.5 min isothermal). Then the temperature was lowered to 60 °C at 30 °/min (5 min isothermal) before the ^{2}D GC separation started by raising the temperature to 75 °C at 1 °/min and finally, to 190 °C at 50 °/min (5 min isothermal). The cryo-jet was actuated from 11.5 min (ca. 0.5 min before the first heart-cut) until 18 min (ca.

1 min after the oven temperature had reached 60 °C). MS detection was performed in positive EI mode at 70 eV. For selected reaction monitoring (SRM) argon (99.999 % purity) was used as collision gas with a collision cell pressure of 1.1 mTorr. Mass resolution in Q1 and Q3 were 0.7 amu. The total cycle time was 300 ms. The optimized SRM transfers and collision energies were (quantifier SRMs are highlighted in bold): 137 → 107 (10 V), 138 → 109 (12 V), and **138** → **120** (8 V) for **1** and **2**; 123.1 → 95.1 (8 V), 138.1 → 119.1 (8 V), and **138.1** → **123.1** (10 V) for **4**; 137 → 109 (8 V), 152 → 124 (8 V), and **152** → **137** (8 V) for **5**; and 127.1 → 95 (8 V), 140 → 112.1 (8 V), and **155.1** → **140** (8 V) for **d-5**. Ion source temperature was held at 230 °C and the transfer line was set at 190 °C. Automated HS-SPME extraction (TriPlus RSH, Thermo Fisher Scientific) of ladybug samples used a 2 cm divinylbenzene/carboxen/polydimethylsiloxane fiber (Supelco, Sigma-Aldrich) with an extraction time of 30 min at a temperature of 50 °C. Life bugs were chilled and then weighted into 20 ml headspace vials with a teflon-lined screw cap. Approximate estimation of concentrations of dimethyl methoxypyrazines was based on relative response factors using **d-5** as an internal standard. Injection of cork extracts (soaks in 10 %vol. ethanol, previously purged and trapped onto LiChrolut® EN (Merck) SPE cartridges, then eluted with dichloromethane and concentrated to a small volume using micro methods [36]) was done in a PTV on-column mode (on-column liner from Thermo Fisher Scientific) after attaching a 1 m × 0.53 mm i.d. phenylmethyl silylated pre-column via a press-fit connector (BGB Analytik AG, Adliswil, Switzerland) and adjusting the chromatographic conditions accordingly.

Instrument control and MS data acquisition was performed via Xcalibur software, version 1.2 (ii), 2.0.7 (i), and 2.2 (iii) (all Thermo Fisher Scientific). NIST library version 2011 was available as mass spectral database (NIST, Gaithersburg, MD, USA). LRIs were calculated using a series of n-alkanes [34].

3.3.4 Nuclear magnetic resonance spectroscopy

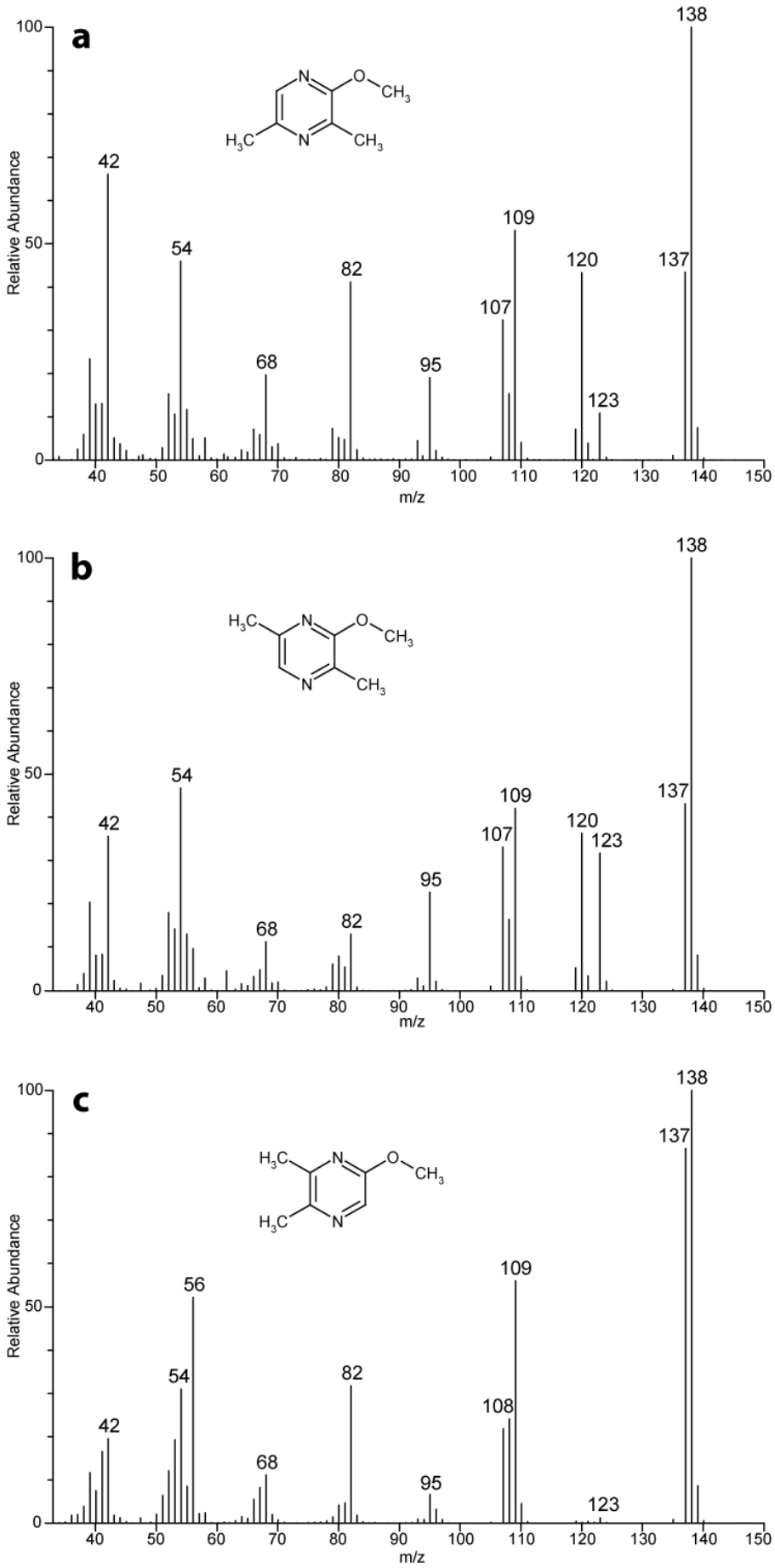
NMR spectroscopy was performed on Bruker Avance DRX 500 spectrometer (Bruker Biospin, Karlsruhe, Germany). All ^{1}H, ^{13}C, and ^{15}N-NMR measurements were performed with standard conditions, using [$^{2}H_{1}$]-chloroform as solvent. The chloroform signals were used as internal standard for ^{1}H- (7.20 ppm) and ^{13}C-NMR (77.20 ppm) spectra, whereas the ^{15}N-NMR spectra were referred to nitromethane (0.0 ppm). All ^{15}N-NMR shift values were determined by heteronuclear multiple bond correlation (HMBC) spectra. The convention of assigning negative values to signals occurring high field of the reference is used here also. The reliable assignment of all ^{1}H, ^{13}C and ^{15}N-signals were received by two-dimensional NMR measurements (COSY-, NOESY-, HSQC-, and HMBC spectra). Raw data were

processed with the MestReNova vers. 8.0 software (Mestrelab Research, Santiago de Compostela, Spain).

3.4 Results and discussion

Based on the reports of **1** as a potent musty compound in wine from cork stoppers [13] and of **2** as a constituent of *Harmonia axyridis* [17] or in *Coccinella septempunctata* [18], chromatographic and mass spectrometric properties of these compounds were at first evaluated for future wine aroma studies. Whereas **1** was commercially available, **2** had to be synthesized as a reference substance. Compounds **1** and **2** showed an almost identical mass spectrum (Figure 3-2a, b). Also, on classical stationary phases often used in GC analysis of aroma compounds (such as a 5 % diphenyl 95 % dimethyl polysiloxane, or a polyethylene glycol type stationary phase), retention indices for compounds **1** (ZB-5 1053, ZB-WAX 1439) and **2** (ZB-5 1058, ZB-WAX 1442) are very similar. This hampers an unambiguous identification by retention index-based GC analysis but also by GC with MS detection, particularly in the situation of real world matrix burdened samples. LRIs for **1** found in literature on a DB-5 are 1054 [9] or 1055 [10], data that compares well with the findings of 1053 on a ZB-5. In general, reliability of retention index information used for compound identification is dependent on a variety of conditions involved in their generation, such as the exact chemical nature of the stationary phase used, the temperature program rate involved, and others. This has been studied in more detail and has been summarized e.g. by Bicchi et al. [37].

Furthermore, when either compound **1** or **2** is analyzed by GC-MS and searching the resulting spectrum against one of the common commercial spectral databases (such as NIST 2011 in this case), the search result yields compound **2** as hit (Figure 3-3). Compound **1** is not listed yet. Looking closer into the mass spectra of compound **2** and into that included in the NIST database, there is an apparent difference that cannot be explained. The NIST spectrum does not show m/z 120. However, m/z 120 is present in the spectrum generated with a quadrupole mass spectrometer as seen in Figure 3-2b as well as in the mass spectrum produced with an ion trap mass spectrometer by Czerny [38]. The mass spectrum obtained for **1** is comparable to data published earlier [13,14,38]. To further complicate an identification solely based on a spectral library comparison is the fact that the constitutional isomer 3-ethyl-2-methoxypyrazine **4** generates an almost identical mass spectrum (Figure 3-2d) and is therefore listed among the search results of the spectral database. Interestingly, **4** also shows similar LRIs (ZB-5 1055, ZB-WAX 1439) as compound **1** or **2** on a phenyl dimethyl polysiloxane or a polyethylene glycol stationary phase, respectively. Thus, an unambiguous identification in a GC-MS analysis is critical and should require sufficient



(continuation on next page)

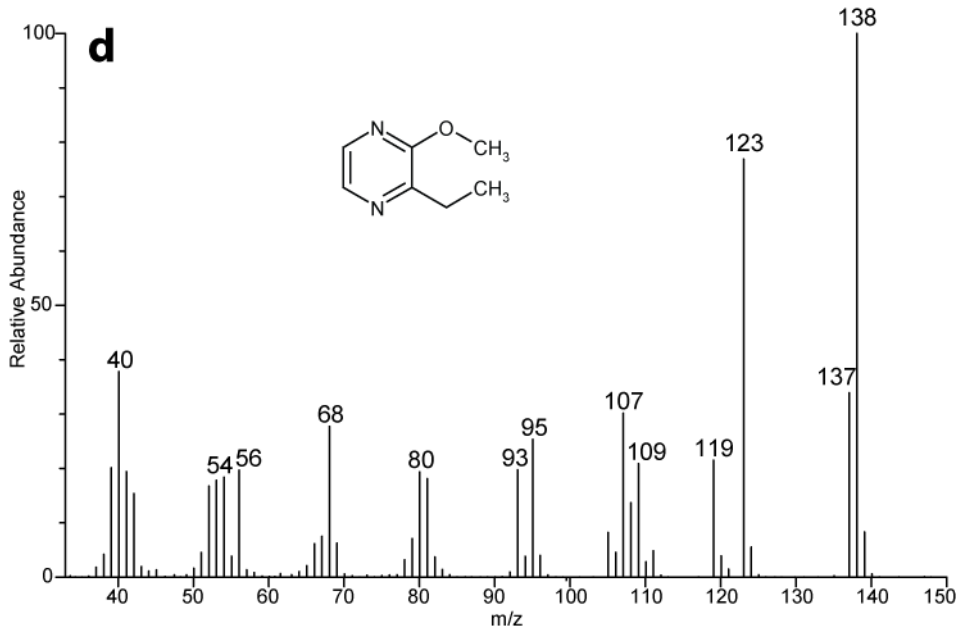


Figure 3-2 Mass spectra of constitutional isomers of methoxypyrazines with molecular formula $C_7H_{10}N_2O$ (compounds **1**-**4**); conditions as described in text

chromatographic separation and reference standards with a known chemical nature. This fundamental problem is not new to the analytical chemist but might be overseen occasionally. In fact, this issue had triggered others to publish a statement on how compound identification should be conducted, particularly in the complex field of aroma analysis [31].

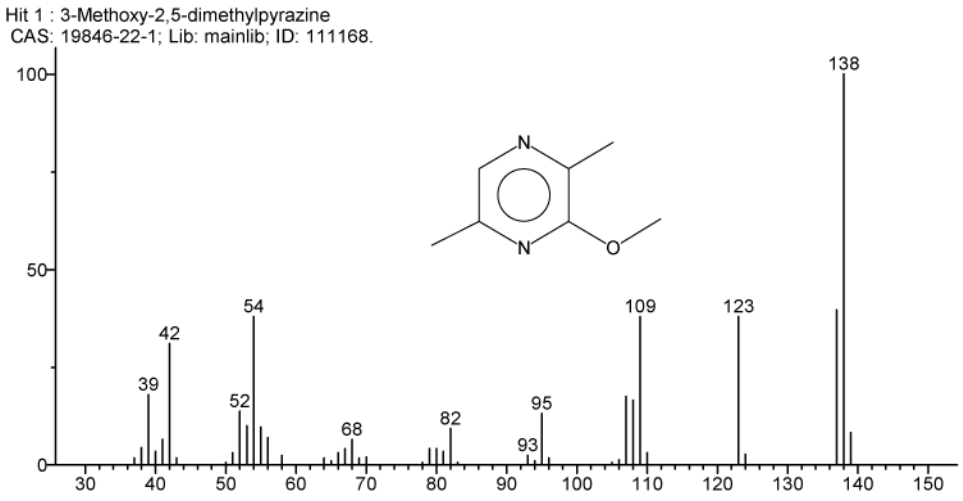


Figure 3-3 Mass spectrum of compound **2** published in NIST database (relative abundance over m/z)

Since a third constitutional isomer is possible, compound **3** was also synthesized for comparison studies. The LRIs of **3** (ZB-5 1079, ZB-WAX 1493) on the investigated stationary phases are different from those of compound **1** and **2**, providing enough separation on the commonly used apolar or polar stationary phases. This might be explained by a shielding effect of the oxygen by the neighboring methyl group in compounds **1** and **2**, which is not possible with structure **3**. Also, the mass spectrum of **3** is somewhat different and would ease

differentiation from compounds **1**, **2**, or **4** (Figure 3-2c) by MS detection. Mass fragmentation of dimethyl methoxypyrazines is proposed in Figure 3-4 with the example of **1**. The major difference in the spectra of compound **3** is the missing fragment ion m/z 120, that can be explained by the loss of H_2O. Such a loss of H_2O is only possible if a methyl group is situated at the adjacent ring carbon bearing the methoxy group and is only compatible with compounds **1** and **2** [39]. Some of the proposed mass fragments given in Figure 3-4 are supported by the study of the synthesized isotopic compounds with deuterium incorporation

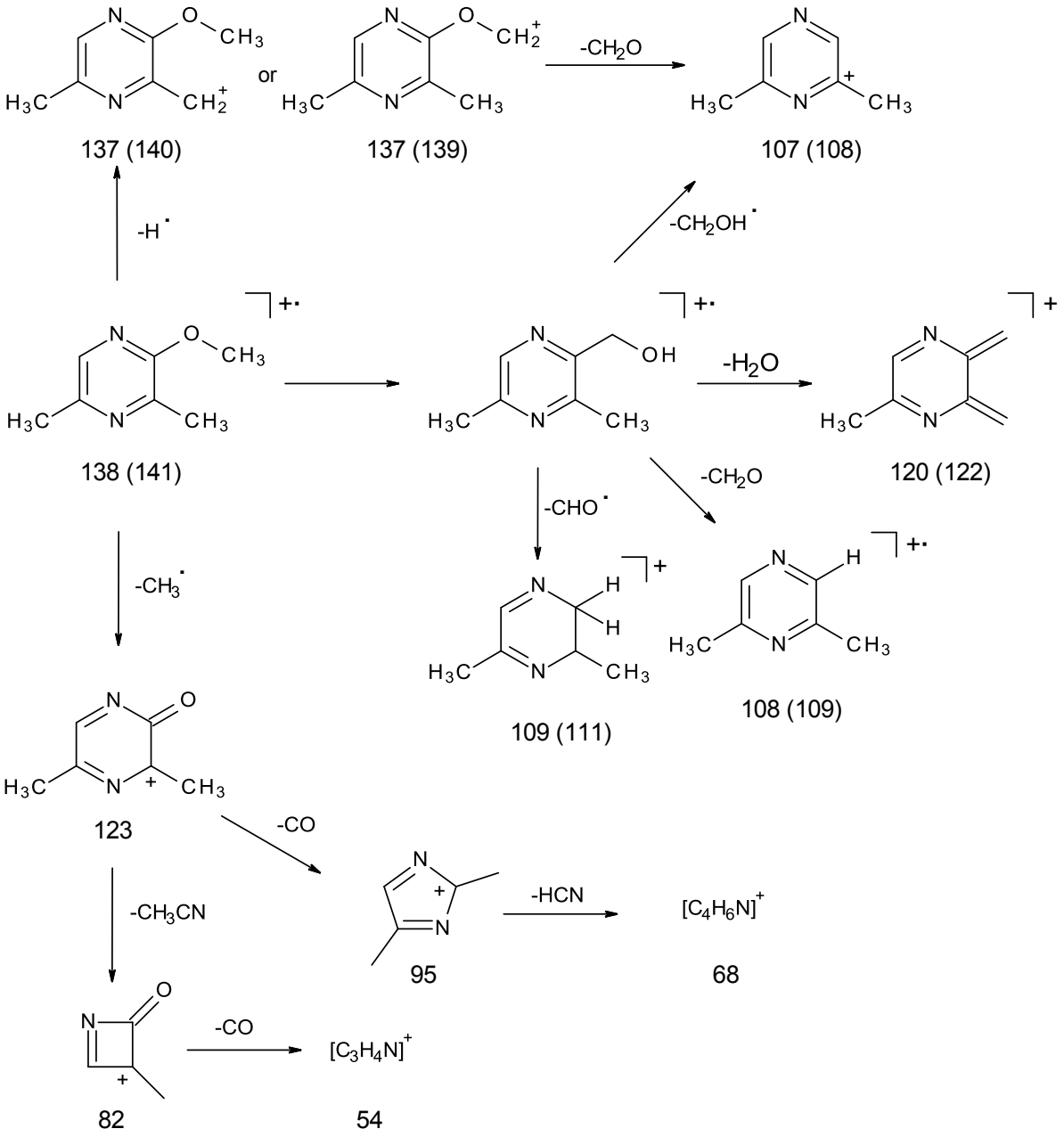


Figure 3-4 Proposed mass fragmentation of **1**. Fragments of **2** and **3** are concordant with a different methyl group arrangement, respectively. With compound **3** the fragment with m/z 120 is not observed (explanation described in text). Fragmentations with m/z values given in parenthesis represent isotopic (deuterated, $^2H_3$) compounds **d-1** and **d-2**

in the methoxy group (–OC[$^2H_3$]; **d-1** and **d-2**; Figure 3-5). Particularly, those fragmentations with m/z values given also in parenthesis represent such instances. As deuteration is in the methoxy group, cleavage of the methoxy group would lead to the mass fragment m/z 107. This fragment is present, however, with minor intensity in comparison to m/z 108. The latter could be explained by a rearrangement forming the proposed alcohol and followed by loss of C[$^2H_2$]OH•. Comparing dimethyl methoxypyrazines (**1**–**3**) with the ethyl methoxypyrazine **4**, a higher abundance for m/z 123 is observed. This can be explained by a favored expulsion of a CH_3• from the alkyl chain rather than from the methoxy group. Interestingly, in **4** a loss of H_2O is occurring with a slightly different mechanism, since m/z 119 is rather observed than the m/z 120 explained before.

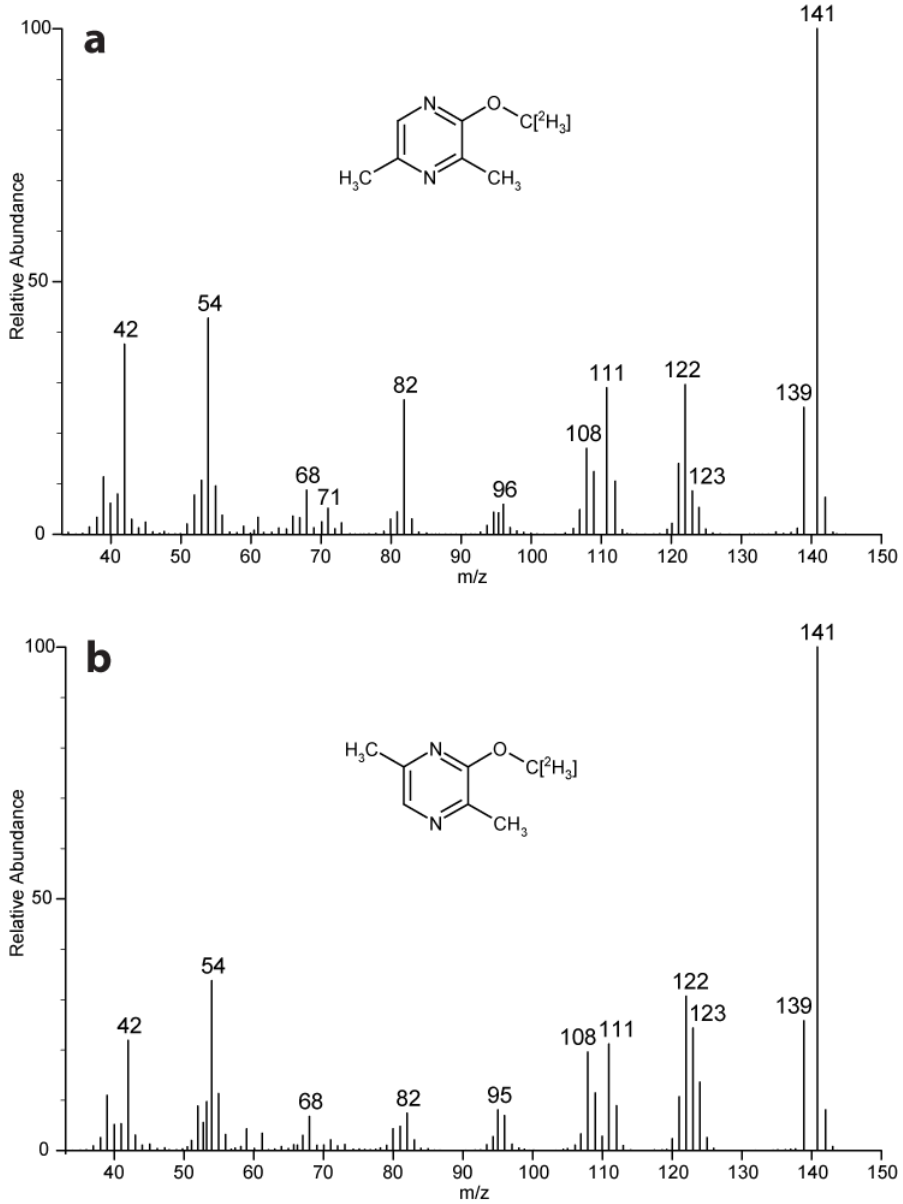


Figure 3-5 Mass spectra of deuterated isotopologues of **1** and **2**; conditions as described in text

Table 3-1 500 MHz ^{1}H-NMR, chemical shifts in parts per million (ppm) and couplings in Hertz (ref. $CDCl_3$ = 7.2 ppm)

Compound	H-6	2-CH_3	3-CH_3	5-CH_3	OCH_3	Couplings
1	7.7 septet	-	2.38 doublet	2.36 doublet	3.88 singlet	$^{4}J_{5\text{-Me},6}$ = 0.7 $^{6}J_{3\text{-Me},6}$ = 0.8
2	7.78 multiplet	2.35 doublet of quartets	-	2.33 quintet	3.89 singlet	$^{4}J_{5\text{-Me},6}$ = 0.8 $^{5}J_{2\text{-Me},6}$ = 0.1 $^{7}J_{2\text{-Me},5\text{-Me}}$ = 0.7
3	7.89 broad	2.45 broad	2.52 broad	-	3.93 singlet	[a]

[a] not determined

Table 3-2 ^{13}C-NMR, chemical shifts in ppm (ref. $CDCl_3$ = 77.2 ppm)

Compound	C-2	C-3	C-5	C-6	2-CH_3	3-CH_3	5-CH_3	OCH_3
1	157.2	143.4	143.9	136.8	-	19.4	20.3	53.6
2	141.1	158.2	147.7	134.4	18.9	-	20.8	53.4
3	140.7	151.5	159.4	127.5	21.8	19.1	-	54.3

Table 3-3 ^{15}N-NMR, chemical shifts in ppm (ref. CH_3NO_2 = 0.0 ppm)

Compound	N-1	N-4
1	-102.6	-44.0
2	-45.2	-100.8
3	[a]	[a]

[a] not determined

The experimental results of the NMR measurements are summarized in Table 3-1, Table 3-2, and Table 3-3. No significant differences of the chemical shifts were found in ^{1}H-NMR spectra of compounds **1** and **2**. In ^{13}C-NMR spectra, a small chemical shift difference of 3.8 ppm was observed only for the ring carbon C-5 that does not guarantee a reliable verification by a NMR database. The unambiguous assignment was achieved by ^{1}H-^{15}N-HMBC measurements, finally (Figure 3-6). In Figure 3-6a, cross peaks were observed from both ^{1}H-NMR signals of methyl groups with the low field shifted nitrogen atom which is in accordance only with the structure of compound **1** where both methyl groups are arranged near N-4. Otherwise, in Figure 3-6b, cross peaks of the methyl groups were observed to both nitrogen atoms. In the latter, this can be explained with the chemical structure of compound

2, only. The unambiguous assignment of the different H-6 positions of the aromatic hydrogen atoms near 7.7 ppm is also seen here clearly in both compounds. Surprisingly, in ^{1}H-NMR spectra of compounds **1** and **2**, multiple patterns were observed for the methyl proton signals. Long-range couplings across up to seven atomic bonds cause the signal splitting. The remarkable coupling constants up to 0.8 Hz were observed as early as 1968 by Cox and Bothner-By [40]. The unambiguous assignment of compound **3** was achieved by ^{1}H-^{13}C-HSQC and HMBC measurements as presented in Figure 3-7. In Figure 3-7b, the methyl protons at 2.45 ppm in position 2 show in addition to the strong 2J and 3J couplings to C-2 and C-3 carbons a weak cross peak to carbon C-6 at 127.5 ppm, whereas the methyl protons in position 3 at 2.52 ppm show a weak 4J long-range coupling to carbon C-5 at 159.4 ppm.

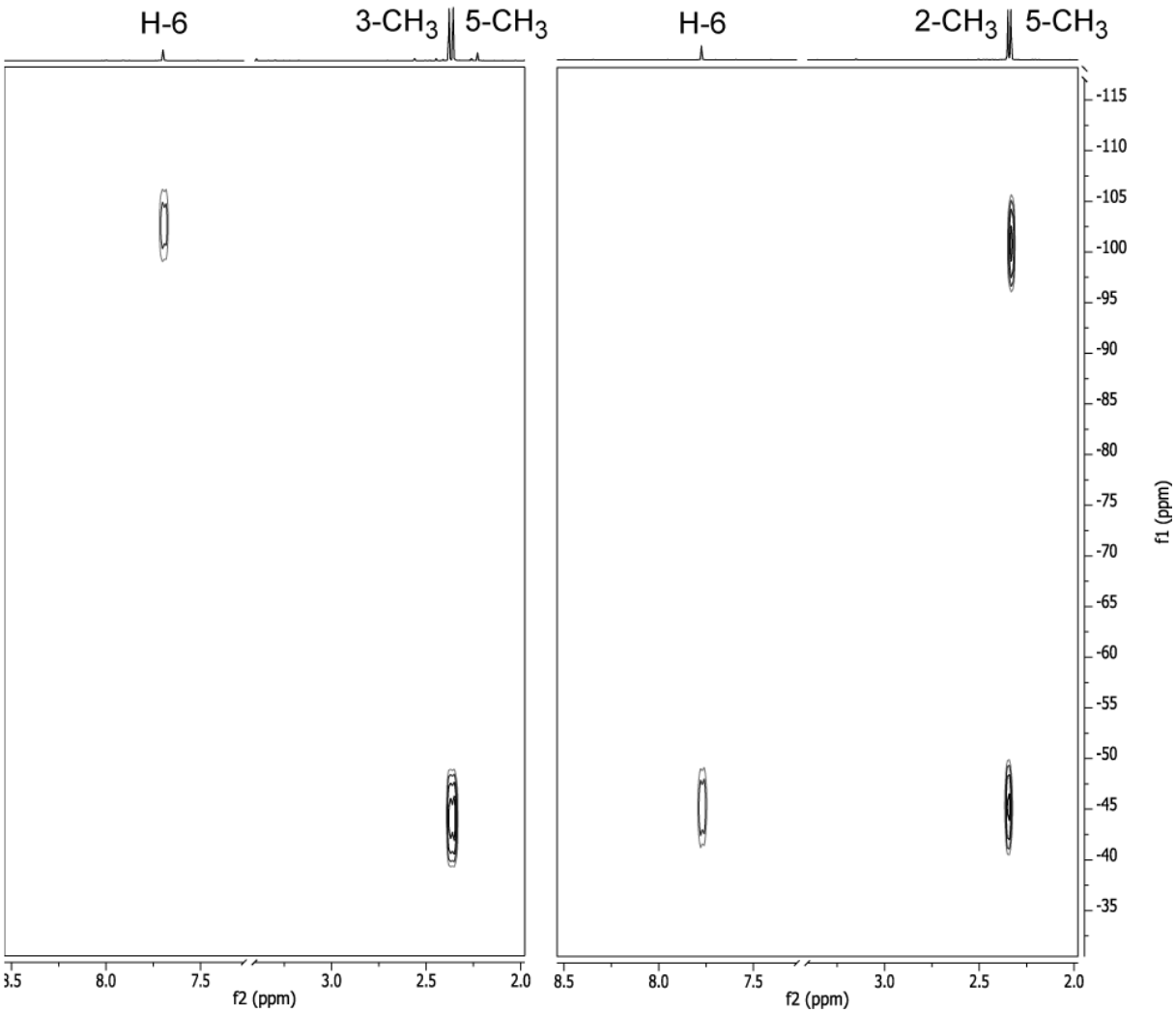


Figure 3-6 Part of ^{1}H-^{15}N-HMBC spectra of compound **1** (6a, left) and **2** (6b, right)

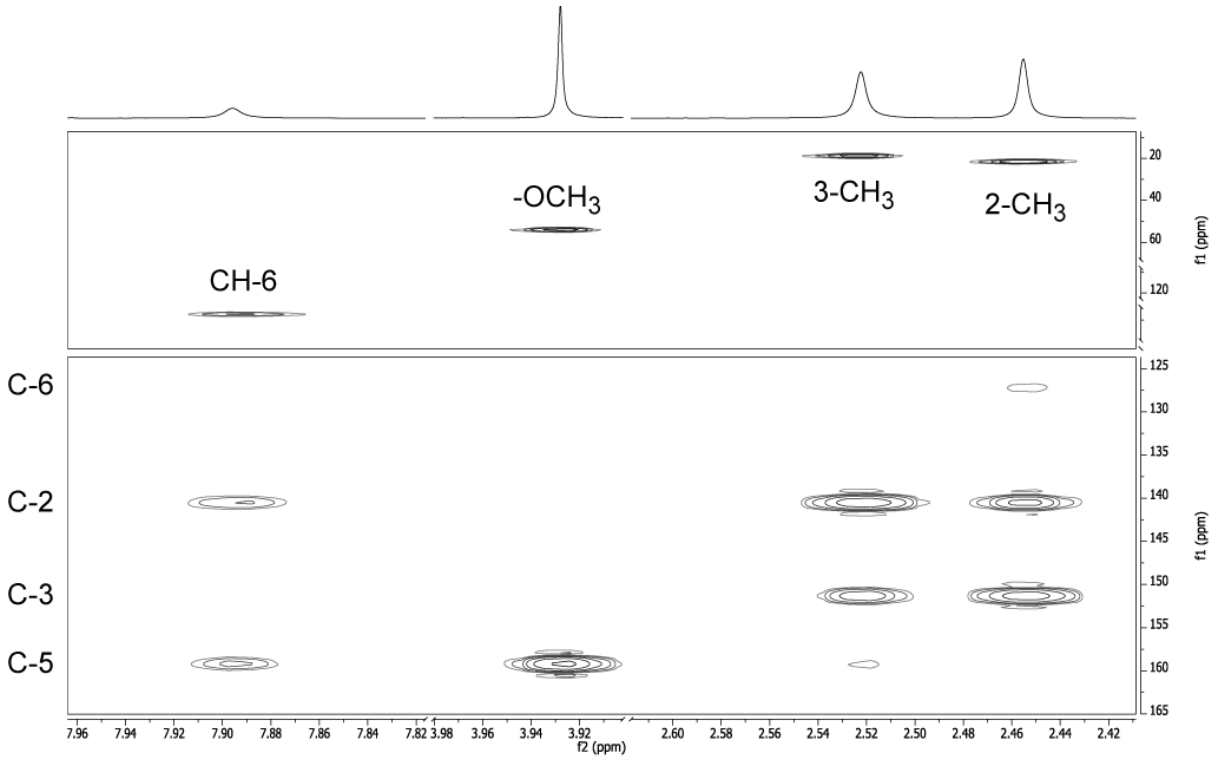


Figure 3-7 Part of ^{1}H-^{13}C-HSQC (7a, top) and HMBC spectra (7b, bottom) of compound **3**

The analytical approach using H/C MDGC-MS-MS finally allowed an unambiguous differentiation of the compounds under investigation. On octakis-(2,3-di-*O*-pentyl-6-*O*-methyl)-γ-cyclodextrin as stationary phase for the ^{2}D separation column (trade name LIPODEX G®), baseline separation of the critical dimethyl- (**1**, **2**) and also alkyl methoxypyrazines (**4**, **5**) could be achieved. An example for the analysis of dimethyl-substituted methoxypyrazines in ladybugs is given in Figure 3-8. Interestingly, in ^{1}D on the polyethylene glycol stationary phase a co-elution of **1**, **2**, **4** and **5** is observed that allowed the use of a single H/C for all these compounds (Figure 3-8a). As an important result, compound **2** that had previously been described as a constituent of *H. axyridis* could not be detected. Instead, compound **1** was clearly identified (Figure 3-8c) with an approximate concentration of some 1-2 ng/g bug. This could also be confirmed for another ladybug species investigated. In *C. septempunctata*, **2** was again absent and **1** could be identified (Figure 3-8c) as seen for *H. axyridis* (however, on a lower concentration of about 0.01-0.1 ng/g bug). In other investigations on suspicious cork samples with moldy cork off-flavors, the earlier identification of **1** by Simpson et al. and Chatonnet et al. could be confirmed [13,14] (see chapter 4).

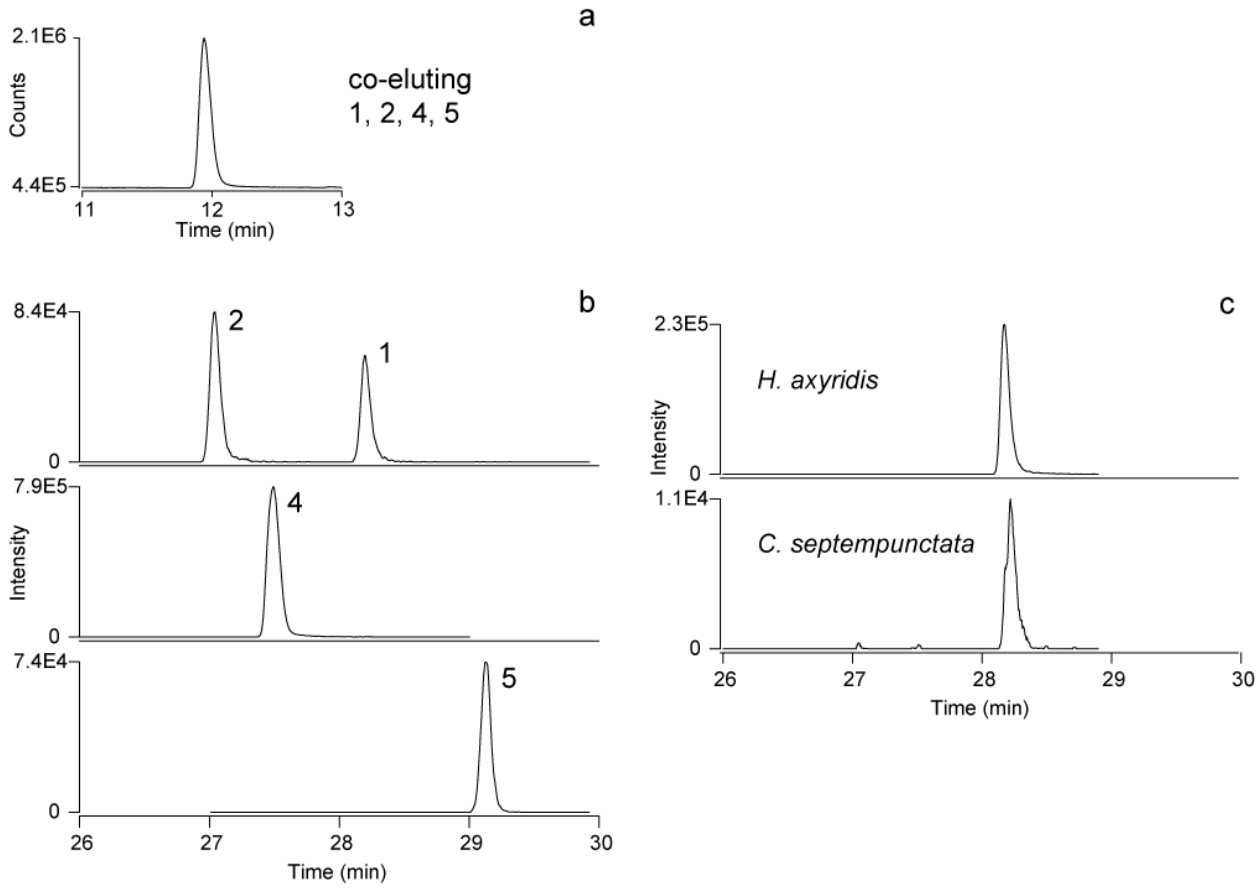


Figure 3-8 H/C MDGC chromatograms after ^{1}D (a; flame ionization detector) and ^{2}D (b; MS-MS) separation of a standard mixture of 3,5-dimethyl-2-methoxypyrazine **1**; 2,5-dimethyl-3-methoxypyrazine **2**; 3-ethyl-2-methoxypyrazine **4**; and 3-isopropyl-2-methoxypyrazine **5**; HS-SPME-H/C MDGC-MS-MS chromatograms of different ladybug species (c); illustrated ion traces represent quantifier SRMs; conditions as described in text

3.5 Conclusion

The unequivocal identification of structurally similar compounds by GC-MS is critical due to their similar spectrometric and often also chromatographic data. In the present analytical study this was shown with the example of alkyl methoxypyrazines **1** to **4**. This work affirms an earlier statement by Molyneux and Schieberle [31] and renders some literature citations describing the occurrence of these compounds questionable. For example, **2** has first been described as a constituent of *H. axyridis*, however, with a tentative assumption dependent upon mass spectrometric identification based solely on comparison with commercial databases [17]. With respect to these findings and the earlier statement for a proper identification [31] such a workflow is not sufficient and should, at best, end with a tentative result. However, analysis of the dimethyl methoxypyrazine compounds described in this work is possible if based on a sufficient chromatographic separation and utilizing structurally verified reference substances. The analytical method proposed in this work (H/C MDGC-MS-MS using the LIPODEX G® column in ^{2}D) provided the necessary separation and had also

proven to be suitable for application to complex matrices. With the examples shown in the present study, 3,5-dimethyl-2-methoxypyrazine **1** could be confirmed to be a constituent in cork samples with a moldy off-flavor. However, the presence of 2,5-dimethyl-3-methoxypyrazine **2** in either ladybug species *H. axyridis* or *C. septempunctata* could not be confirmed. Instead, 3,5-dimethyl-2-methoxypyrazine **1** was identified for the first time in these ladybug species.

3.6 References

1. Maga JA, Sizer CE (1973) Pyrazines in foods. Review. Journal of Agricultural and Food Chemistry 21 (1):22-30.
2. Fors S (1983) Sensory properties of volatile maillard reaction products and related compounds. In: The Maillard Reaction in Foods and Nutrition, vol 215. ACS Symposium Series, vol 215. AMERICAN CHEMICAL SOCIETY, pp 185-286.
3. Buttery RG, Seifert RM, Guadagni DG, Ling LC (1969) Characterization of some volatile constituents of bell peppers. Journal of Agricultural and Food Chemistry 17 (6):1322-1327.
4. Murray KE, Shipton J, Whitfield FB (1970) 2-Methoxypyrazines and the flavor of green peas (*Pisum sativum*). Chemistry & Industry (London, United Kingdom) (27):897-898.
5. Cronin DA, Stanon P (1976) 2-Methoxy-3-*sec*-butylpyrazine. An important contributor to carrot aroma. Journal of the Science of Food and Agriculture 27 (2):145-151.
6. Allen MS, Lacey MJ, Harris RLN, Brown WV (1991) Contribution of methoxypyrazines to Sauvignon blanc wine aroma. American Journal of Enology and Viticulture 42 (2):109-112.
7. Mottram DS, Patterson RLS, Warrilow E (1984) 2,6-Dimethyl-3-methoxypyrazine: a microbiologically-produced compound with an obnoxious musty odor. Chemistry & Industry (London, United Kingdom) (12):448-449.
8. Yasuhara A, Yamanaka Y, Ogawa T (1986) Volatile compounds in machine cutting-fluid emulsion. Agricultural and Biological Chemistry 50 (7):1765-1770.
9. Burdack-Freitag A, Schieberle P (2010) Changes in the key odorants of italian hazelnuts (*Coryllus avellana* L. Var. Tonda Romana) induced by roasting. Journal of Agricultural and Food Chemistry 58 (10):6351-6359.
10. Czerny M, Grosch W (2000) Potent odorants of raw arabica coffee. Their changes during roasting. Journal of Agricultural and Food Chemistry 48 (3):868-872.

11. Jezussek M, Juliano BO, Schieberle P (2002) Comparison of key aroma compounds in cooked brown rice varieties based on aroma extract dilution analyses. Journal of Agricultural and Food Chemistry 50 (5):1101-1105.

12. Ferreira V, San Juan F, Escudero A, Cullere L, Fernandez-Zurbano P, Saenz-Navajas MP, Cacho J (2009) Modeling quality of premium spanish red wines from gas chromatography-olfactometry data. Journal of Agricultural and Food Chemistry 57 (16):7490-7498.

13. Simpson RF, Capone DL, Sefton MA (2004) Isolation and identification of 2-methoxy-3,5-dimethylpyrazine, a potent musty compound from wine corks. Journal of Agricultural and Food Chemistry 52 (17):5425-5430.

14. Chatonnet P, Fleury A, Boutou S (2010) Origin and incidence of 2-methoxy-3,5-dimethylpyrazine, a compound with a "fungal" and "corky" aroma found in cork stoppers and oak chips in contact with wines. Journal of Agricultural and Food Chemistry 58 (23):12481-12490.

15. Mihara S, Masuda H, Tateba H, Tuda T (1991) Olfactive properties of 3-substituted 5-alkyl-2-methylpyrazines. Journal of Agricultural and Food Chemistry 39 (7):1262-1264.

16. Mihara S, Masuda H (1988) Structure-odor relationships for disubstituted pyrazines. Journal of Agricultural and Food Chemistry 36 (6):1242-1247.

17. Cai L, Koziel JA, O'Neal ME (2007) Determination of characteristic odorants from *Harmonia axyridis* beetles using in vivo solid-phase microextraction and multidimensional gas chromatography-mass spectrometry-olfactometry. Journal of Chromatography A 1147 (1):66-78.

18. Botezatu AI, Kotseridis Y, Inglis D, Pickering GJ (2013) Occurrence and contribution of alkyl methoxypyrazines in wine tainted by *Harmonia axyridis* and *Coccinella septempunctata*. Journal of the Science of Food and Agriculture 93 (4):803-810.

19. Jagella T, Grosch W (1999) Flavour and off-flavour compounds of black and white pepper (*Piper nigrum* L.). Part 1. Evaluation of potent odorants of black pepper by dilution and concentration techniques. European Food Research and Technology 209 (1):16-21.

20. Drake MA, Miracle RE, McMahon DJ (2010) Impact of fat reduction on flavor and flavor chemistry of Cheddar cheeses. Journal of Dairy Science 93 (11):5069-5081.

21. Schirack AV, Drake MA, Sanders TH, Sandeep KP (2006) Characterization of aroma-active compounds in microwave blanched peanuts. Journal of Food Science 71 (9):C513-C520.

22. Shahidi F, Aishima T, Abou-Gharbia HA, Youssef M, Shehata AAY (1997) Effect of processing on flavor precursor amino acids and volatiles of sesame paste (Tehina). Journal of the American Oil Chemists' Society 74 (6):667-678.

23. Silva CL, Camara JS (2013) Profiling of volatiles in the leaves of Lamiaceae species based on headspace solid phase microextraction and mass spectrometry. Food Research International 51 (1):378-387.

24. Poehlmann S, Schieberle P (2013) Characterization of the aroma signature of styrian pumpkin seed oil (*Cucurbita pepo* subsp. *pepo* var. *Styriaca*) by molecular sensory science. Journal of Agricultural and Food Chemistry 61 (12):2933-2942.

25. Schmarr HG, Sang W, Ganß S, Koschinski S, Meusinger R (2011) New insights into the synthesis and characterization of 2-methoxy-3-alkylpyrazines and their deuterated isotopologues. Journal of Labelled Compounds & Radiopharmaceuticals 54 (8):438-440.

26. Schmarr H-G, Ganß S, Koschinski S, Fischer U, Riehle C, Kinnart J, Potouridis T, Kutyrev M (2010) Pitfalls encountered during quantitative determination of 3-alkyl-2-methoxypyrazines in grape must and wine using gas chromatography-mass spectrometry with stable isotope dilution analysis. Comprehensive two-dimensional gas chromatography-mass spectrometry and on-line liquid chromatography-multidimensional gas chromatography-mass spectrometry as potential loopholes. Journal of Chromatography, A 1217 (43):6769-6777.

27. Ventura F, Quintana J, Gomez M, Velo-Cid M (2010) Identification of alkyl-methoxypyrazines as the malodorous compounds in water supplies from northwest spain. Bulletin of Environmental Contamination and Toxicology 85 (2):160-164.

28. Schulz S, Fuhlendorff J, Reichenbach H (2004) Identification and synthesis of volatiles released by the myxobacterium *Chondromyces crocatus*. Tetrahedron 60 (17):3863-3872.

29. Dickschat JS, Reichenbach H, Wagner-Doebler I, Schulz S (2005) Novel pyrazines from the myxobacterium *Chondromyces crocatus* and marine bacteria. European Journal of Organic Chemistry (19):4141-4153.

30. Pickering G, Lin J, Riesen R, Reynolds A, Brindle I, Soleas G (2004) Influence of *Harmonia axyridis* on the sensory properties of white and red wine. American Journal of Enology and Viticulture 55 (2):153-159.

31. Molyneux RJ, Schieberle P (2007) Compound identification: A Journal of Agricultural and Food Chemistry perspective. Journal of Agricultural and Food Chemistry 55 (12):4625-4629.

32. Jones RG (1949) Pyrazines and related compounds. I. A new synthesis of hydroxypyrazines. Journal of the American Chemical Society 71:78-81.

33. Karmas G, Spoerri PE (1952) The preparation of hydroxypyrazines and derived chloropyrazines. Journal of the American Chemical Society 74:1580-1584.

34. van Den Dool H, Dec. Kratz P (1963) A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography. Journal of Chromatography A 11 (0):463-471.

35. Liebich HM, Gesele E, Wöll J (1998) Urinary organic acid screening by solid-phase microextraction of the methyl esters. Journal of Chromatography, B: Biomedical Sciences and Applications 713 (2):427-432.

36. Bemelmans JMH (1979) Review of isolation and concentration techniques. In: Land DG, Nursten HE (eds) Progress in Flavour Research. Applied Science, London, UK, pp 79-88.

37. Bicchi C, Binello A, D'Amato A, Rubiolo P (1999) Reliability of van den Dool retention indices in the analysis of essential oils. Journal of Chromatographic Science 37 (8):288-294.

38. Czerny M (1999) Untersuchungen über den Beitrag von Carbonylverbindungen, Pyrazinen und Furanonen zum Aroma von Arabica-Kaffee. Einfluss der Provenienz und des Röstgrades. Dissertation, Technische Universität München, München

39. Kolor MG, Rizzo DJ (1971) Electron-impact induced skeletal re-arrangement of 2-methoxy-3-methylpyrazine and 2-methylthio-3-methylpyrazine. Organic Mass Spectrometry 5 (8):959-966.

40. Cox RH, Bothner-By AA (1968) Proton nuclear magnetic resonance of di- and trisubstituted pyrazines and their cations. Journal of Physical Chemistry 72 (5):1642-1645.

3.7 Appendix

Observation of an "overdeuteration" during the synthesis of 3,5-dimethyl-2-[$^2H_3$]-methoxypyrazine (d-1) using [$^2H_4$]-methanol

The synthesis of **d-1** as described in chapter 3.3.2 was first done using [$^2H_4$]-methanol instead of [$^2H_3$]-methanol for the preparation of the sodium methoxide solution. [$^2H_3$]-methanol was later used due to the following observations.

Using [$^2H_4$]-methanol the product showed in GC-MS analysis (see system (i) in chapter 3.3.3) a broadened peak in comparison to the non-deuterated standard and a disturbed mass spectrum (Figure 3-9). Instead of a clear molecule ion (m/z 141, M^+) a cluster of m/z was observed that varied between M^+ and M^++6. Similar clusters were observed for the lower fragments. Additionally, the m/z intensities varied over the entire peak width and the extracted ion chromatograms of M^+ to M^++6 revealed a shifting retention time of the corresponding peaks (Figure 3-10).

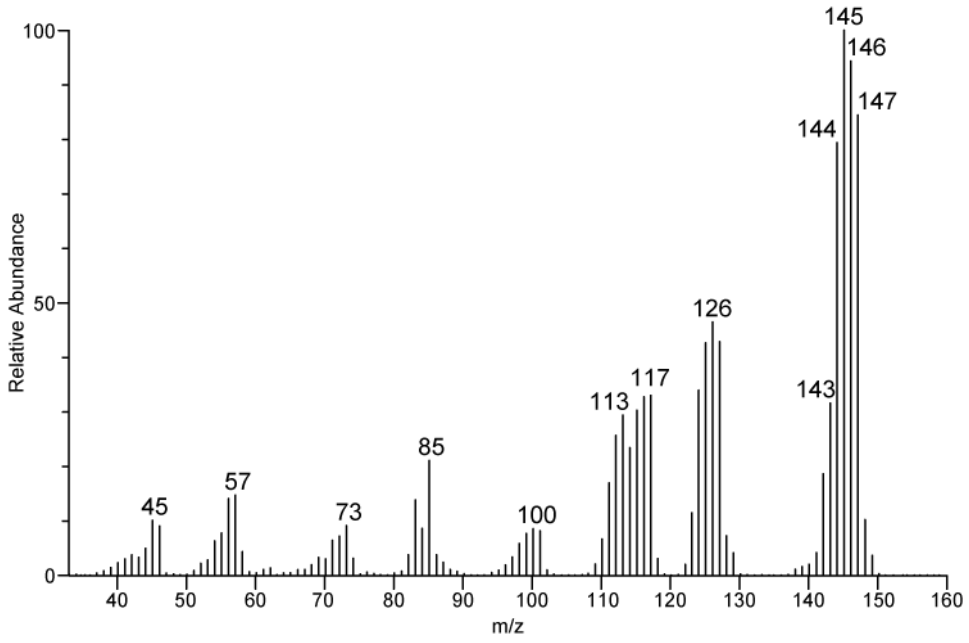


Figure 3-9 Mass spectrum of the reaction product (averaged over entire peak)

These observations indicated that a mixture of compounds with different numbers of deuterium atoms was synthesized. A possible reason could be an uncontrolled proton exchange in the methyl groups that is supported by the neighbored basic N in the pyrazine ring. A proposed mechanism is shown in Figure 3-11. A closer look into the reaction controls also revealed a deuteration of the starting material 3,5-dimethyl-2-chloropyrazine. Considering the observed M^+ the reaction product contains probably compounds with a deuteration number between [$^2H_3$] and [$^2H_9$]. In the extracted ion chromatograms (Figure 3-10) a descending elution order of the compounds according to their number of incorporated

deuterium was observed (inverse isotopic effect). A GC separation of the different deuterated compounds was finally achieved using a 90 m × 0.25 mm i.d. fused silica capillary coated with 1 µm of 5 % phenylmethylpolysiloxane (Restek, Bad Homburg, Germany). The corresponding chromatogram is shown in Figure 3-12.

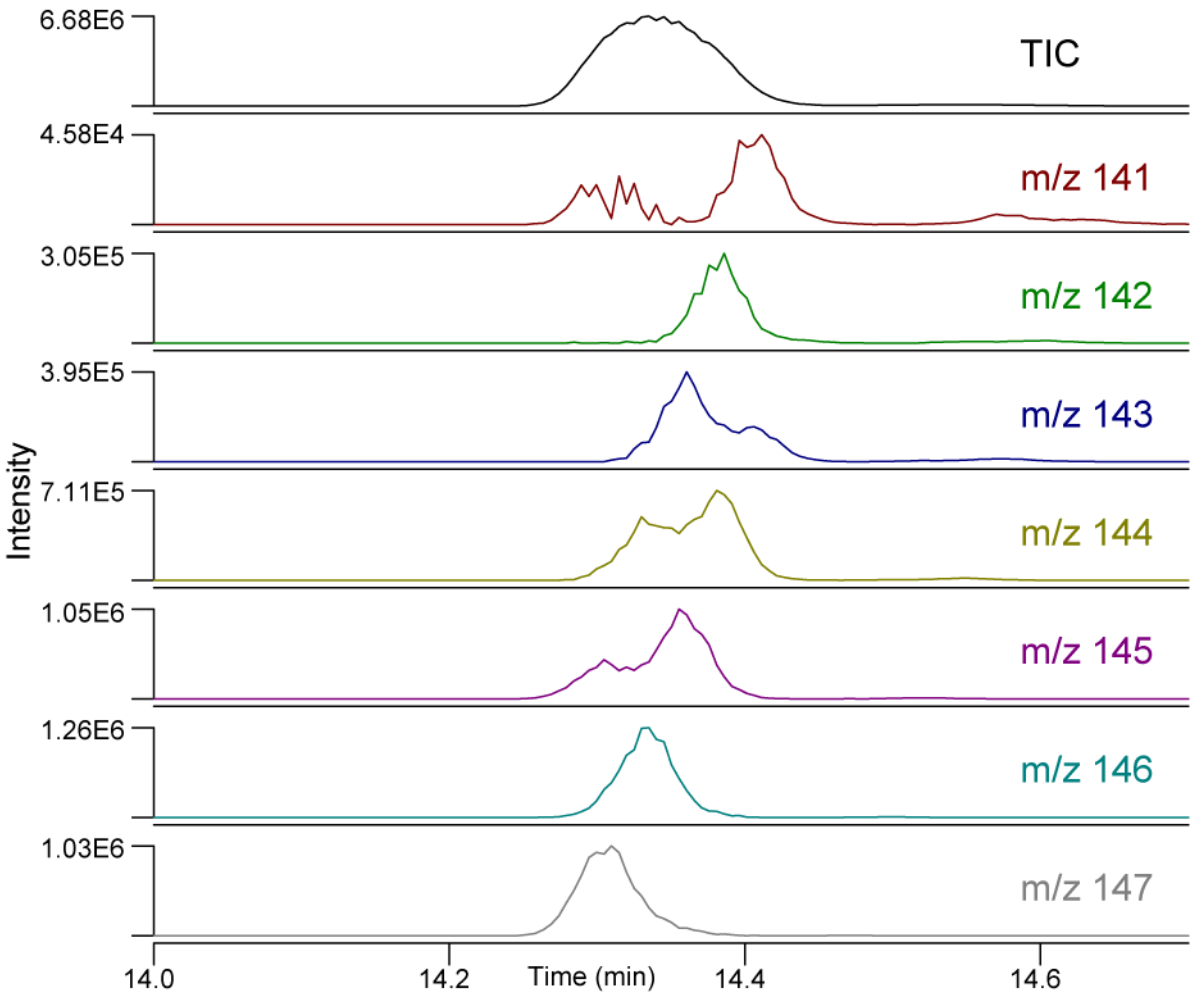


Figure 3-10 Extracted ion chromatograms of M^+ to M^++6 (shoulders derive from fragments of higher deuterated compounds due to C-^{2}H cleavage; see also Figure 3-4)

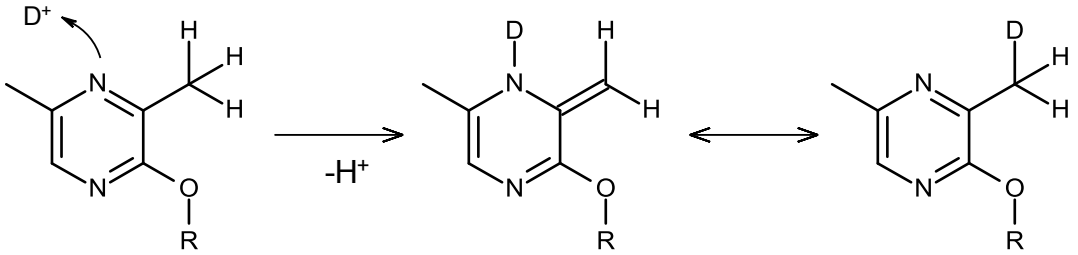


Figure 3-11 Hypothesized proton exchange mechanism in γ-position to the aromatic N leading to a deuteration in methyl group

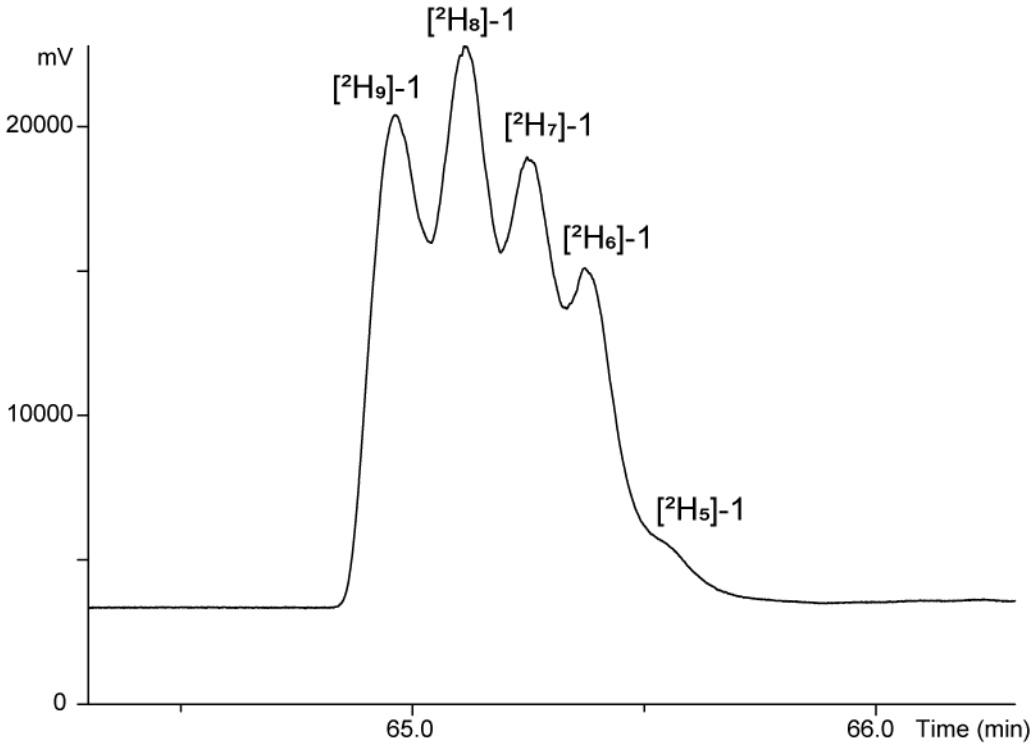


Figure 3-12 GC separation of the mixture of different deuterated compound **1** (analytical column: RXI5Sil-MS, 60 + 30 m coupled in series, 0.25 mm i.d., 1 µm d_f; carrier: 240 kPa H_2; oven temperature program: 40°/2 min//1°/min//250°/5 min; detector: FID)

Furthermore, in the ^{1}H-NMR spectrum of the product an almost complete deuteration of the methyl group in position 3 could be determined as no peak for CH_3 and only small amounts of $CH[^2H_2]$ and $CH_2[^2H_1]$ could be detected. Whereas the methyl group in position 5 is observed as multiple peaks representing CH_3, $CH_2[^2H_1]$, and $CH[^2H_2]$. The methyl group in position 3 is probably favored in the proton exchange mechanism. This could be explained by the neighbored methoxy group that possibly plays an additional role in the proposed mechanism.

The described co-synthesis is further supported by similar observations with the synthesis of 3-isobutyl-2-[$^2H_3$]-methoxypyrazine. During the synthesis of 3-*sec*-butyl-2-[$^2H_3$]-methoxypyrazine and 3-isopropyl-2-[$^2H_3$]-methoxypyrazine this effect may be existent but is difficult to observe as there is only one proton available in γ-position to the aromatic N. The fragment M^++1 is difficult to differentiate from the natural isotopic pattern and, additionally, the adjacent methyl group could be a steric hindrance.

4 Characterization of atypical off-flavor compounds in natural cork stoppers by multidimensional gas chromatographic techniques

Adapted with permission from P. Slabizki, C. Fischer, C. Legrum, H.-G. Schmarr, Characterization of atypical off-flavor compounds in natural cork stoppers by multidimensional gas chromatography techniques, *Journal of Agricultural and Food Chemistry*, **2015**, 63, 7840-7848, Copyright (2015) American Chemical Society.

4.1 Abstract

Natural cork stoppers with sensory deviations other than the typical cork taint were sub-grouped according to their sensory descriptions and compared with unaffected control cork stoppers. The assessment of purge and trap extracts obtained from corresponding cork soaks was performed by heart-cut multidimensional gas chromatography olfactometry (MDGC-O). The identification of compounds responsible for atypical cork taint detected in MDGC-O was further supported with additional multidimensional GC analysis in combination with mass spectrometric detection. Geosmin and 2-methylisoborneol were mainly found in cork stoppers described as moldy and cellarlike; 3-isopropyl-2-methoxypyrazine and 3-isobutyl-2-methoxypyrazine in cork stoppers described with green attributes. Across all cork sub-groups the impact compound for the typical cork taint, 2,4,6-trichloroanisole, was present and is therefore a good marker for cork taint in general. Another potent aroma compound, 3,5-dimethyl-2-methoxypyrazine (MDMP), was also detected in each sub-group obviously playing an important role with regard to the atypical cork taint. Sensory deviations possibly affecting the wine could be generated by MDMP and its presence should thus be monitored in a routine quality control.

4.2 Introduction

Cork taint is one of the most known sensory defects in wine and is related to the musty smelling substance 2,4,6-trichloroanisole (TCA), that is considered to be the primary responsible compound [1]. Due to substantial improvements in the processing of natural cork, such as the avoidance of hypochlorite as bleaching agent, and rigorous quality

management with respect to microbial contamination, today the typical TCA-based corky off-flavor is of reduced importance in the cork industry. Still, some wineries using natural cork stoppers report a sensory alteration of their wines that can be described with a reduced fruitiness, moldy or musty notes, however not resembling the typical TCA-based off-flavor. Customers often ascribe this phenomenon to the wine as such and not to a problem originating from the cork. Whereas the typical cork taint was regarded as an unavoidable risk when using natural cork stoppers, the before described *atypical* off-flavor is a new situation. In the first instance it poses a problem to the winery if the problem cannot be traced to the cork stopper. Up to now, this vague sensory alteration lacked any substantial information and therefore quality control in the cork industry may only rely on sensory evaluation of cork lots. Without a clear correlation of affected wines to the atypical off-flavor financial losses for the wine industry cannot even be estimated.

Identification of compounds responsible for musty, earthy off-flavors in wine and cork were already studied by several groups [2-5] and have also been reviewed in a number of publications [6-9]. Amongst the most important cork off-flavor compounds are well-known substances such as geosmin (GSM), 2-methylisoborneol (MIB), guaiacol, 1-octen-3-ol, 1-octen-3-one, 3-isopropyl-2-methoxypyrazine (IPMP) and 3,5-dimethyl-2-methoxypyrazine (MDMP).

GSM and MIB are well known for earthy off-odors in water supplies and marine foods [10,11], possibly originating from microorganisms [12,13]. Microorganisms isolated from cork were able to produce GSM and MIB as well as guaiacol, TCA and MDMP [5]. MDMP is described with the unpleasant sensory attributes “wet cardboard”, “musty”, “moldy”, “dusty”, “earthy” and was determined in cork for the first time by Simpson et al. causing a “fungal must” taint in wine [4]. It has also been found as a malodorous compound in water supplies [14] and seems to be generated by bacteria [15,16]. Simpson and Chatonnet propose that MDMP is the most important substance affecting cork stoppers and thus wine next to TCA [4,16]. Other alkyl methoxypyrazines like IPMP and 3-isobutyl-2-methoxypyrazine (IBMP) with a vegetative, green odor occur in many vegetables as aroma relevant compounds [17] and also in *V. vinifera* varieties [18]. However, they can also contribute to earthy off-flavors in water [19], fish [20] and other foodstuffs [6]. IPMP is also known as major component contributing to the so-called “ladybug taint” in wine, associated with beetles that are harvested together with the grapes [21].

Further known substances capable of producing corky off-flavors in wine are other haloanisoles like 2,4,6-tribromoanisole (TBA), 2,3,4,6-tetrachloroanisole (TeCA) and pentachloroanisole (PCA) [8,22,23]. Although their sensory characteristics are rather similar to TCA, they originate from the microbial degradation of halophenols (in pesticides or flame

retardants) that then produce the potent haloanisoles. These compounds represent a contamination of the wine cellar and not the cork itself. However, migration may occur depending on storage and wine production conditions. Halogenation of phenolic compounds with further microbial transformation into potent haloanisoles is a well-known problem in general and can particularly be found in the wood processing industry [24,25]. In plant anatomy, cork is part of the periderm in the bark system of the cork oak tree (*Quercus suber* L.) and consists primarily of suberin, lignin and polysaccharides [26]. As a consequence of the halogenation of lignin Kugler and Rapp detected various chloroguaiacols and other chlorophenolic compounds in cork [3].

On the basis of the substantiated state of knowledge on the typical cork off-flavor as well as other musty substances, compounds particularly responsible for the atypical cork taint, an off-flavor that had not been fully characterized so far, should be identified. Cork samples that had been considered off-odorous should be submitted to a purge and trap extraction followed by gas chromatography-olfactometry analysis (GC-O). Detected off-flavor regions in the chromatograms deviant from the control samples should be further processed by a set of multidimensional gas chromatographic methods to characterize the structural identity of the underlying chemical substances.

4.3 Materials and Methods

4.3.1 Chemicals

Reference compounds used were: 2,4,6-trichloroanisole, pentachloroanisole, 2,4,6-tribromoanisole, geosmin (100 µg/ml in methanol), 2-methylisoborneol (10 mg/ml in methanol), 3-isopropyl-2-methoxypyrazine, 3-isobutyl-2-methoxypyrazine (Sigma-Aldrich, Steinheim, Germany); 2,3,4,6-tetrachloroanisole, 3,5-dichlorocatechol (LGC Promochem, Wesel, Germany); guaiacol (Fluka, Buchs, Switzerland), 3,5-dimethyl-2-methoxypyrazine (Bellen Chemistry Co. Ltd., Beijing, China); 1-octen-3-one (Alfa Aesar, Karlsruhe Germany); 1-octen-3-ol (BASF, Ludwigshafen, Germany); 2,6-dichloroanisole (Riedel-de Haën, Seelze, Germany); and 3,4,6-trichloroveratrole (kindly supplied by Prof. Juha Knuutinen, University of Jyväskylä, Finland) and chlorophenolics standard mixtures regulated in US EPA method 1653A were from Cambridge Isotope Laboratories, (Andover, MA, USA) comprising pentachlorophenol, tetrachlorocatechol, tetrachloroguaiacol, 2,3,4,6-tetrachlorophenol, 3,4,5-trichlorocatechol, 3,4,6-trichlorocatechol, 3,4,5-trichloroguaiacol, 3,4,6-trichloroguaiacol, 4,5,6-trichloroguaiacol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol in methanol (EM-4181); 3,4,5-trichlorosyringol in acetone (EM-4182); 4-chlorocatechol, 4-chloroguaiacol, 4-chlorophenol, 3,4-dichlorocatechol, 3,6-dichlorocatechol, 4,5-dichlorocatechol, 3,4-dichloroguaiacol, 4,5-dichloroguaiacol, 4,6-dichloroguaiacol, 2,4-dichlorophenol, 2,6-

dichlorophenol in methanol (EM-4183); 2-chlorosyringaldehyde, 5-chlorovanillin, 6-chlorovanillin, 2,6-dichlorosyringaldehyde, 5,6-dichlorovanillin in acetone (EM-4184).

Reagents used for synthesizing further references were trimethylsilyldiazomethane (2 M in diethyl ether; Sigma-Aldrich), 1,3-propylene glycol and *p*-toluenesulphonic acid (Fluka). Commercial chemicals were usually of analytical grade and used as such.

4.3.2 Synthesis of reference compounds

Based on multicomponent standard mixes containing chlorophenolic substances (EM-4181, EM-4182, EM-4183, EM-4184), the corresponding *O*-methylated derivatives were synthesized using trimethylsilyldiazomethane (TMSD) according to Ranz et al. [27]. An aliquot (40 µl) of the standard mixture (EM-4181 to EM-4183) with 250–1000 µg/ml of the chlorophenolic substances were mixed with TMSD (40 µl of 2 M in diethyl ether) and methanol (160 µl). After 2 h at 40 °C the reaction was complete (as monitored by GC-mass spectrometry; GC-MS). 3,5-Dichlorocatechol was methylated accordingly. Chlorophenolic substances containing a carbonyl group (standard mixture EM-4184) yielded mixtures of reaction products. In this case the carbonyl group was protected as a cyclic acetal using 1,3-propylene glycol. Briefly, 1,3-propylene glycol (100 µl), a catalytic amount of *p*-toluene sulphonic acid and molecular sieve were added to 50 µl of the standard mixture EM-4184 and kept at 45 °C overnight. The reaction mixture was then washed with a $NaHCO_3$ solution (4.8 g/l) and extracted with dichloromethane. The organic phase was used for *O*-methylation as described above. Thereafter, the carbonyl group was deprotected by adding 3 M hydrochloric acid (250 µl) to the organic solution, with agitating and heating at 50 °C until completion of the reaction (monitored by GC-MS). The dichloromethane phase was separated and used for characterization by GC. Retention indices (LRI), mass spectral data and odor descriptions of the *O*-methylated derivatives are listed in Table 4-2.

4.3.3 Pre-selection of conspicuous cork stoppers

In the industrial quality control process of raw natural cork stoppers (before processing, e.g. coating, imprinting) sensory evaluation of cork stoppers was performed by three panelists experienced for years in assessing the odor of cork stoppers. A consistent vocabulary on the odor descriptions of the cork stoppers had early been determined by repeatedly sniffing about 100 exemplary cork stoppers and comparing their individual odor descriptions. Technically, cork stoppers were moisturized by dipping in purified water, put in screw top jars and left overnight at room temperature. After sniffing the supernatant air, cork stoppers were classified in a first step by their odor into four classes: no deviant odor (class 1), slightly deviant odor (class 2), strongly deviant odor (class 3A), and typical TCA taint (class 3B).

Whereas cork stoppers of class 2 could be clearly distinguished from inconspicuous cork stoppers, the off-odor description was not further defined, also due to a high variability of subtle off-flavor notes. For the study of the atypical cork taint, strongly deviant cork stoppers of class 3A were further sorted into the sub-groups *musty* (dusty-musty), *moldy* (mildewed, MIB-like, earthy, mushroom), *cellarlike* (wet-cloth, musty wet cellar), *earthy* (woody, mossy, earthy), *green* (vegetative, bell pepper, pea). The definition of these sub-groups was done prior to analysis and was based on common and dominating subtle sensory notes (nuances). Cork stoppers of class 1 were used as inconspicuous control sample.

4.3.4 Extraction of volatiles from natural cork stoppers

The extraction of cork stoppers followed a standard quality control procedure applied in the cork industry to monitor TCA. In this case, six cork stoppers of each group were soaked in an aqueous solution of 10 %vol. ethanol (absolute) for 24 h at 40 °C, repeated three times. The combined cork soaks were transferred into a wide neck glass bottle with a GLS 80 thread and a PTFE screw cap with connection ports (Bohlender GmbH, Grünsfeld, Germany). Volatiles were purged by bubbling nitrogen (purified by an activated charcoal in-line filter) with a metal-frit sparger through the solution; similar to a set-up previously described [28]. The purged gas passed a SPE tube containing Lichrolut® EN (0.4g; Merck, Darmstadt, Germany) as sorbent *via* a self-made glass adapter. Each cork soak was purged for 7 h at 37 °C with a nitrogen flow of about 100 ml/min. The necessary purge time was evaluated in a preliminary experiment, changing the SPE cartridge every 100 min. Breakthrough of compounds was evaluated with a second cartridge connected in series. Trapped volatiles were then eluted with dichloromethane and the organic phase was kept at -25 °C over night to remove moisture. Extracts obtained were finally concentrated to a volume of about 0.1 ml using a microdistillation apparatus according to Bemelmans [29]. These extracts containing the purgeable volatiles from the cork soaks were used for further analysis by various GC approaches.

4.3.5 Detection of off-flavor compounds by heart-cut multidimensional gas chromatography-olfactometry

GC-O experiments (*sniffings*) were carried out by two panelists in separate runs using heart-cut multidimensional GC (H/C MDGC) utilizing a “Moving Capillary Stream Switching” (MCSS) device described previously [30]. Two GC instruments (model 8560, Mega II series) from C.E. Instruments (today ThermoFisher Scientific, Dreieich, Germany) were connected via a heated transfer line. The GC in the first dimension (^{1}D) was equipped with the MCSS device allowing heart-cutting of GC fractions. For a first evaluation of odorous elution zones

(odor or aroma events), sniffing occurred after separation in ^{1}D. Later, individual odor events were transferred onto the second dimension (^{2}D) separation column with a transfer window of the ^{1}D linear retention index (LRI) ±25 units. The basic components of the MDGC system are outlined hereafter.

1st Dimension system: The ^{1}D column configuration consisted of a 1 m × 0.32 mm i.d. phenylmethylsilylated pre-column (BGB Analytik, Rheinfelden, Germany) which was connected via a press-fit to a fused silica capillary column (20 m × 0.25 mm i.d.) coated with 0.5 µm of a polyethylene glycol phase (ZB-Wax, Phenomenex, Aschaffenburg, Germany). The carrier gas used was helium with an inlet pressure of 70 kPa (P1) and a midpoint pressure of 47 kPa (P2). The actual inlet pressure for the ^{2}D column was given by the read-out on the pressure gauge P2* and was at 30 kPa. A flame ionization detector (FID) was set to 250 °C and used as ^{1}D monitor detector when sniffing was after ^{2}D separation. For sniffing after ^{1}D separation a sniffing adapter was installed instead of the FID, keeping the detector base temperature at 250 °C. Cork extracts (3 µl) were injected using a cold on-column injector. The oven temperature was programmed from 40 °C (5 min isothermal), at 8 °/min to 250 °C (15 min isothermal). Control of the MCSS system as well as data processing was achieved by the Chromcard data acquisition software, version 2.2 (ThermoFisher).

2nd Dimension system: A deactivated fused silica capillary (1.5 m × 0.25 mm i.d., Phenomenex) was guided through a heated transfer line (200 °C), connecting the two GC ovens. The transfer capillary from the first oven was connected via a press-fit to the ^{2}D analytical column (11 m × 0.32 mm i.d. fused silica capillary) with 1 µm of a 5 % phenylmethylpolysiloxane (DB-5, Agilent, Waldbronn, Germany). At the end of the analytical column the eluent flow was split via a Y-splitter connecting a deactivated fused silica capillary of 0.4 m × 0.25 mm i.d. (BGB Analytik) with the sniffing port and a 0.6 m × 0.22 mm i.d. (SGE, Victoria, Australia) leading to the FID, respectively. The sniffing port (detector base) was set to 250 °C and the FID was set to 280 °C. The oven temperature program was initiated via external event activation 1 s after the start signal of the first dimension oven. The oven temperature was raised from 35 °C (32 min isothermal) at 10 °/min to 280 °C (5 min isothermal). LRIs were calculated using a series of *n*-alkanes (C10-C25). For the determination of LRIs after ^{2}D separation alkanes injected into ^{1}D were fully transferred into ^{2}D. Keeping the oven temperature in ^{2}D at 35 °C allowed cold trapping from C10 on and following this transfer oven temperature programming was done as in the analytical run.

4.3.6 Identification of odorous compounds

Retention indices after ^{1}D and ^{2}D separation of odor events, as well as their odor descriptions, were compared with those of reference compounds. Thus, tentatively identified

substances were verified by injection of reference material applying MDGC conditions as described in the previous section. The identification was further supported by additional analyses via MDGC-MS-MS and GC×GC-MS (described below). Odor events that could not be identified in this way demanded further specific sample enrichment and mass spectrometric detection. For this purpose, compounds eluting in the dedicated odor event zone (after H/C and ^{2}D separation) were trapped using thermodesorption tubes packed with polydimethylsiloxane (PDMS) foam (Gerstel, Mühlheim an der Ruhr, Germany). PDMS foam was chosen due to its high capacity and known moderate bleed characteristics in thermodesorption applications compared to e.g. Tenax or other polymer based sorbents. Good sorption properties for various aroma compounds have been demonstrated with solid phase microextraction (SPME) and (stir bar sorptive extraction) SBSE applications [31]. For higher sensitivity the Y-splitter was removed and a deactivated fused silica capillary of 0.6 m × 0.32 mm i.d. (BGB Analytik) was directly connected to the ^{2}D analytical column, guided through the heated detector base and connected with the trap on top of the detector base with a homemade press-fit type adapter made of a 6 mm i.d. polyethylene tube.

In a separate GC (HP6890, Agilent) equipped with a MPS2 autosampler, a thermodesorption unit (TDU) and a cold injection system (CIS 4; all Gerstel), the trapped components were thermodesorbed at 250 °C for 10 min (in splitless mode) with a purge flow of 60 ml/min. The transferline between TDU and CIS was set to 300 °C. During thermodesorption the CIS was set to -100 °C and operated in solvent vent mode. After thermodesorption the CIS temperature was raised at 12 °/s to 280 °C (1.5 min isothermal), with closed split valve for 2 min. The analytical column was a 30 m × 0.25 mm i.d. fused silica capillary column coated with 0.25 µm of a 5 % phenylmethylpolysiloxane (ZB-5, Phenomenex). Carrier gas used was helium at a constant flow rate of 1.2 ml/min. The oven temperature was raised from 50 °C (2 min isothermal) at 10 °/min to 280 °C (5 min isothermal). MS acquisition was done with an Agilent MSD 5975 at 70 eV (EI+) in full scan mode from *m/z* 29 to 400. Ion source and quadrupole were heated at 230°C and 150°C, respectively. The MS transferline was set to 280 °C. Instrument control and MS data acquisition was performed via MSD ChemStation (version E.02.02.1431, Agilent) and Gerstel Maestro (version 1.4.23.11). Identification of unknowns was then based on mass spectra matching NIST library, LRIs, reference substances, and also applying deconvolution algorithms (AMDIS software).

4.3.7 Methods for specific compound identification

(i) Determination of TCA, TBA and TeCA

Confirmation of haloanisoles in cork stoppers was done by analyzing the cork soaks, before purging, with the HS-SPME-MDGC-ECD system described in an earlier work [32].

(ii) Determination of alkyl methoxypyrazines

Detection of the alkyl methoxypyrazines (MDMP, IPMP, IBMP) was achieved with the H/C enantio-MDGC-MS-MS system described previously [33]. Injection of the cork extracts (3 µl) was done in PTV on-column mode (simile on-column liner, ThermoFisher Scientific) after attaching a 1 m × 0.53 mm i.d. phenylmethylsilylated pre-column (BGB Analytik) via a press-fit connector. PTV on-column injection was done at 50 °C (PTV temperature; 1 min hold), then raised at 14.5 °/s to 190 °C (0.5 min hold) and to 270 °C at 10 °/s (hold during remaining runtime). The splitless time was 2 min. Temperature program, cut-windows and cryo-time were adjusted accordingly.

(iii) Determination of GSM and MIB

Confirmation of GSM and MIB was done with the before mentioned H/C MDGC-MS-MS system, modified as follows. The original 15 m ^{1}D column was replaced by a 30 m × 0.25 mm i.d. fused silica capillary column, coated with 0.25 µm of Stabilwax-MS (Restek, Bad Homburg, Germany). Inlet pressure was 197 kPa and midpoint pressure was 121 kPa (Deans' Switch). A ^{1}D column backflush (releasing the effluent through the PTV in split mode) was initiated after heart-cutting (24 min) by lowering the PTV inlet pressure to 15 kPa. Oven temperature was programmed from 50 ˚C (5 min isothermal) at 10 ˚/min to 130 ˚C (0 min hold) and at 5 ˚/min to 179 °C (155 °C for MIB). Then the temperature was lowered to 100 °C (80 °C for MIB) at 30 °/min (2 min isothermal) before the ^{2}D-GC separation started by raising the temperature to 150 °C at 5˚/min and at 30 ˚/min to 190 ˚C (5 min hold). Cut-windows and cryo-time were adjusted accordingly. The optimized MS-MS transfers and collision energies were 182 → 112 (12 V), 182 → 97 (12 V) and 112 → 97 (12 V) for GSM and 107 → 65 (20 V), 107 → 91 (12 V), 135 → 91 (15 V), 135 → 107 (8 V) and 150 → 107 (12 V) for MIB, respectively.

4.3.8 Additional compound identification supported by comprehensive multidimensional GC

A comprehensive multidimensional GC system (GC×GC) equipped with a PTV injector and a dual-jet carbon dioxide modulator (Trace GC×GC Ultra, ThermoFisher Scientific) was coupled to a quadrupole MS (DSQ, ThermoFisher Scientific). The analytical column system consisted of a 1 m × 0.53 mm i.d. phenylmethylsilylated fused silica capillary (BGB Analytik) used as a pre-column, a 30 m × 0.25 mm i.d. fused silica capillary column coated with a polyethylene glycol phase of 0.5 µm (ZB-Wax, Phenomenex) as ^{1}D column, and a 2 m × 0.15 mm i.d. fused silica capillary coated with 0.25 µm of a 5 % phenyldimethyl-polysiloxane phase (BPX-5, SGE) as ^{2}D column. Helium was used as carrier gas at a

constant flow of 1.2 ml/min. Injection (3 µl) was done in PTV on-column mode at 50 °C (PTV temperature; 1 min hold), then raised at 14.5 °/s to 260 °C (1 min hold) and to 270 °C at 10 °/s (hold during remaining runtime). The splitless time was 2 min. The oven temperature was programmed from 50 °C (5 min isothermal) to 250 °C at 4.5 °/min (5 min hold). Cryo-modulation occurred on the last section of the ^{1}D column and was started after a delay time of 6 min. The modulation period was 6 s. The MS transfer line was set to 250 °C and the ion source was set to 240 °C. MS data acquisition was done in electron ionization (EI+) mode at 70 eV. The mass scan range was segmented into *m/z* 40 – 250 from 5 to 30 min and *m/z* 60 – 350 after 30 min. The scan rates were 16 and 13 Hz, respectively. Data acquisition was done with Xcalibur software (version 1.4, ThermoFisher Scientific) and processing of two-dimensional data was done with HyperChrom software version 2.5 (ThermoFisher Scientific). Compound identification was based on mass spectra matching NIST library, LRI, reference substances, and applying deconvolution algorithms (AMDIS software) when required.

4.4 Results and Discussion

4.4.1 Sample selection and preparation

About 150 000 individual natural cork stoppers were sensorially evaluated by trained panelists. As described in the Material and Methods section, rejected cork stoppers were described either with a slightly deviant odor (class 2: 1.15 %), a strong deviant odor (class 3A: 0.35 %) or a typical TCA taint (class 3B: 0.04 %). The cork stoppers classified as class 3B (the typical TCA taint) represent only a minor part of those classified with adverse sensory properties. Within the strongly deviant cork group (class 3) 3B is about a tenth of its sum. Already indicated by these numbers, the atypical off-odor seems to be a problem even more crucial to the industry than the typical TCA taint. Only cork stoppers from class 3A were further investigated (additionally subdivided into five groups) to determine the substantial base underlying these off-odors.

The basic workflow of the various analytical approaches applied in this investigation is illustrated in Figure 4-1. Based on established analytical procedures, known haloanisoles responsible for musty cork taint (TCA, TBA, TeCA) were analyzed by HS-SPME-MDGC-ECD from cork soaks. In order to target hitherto unknown compounds, volatiles were purged from pooled cork soaks and trapped on a sorbent material. Purge time for an individual extraction step was determined by detecting off-odors from trap extracts (each taken after 100 min) with sensory evaluation. After 400 min (four consecutive traps), off-odors could still be detected in the GC-O experiment. For practical reasons (working day), the overall purge time was set to 7 h for all samples.

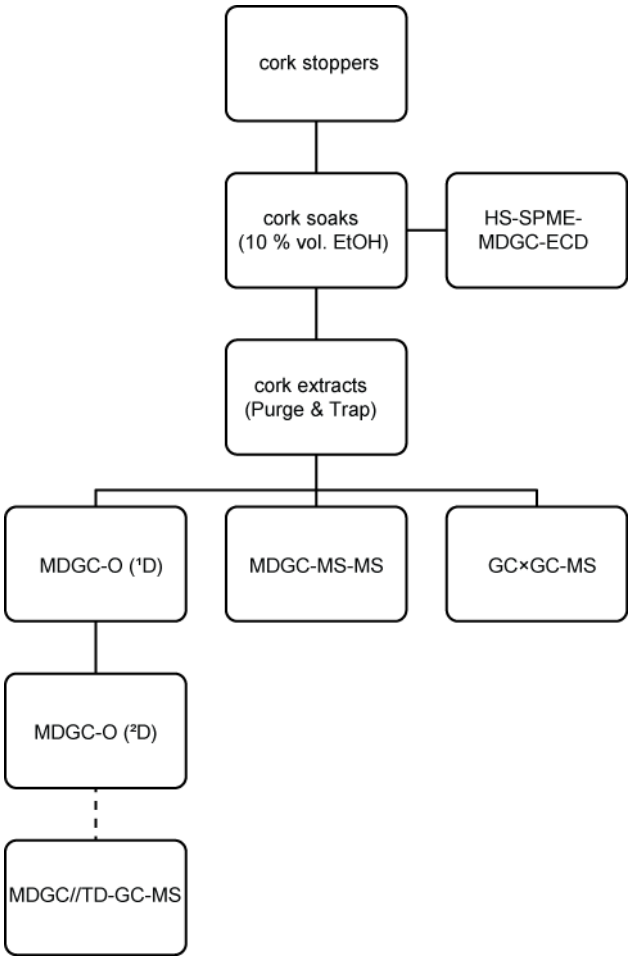


Figure 4-1 Workflow for the identification of compounds responsible for atypical cork taint

4.4.2 Olfactometric analysis and identification of compounds responsible for off-odors

Considering the complexity of a concentrated extract of a natural material such as cork co-elution problems are to be expected when using standard one-dimensional GC-O. Therefore, the GC-O system was based on H/C MDGC-O with at first a ^{1}D GC-O experiment for detecting odorous elution zones in general. Such odor events were then individually transferred onto a second analytical column with different separation properties. The thus reduced matrix allowed in the following ^{2}D GC-O experiment a more reliable detection of odorous events as visualized with the example of Figure 4-2. The odor event with a ^{1}D LRI of 1442 that had been described with the sensory attributes “nutty, musty, dusty” actually resulted in five odor events after ^{2}D separation and a number of additional non-odorous compounds producing a FID signal. This result reveals that GC-O odor events (particularly after a ^{1}D separation only) may be generated from a number of individual flavor compounds. Synergistic, antagonistic or even suppressive effects can then influence the resulting odor sensation. The approach with H/C MDGC-O thus provides a much higher resolution of odor events, eventually resulting in the sniffing of single components.

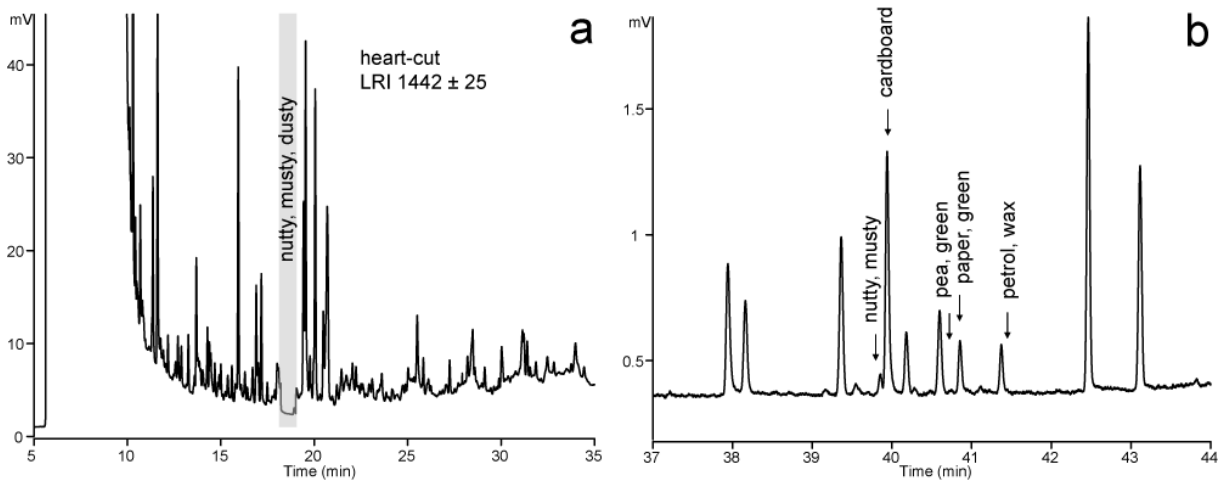


Figure 4-2 Enhanced resolution for determination of odorous zones responsible for cork taint after MDGC-O. (a) ^{1}D FID-chromatogram with transfer of odor event. (b) Resulting ^{2}D FID-chromatogram with indication of multiple odor events. Conditions were as described in the text

In general, sniffing of the cork extracts under investigation resulted in about 35-40 odor events per sample after ^{1}D separation. Compared to the extract from the control sample (class 1 cork stoppers), 4-8 odor events (depending on sub-group) were different with regard to musty or vegetative off-odors. The MDGC-O results after ^{1}D and ^{2}D sniffing are summarized in Table 4-1. Compounds responsible for the individual odor events were tentatively identified by comparing the odor descriptions and LRIs on two stationary phases (ZB-Wax, DB-5) with authentic reference substances. Further confirmation was achieved by target analysis using already established analytical methods such as MDGC-MS-MS or MDGC-ECD. The latter allows direct quantification of TCA (also TBA and TeCA) from the original cork soaks. Based on a previous work [2] suggesting guaiacol and 1-octen-3-one as compounds negatively affecting cork aroma, these two compounds could be confirmed in the study using GC×GC-MS as a supplementary method (see also Figure 4-1).

Although the investigated cork stoppers had been described with a musty odor deviant from TCA, this typical cork taint compound was perceived in all sub-groups, including even the unaffected control cork stoppers. The intensity of the perceived TCA was highest in the moldy, musty and cellarlike sub-groups. This corresponds well with the analytical data obtained from the original cork soaks with TCA concentrations at 20 ng/l (moldy), 54 ng/l (musty) or at 46 ng/l (cellarlike). The control sample was determined with <0.4 ng/l (limit of quantification), whereas 0.7 and 11 ng/l were found in the green and earthy sub-group, respectively.

Table 4-1 MDGC-O analysis of atypical off-flavor compounds

LRI [a] ZB-Wax	LRI [a] DB-5	Odor description	Odor description of cork stoppers [b] (sub-groups of class 3A)					Compound	Confirmation by
			control [c]	musty	moldy	cellarlike	earthy	green	
1313	999	mushroom, metallic	+	++	+	+	+	+	1-octen-3-one	GC×GC-MS
1441	1067	musty, moldy, nutty, earthy	(+)	+++	++	++	++	+	MDMP	MDGC-MS-MS, GC×GC-MS
1443	1102	green, pea, earthy	+	+	-	-	+	+++	IPMP	MDGC-MS-MS, GC×GC-MS
1536	1188	green, bell pepper	(+)	-	(+)	-	-	++	IBMP	MDGC-MS-MS
1618	1197	earthy, musty, camphoraceous	-	-	+++	+	-	(+)	MIB	MDGC-MS-MS
1736	1212	musty, TCA-like	-	-	-	+	-	-	unknown A [d]	
1865	1344	musty, TCA-like	+	++	++	+++	+	+	TCA	MDGC-ECD, GC×GC-MS
1889	1432	earthy, moldy, beetroot	-	-	+++	+++	+	++	GSM	MDGC-MS-MS
1908	1086	smoke, sweet, medicine	(+)	+	+	+	+	+	guaiacol	GC×GC-MS
2098	1435	musty, TCA-like	-	+	+	+	-	-	unknown B [d]	
2108	1530	musty, TCA-like	-	+	(+)	-	-	-	unknown C [d]	
2272	1474	musty, TCA-like	-	-	-	+	+	-	unknown D	
2272	1697	moldy, GSM-like	-	+	-	+	-	-	unknown E	

[a] LRI, linear retention index.

[b] -, not perceived; (+), perceived only by one panelist; +, weak; ++, strong; +++, very strong.

[c] class 1 cork stoppers.

[d] identification as described in text.

Another substance that was perceived in each extract was MDMP. This substance was first described as a compound associated with “fungal must” in wine and found in cork stoppers by Simpson et al. [4] and was later confirmed by Prat et al. [5] and Chatonnet et al. [16]. Besides, MDMP has not yet been described in other studies with respect to corky off-flavor. However, it was found among other alkyl methoxypyrazines as a potent and contributing off-flavor compound in wine related with the so-called “ladybug taint”. Originally, this off-flavor was associated with a structural isomer of MDMP, the 2,5-dimethyl-3-methoxypyrazine (DMMP) [16]. Since retention properties on common stationary phases used for GC are almost identical for both isomers with furthermore non-distinguishable mass spectra, an unequivocal identification is critical. Such identification presupposes a chromatographic separation of MDMP and DMMP and also the availability of fully characterized standards as was outlined recently [33]. The origin of MDMP in cork is not yet fully understood. There is some evidence that microorganisms may produce MDMP [5,15,16]. Prat et al. [5] isolated microorganisms from cork and inoculated sterilized cork granules with bacterial or fungal suspensions. Resulting cork samples that were sensorially evaluated and described with vegetative and musty-earthy attributes contained MDMP, as was confirmed by GC-MS analysis. Chatonnet et al. [16] identified a bacterium capable of synthesizing high amounts of MDMP that is widespread in soil. A hypothetical contamination may occur during storage of cork barks or during cork stopper production in a process that has yet to be determined.

Besides MDMP and TCA also 1-octen-3-one and guaiacol were present in all cork extracts and showed no distinct difference to the control. Both 1-octen-3-one and its reduced version 1-octen-3-ol are common metabolites of molds [34], known to derive from enzymatic oxidation of unsaturated fatty acids. The ketone has a very low odor threshold in wine (20 ng/l dry white wine) [2] that is 1000 times lower than the corresponding alcohol. Therefore it is an important contributor to the off-flavor of affected wines or corks. It is well known that widespread fungi like *Penicillium* species or *Botrytis cinerea* produce 1-octen-3-one and, among others, GSM and MIB [35]. During the production of cork stoppers the contamination with molds occurs and thus contamination with such compounds can be explained. Guaiacol has already been described as a flavor relevant compound affecting cork stoppers [2]. However, its odor threshold in wine is comparable to 1-octen-3-ol and its flavor contributes to smoky or medicinal attributes. Its production by degradation of vanillic acid with microorganisms isolated from cork has been described earlier [36].

Within the moldy and cellarlike cork sub-groups GSM and MIB were the major contributing compounds together with increased presence of TCA and also MDMP. Two other potent alkyl methoxypyrazines, IPMP and IBMP, were primarily found in cork stoppers with a green odor description. IPMP and IBMP are known for musty, earthy off-flavors in water and some foodstuffs [6,19,20]. Allen et al. [37] have already hypothesized the contamination of cork

with IPMP in a situation where individual IPMP concentrations were found in different bottles of the same wine. Again, a synthesis by microorganisms was described earlier [38]. Migration of IPMP or IBMP from cork to wine and its potential for a negative effect on the wine sensory properties has not been studied yet. One may assume similar defects as seen with the ladybug taint. However, if there is a negative influence, this cork stopper class can clearly be differentiated from musty, moldy type notes experienced with the other sub-groups.

Further known compounds contributing to typical cork taint, like the haloanisoles TBA, TeCA, and PCA, were not detected by MDGC-O or further GC analyses, besides traces of PCA found in some extracts with GC×GC-MS. In principle, this could be expected as they are related to an anthropogenic entry into the environment (cellar and winery). The detection of traces of PCA in some cork stoppers may indicate that there might still be residues of pentachlorophenol, a biocide extensively used a few years ago also in cork forests [3,39-41].

4.4.3 Identification of unknown compounds

In addition to the before discussed odor events there were five which remained unknown (unknowns A-E) after MDGC-O and comparison of LRIs of suspicious compounds known from literature. As a first identification step a visual comparison of GC×GC-MS chromatograms (control versus affected cork stoppers) in the conspicuous retention time ranges of the unknown compounds was performed. In some samples recurring differences in the peak patterns corresponding to the retention of unknowns A-C could be detected and are exemplarily shown in Figure 4-3. A first comparison (NIST database) and interpretation of mass spectra obtained for the three highlighted peaks indicated the presence of chlorinated phenolic compounds. For further identification the corresponding elution zones after MDGC were trapped and analyzed by thermal desorption (TD) GC-MS as described in the Method section (MDGC//TD-GC-MS). Trace level mass spectra interpretation was hindered by bleed produced from the sorbent used (PDMS). Subtraction of data obtained from a blank and application of deconvolution algorithms (AMDIS) finally yielded the corresponding mass spectra presented in Figure 4-4 that could also be observed with GC×GC-MS.

Conclusive information for the remaining unknowns D and E could not be achieved. Based on the hypothesis of the presence of chlorophenolic compounds, structurally related compounds present in commercially available environmental contaminants standard mixtures were investigated. On the background that chlorophenols are *O*-methylated by microorganisms (as is known from the generation of TCA) their *O*-methylated derivatives were synthesized. In the case of aldehydes (5-chloro-, 6-chloro- and 5,6-chlorovanillin and 2-chloro- and 2,6-chlorosyringaldehyde) an intermediate protection of the carbonyl function

was necessary before *O*-methylation. The corresponding chromatographic (LRI on two different stationary phases), mass spectrometric and sensory (GC-O) data were determined and are summarized in the Appendix in Table 4-2 (LRIs and MS data of chlorophenols are presented in Table 4-3). Since the synthesis of the *O*-methylated derivatives was based on a mixture of phenols, the correct assignment particularly of isomeric compounds of the *O*-methylated products was achieved by comparison of their LRIs with data published in literature [42-45]. Based on the comparison of the two LRIs from MDGC-O, mass spectrometric and sensory information, 2,6-dichloroanisole (unknown A), 3,5-dichloroveratrole (unknown B) and 3,4,6-trichloroveratrole (unknown C) could be identified and were verified with individual reference substances. Considering their chemical structure being similar to TCA this may explain their comparable sensory properties.

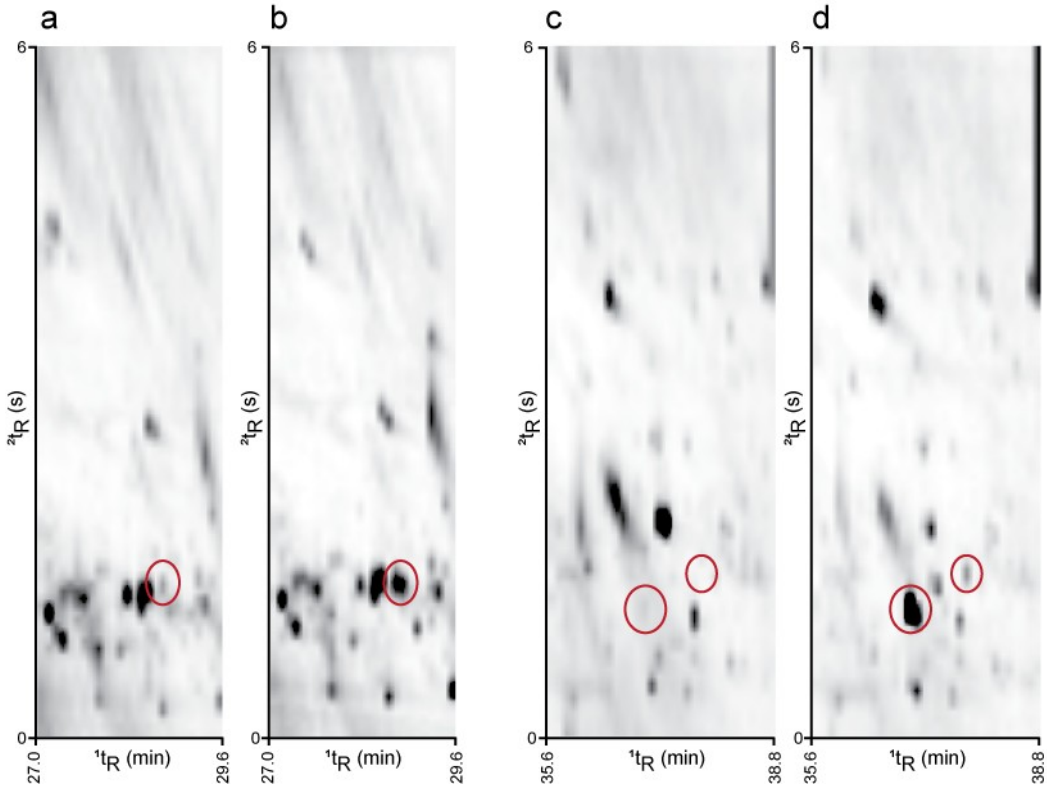


Figure 4-3 Comparison of GC×GC chromatograms (sections) of cork extracts. (a, c) inconspicuous control samples; (b, d) tainted cork samples. Indication of differences within peak patterns correspond to unknown odor events

Among the chlorinated compounds detected in cork stoppers were dichloroanisoles [1,46] and chloroveratroles [1]. A mono- and a dichloroveratrole (unassigned isomers) were also detected in the volatile fraction of microorganism cultures isolated from cork [47]. However, an exact identification of chloroveratrole isomers as well as sensory descriptions were not given earlier, so to the best of the author's knowledge, 3,4,6-trichloroveratrole and 3,5-dichloroveratrole are described here for the first time as a constituent of cork stoppers with off-flavor and were characterized by GC-O. Since Kugler and Rapp [3] found relatively high

amounts of chloroguaiacols and 6-chlorovanillin in cork stoppers, these compounds were also considered but could not be identified in the analyzed samples.

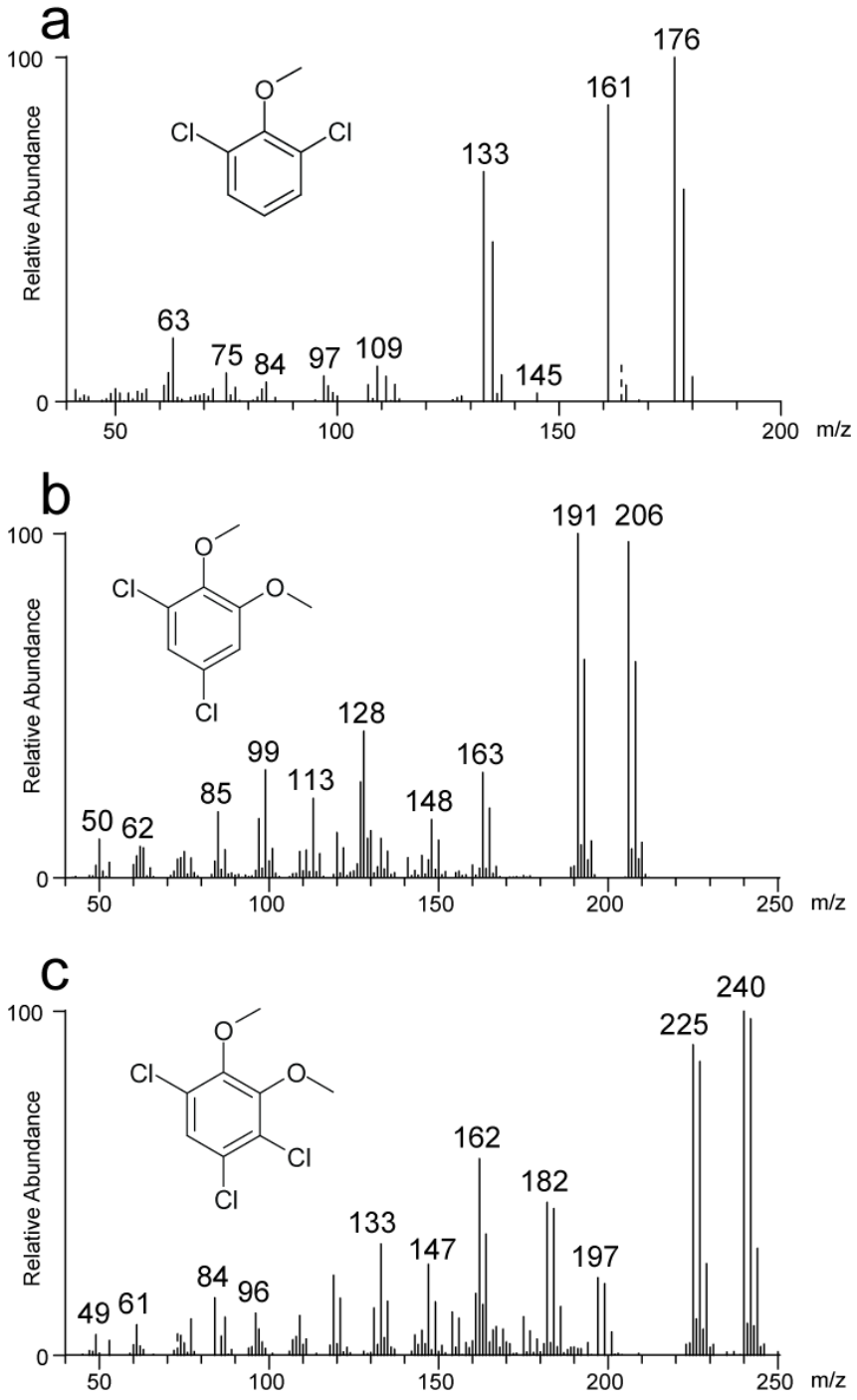


Figure 4-4 Mass spectra of chlorinated compounds identified after MDGC//TD-GC-MS analysis. (a) unknown A (2,6-dichloroanisole); (b) unknown B (3,5-dichloroveratrole); (c) unknown C (3,4,6-trichloroveratrole). For detailed information, see text

The overall contribution of chlorinated compounds to today's cork taint situation was relativized in a review by Sefton and Simpson [9] due to the discontinuation of chlorine bleaching in the cork industry. However, in the here described results such chlorinated compounds were found, probably originating from the chlorination of lignin, then putting up

the question of their origin. This is in accordance with the results of Sponholz and Muno [39]. They found trace amounts of pentachlorophenol and trichlorophenol even in non-chlorinated cork stoppers and suggested an environmental influence. In addition to the before mentioned compounds, isomers of dichlorophenol and dichloroanisole could be detected by a targeted screening of the GC×GC chromatograms based on data published in Table 4-2 and Table 4-3. However, they were not perceived by MDGC-O. In general, such chlorinated substances are well known to the wood processing industry as they are produced during the pulp bleaching process [24,25,48]. Chlorinated lignin is degraded to chlorocatechols and -guaiacols. Various microorganisms are then able to metabolize them to the corresponding *O*-methylated compounds producing chloroveratroles [48,49].

4.5 Conclusion

In conclusion across the individual sub-groups with atypical cork taints the well known TCA was present besides the hitherto not often described MDMP. Obviously, TCA is a good marker for cork taint in general but analytical monitoring has to be extended by MDMP, particularly for the detection of atypical cork taint. Specific sensory sub-groups showed high intensities of GSM and MIB (moldy and cellarlike) or of IPMP and IBMP (green), probably contributing with their potent individual flavor notes. Other compounds like 1-octen-3-one, guaiacol and chlorinated compounds discussed above seem to play a minor role possibly contributing to subtle sensory deviations. Future studies should investigate potential synergisms as mostly mixtures of different compounds classes were found. The presented results are in good agreement with compounds identified by Prat et al. [5] who also concluded that the diversity of microorganisms possibly found on the surface of corks generate their individual mix of substances. Based on some preliminary sensory trials performed in the laboratory of the Dienstleistungszentrum Ländlicher Raum Rheinpfalz, the reduced fruitiness perceived in wines seems to be associated with MDMP migrating from affected cork stoppers into the wine. In a future work this should be investigated in more detail. In this respect further sensory studies based on recombination experiments are necessary to fully elucidate the role of the compounds described here.

4.6 References

1. Buser HR, Zanier C, Tanner H (1982) Identification of 2,4,6-trichloroanisole as a potent compound causing cork taint in wine. Journal of Agricultural and Food Chemistry 30 (2):359-362.

2. Amon JM, Vandepeer JM, Simpson RF (1989) Compounds responsible for cork taint in wine. Australian & New Zealand Wine Industry Journal 4 (1):62-69.

3. Kugler D, Rapp A (1997) Bildung und Entwicklung von Inhaltstoffen in Korkborke während des Herstellungsprozesses von Flaschenkorken. Deutsche Lebensmittel-Rundschau 93 (6):174-177.

4. Simpson RF, Capone DL, Sefton MA (2004) Isolation and Identification of 2-Methoxy-3,5-dimethylpyrazine, a Potent Musty Compound from Wine Corks. Journal of Agricultural and Food Chemistry 52 (17):5425-5430.

5. Prat C, Trias R, Cullere L, Escudero A, Antico E, Baneras L (2009) Off-Odor Compounds Produced in Cork by Isolated Bacteria and Fungi: A Gas Chromatography-Mass Spectrometry and Gas Chromatography-Olfactometry Study. Journal of Agricultural and Food Chemistry 57 (16):7473-7479.

6. Maga JA (1987) Musty/earthy aromas. Food Reviews International 3 (3):269-283.

7. Simpson RF (1990) Cork Taint in Wine: A Review of the Causes. Australian and New Zealand Wine Industry Journal 5:286-296.

8. Chatonnet P, Guimberteau G, Dubourdieu D, Boidron J (1994) Nature et origine des odeurs de “moisi” dans les caves. Incidences sur la contamination des vins. Journal International des Sciences de la Vigne et du Vin 28:131-151.

9. Sefton MA, Simpson RF (2005) Compounds causing cork taint and the factors affecting their transfer from natural cork closures to wine – a review. Australian Journal of Grape and Wine Research 11 (2):226-240.

10. Lovell RT, Broce D (1985) Cause of musty flavor in pond-cultured penaeid shrimp. Aquaculture 50 (1-2):169-174.

11. Watson SB, Brownlee B, Satchwill T, Hargesheimer EE (2000) Quantitative analysis of trace levels of geosmin and MIB in source and drinking water using headspace SPME. Water Research 34 (10):2818-2828.

12. Gerber NN, Lechevalier HA (1965) Geosmin, a earthy-smelling substance isolated from actinomycetes. Applied Microbiology 13 (6):935-938.

13. Dickschat JS, Martens T, Brinkhoff T, Simon M, Schulz S (2005) Volatiles released by a Streptomyces species isolated from the North Sea. Chemistry & Biodiversity 2 (7):837-865.

14. Ventura F, Quintana J, Gomez M, Velo-Cid M (2010) Identification of alkyl-methoxypyrazines as the malodorous compounds in water supplies from northwest spain. Bulletin of Environmental Contamination and Toxicology 85 (2):160-164.

15. Mottram DS, Patterson RLS, Warrilow E (1984) 2,6-Dimethyl-3-methoxypyrazine: a microbiologically-produced compound with an obnoxious musty odor. Chemistry & Industry (London, United Kingdom) (12):448-449.

16. Chatonnet P, Fleury A, Boutou S (2010) Origin and incidence of 2-methoxy-3,5-dimethylpyrazine, a compound with a “fungal” and “corky” aroma found in cork stoppers and oak chips in contact with wines. Journal of Agricultural and Food Chemistry 58 (23):12481-12490.

17. Murray KE, Whitfield FB (1975) Occurrence of 3-alkyl-2-methoxypyrazines in raw vegetables. Journal of the Science of Food and Agriculture 26 (7):973-986.

18. Lacey MJ, Allen MS, Harris RLN, Brown WV (1991) Methoxypyrazines in Sauvignon blanc grapes and wines. American Journal of Enology and Viticulture 42 (2):103-108.

19. Sung Y-H, Li T-Y, Huang S-D (2005) Analysis of earthy and musty odors in water samples by solid-phase microextraction coupled with gas chromatography/ion trap mass spectrometry. Talanta 65 (2):518-524.

20. Miller A, 3rd, Scanlan RA, Lee JS, Libbey LM, Morgan ME (1973) Volatile compounds produced in sterile fish muscle (*Sebastes melanops*) by *Pseudomonas perolens*. Applied Microbiology 25 (2):257-261.

21. Pickering G, Lin J, Riesen R, Reynolds A, Brindle I, Soleas G (2004) Influence of *Harmonia axyridis* on the Sensory Properties of White and Red Wine. American Journal of Enology and Viticulture 55 (2):153-159.

22. Chatonnet P, Bonnet S, Boutou S, Labadie M-D (2004) Identification and Responsibility of 2,4,6-Tribromoanisole in Musty, Corked Odors in Wine. Journal of Agricultural and Food Chemistry 52 (5):1255-1262.

23. Schäfer V, Jung R (2010) Nicht immer ist es der Korken... Der Deutsche Weinbau 22:14-18.

24. Knuutinen J (1982) Analysis of chlorianted guaiacols in spent bleach liquor from a pulp mill. Journal of Chromatography, A 248 (2):289-295.

25. Brownlee BG, MacInnis GA, Noton LR (1993) Chlorinated anisoles and veratroles in a Canadian river receiving bleached kraft pulp mill effluent. Identification, distribution, and olfactory evaluation. Environmental Science and Technology 27 (12):2450-2455.

26. Pereira H (1988) Chemical composition and variability of cork from *Quercus suber* L. Wood Science and Technology 22 (3):211-218.

27. Ranz A, Korpecka J, Lankmayr E (2008) Optimized derivatization of acidic herbicides with trimethylsilyldiazomethane for GC analysis. Journal of Separation Science 31 (4):746-752.

28. Campo E, Ferreira V, Escudero A, Cacho J (2005) Prediction of the Wine Sensory Properties Related to Grape Variety from Dynamic-Headspace Gas Chromatography−Olfactometry Data. Journal of Agricultural and Food Chemistry 53 (14):5682-5690.

29. Bemelmans JMH (1979) Review of Isolation and Concentration Techniques. In: Land DG, Nursten HE (eds) Progress in Flavour Research. Applied Science, London, UK, pp 79-88.

30. Schmarr H-G, Ganß S, Sang W, Potouridis T (2007) Analysis of 2-aminoacetophenone in wine using a stable isotope dilution assay and multidimensional gas chromatography–mass spectrometry. Journal of Chromatography, A 1150 (1–2):78-84.

31. Seethapathy S, Gorecki T (2012) Applications of polydimethylsiloxane in analytical chemistry: A review. Analytica Chimica Acta 750:48-62.

32. Slabizki P, Schmarr H-G (2013) Analysis of corky off-flavour compounds at ultra trace level with multidimensional gas chromatography-electron capture detection. Journal of Chromatography, A 1271 (1):181-184.

33. Slabizki P, Legrum C, Meusinger R, Schmarr H-G (2014) Characterization and analysis of structural isomers of dimethyl methoxypyrazines in cork stoppers and ladybugs (*Harmonia axyridis* and *Coccinella septempunctata*). Analytical and Bioanalytical Chemistry 406 (25):6429-6439.

34. Kaminski E, Stawicki S, Wasowicz E (1974) Volatile Flavor Compounds Produced by Molds of Aspergillus, Penicillium, and *Fungi imperfecti*. Applied Microbiology 27 (6):1001-1004.

35. La Guerche S, Dauphin B, Pons M, Blancard D, Darriet P (2006) Characterization of some mushroom and earthy off-odors microbially induced by the development of rot on grapes. Journal of Agricultural and Food Chemistry 54 (24):9193-9200.

36. Alvarez-Rodriguez ML, Belloch C, Villa M, Uruburu F, Larriba G, Coque J-JR (2003) Degradation of vanillic acid and production of guaiacol by microorganisms isolated from cork samples. FEMS Microbiology Letters 220 (1):49-55.

37. Allen MS, Lacey MJ, Boyd SJ (1995) Methoxypyrazines in Red Wines: Occurrence of 2-Methoxy-3-(1-methylethyl)pyrazine. Journal of Agricultural and Food Chemistry 43 (3):769-772.

38. Gallois A, Kergomard A, Adda J (1988) Study of the biosynthesis of 3-isopropyl-2-methoxypyrazine produced by *Pseudomonas taetrolens*. Food Chemistry 28 (4):299-309.

39. Sponholz WR, Muno H (1994) Der Korkton - ein mikrobiologisches Problem? Wein-Wissenschaft, Wiesbaden 49 (1):17-22.

40. Simpson RF, Sefton MA (2007) Origin and fate of 2,4,6-trichloroanisole in cork bark and wine corks. Australian Journal of Grape and Wine Research 13 (2):106-116.

41. Alvarez-Rodriguez ML, Recio E, Coque JJR (2009) The analysis of natural cork stoppers in transversal sections as an effective tool to determine the origin of the taint by 2,4,6-trichloroanisole. European Food Research and Technology 230 (1):135-143.

42. Korhonen IOO (1984) Gas-liquid chromatographic analyses. XXXI. Retention increments of isomeric chlorophenols on low-polarity (SE-30) and polar (FFAP) capillary columns. Journal of Chromatography 315:185-200.

43. Korhonen IOO (1984) Gas-liquid chromatographic analyses. XXVIII. Capillary column studies of chlorinated anisoles. Journal of Chromatography 294:99-116.

44. Korhonen IOO, Knuutinen J, Jaaskelainen R (1984) Gas-liquid chromatographic analyses. XXIV. Capillary column studies of the chlorinated veratroles (1,2-dimethoxybenzenes). Journal of Chromatography 287 (2):293-303.

45. Spadone JC, Takeoka G, Liardon R (1990) Analytical investigation of Rio off-flavor in green coffee. Journal of Agricultural and Food Chemistry 38 (1):226-233.

46. Pollnitz AP, Pardon KH, Liacopoulos D, Skouroumounis GK, Sefton MA (1996) The analysis of 2,4,6-trichloroanisole and other chloroanisoles in tainted wines and corks. Australian Journal of Grape and Wine Research 2 (3):184-190.

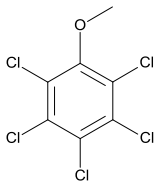
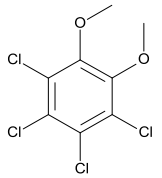
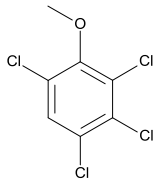
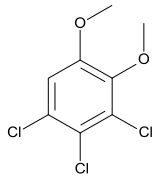
47. Caldentey P, Fumi MD, Mazzoleni V, Careri M (1998) Volatile compounds produced by microorganisms isolated from cork. Flavour and Fragrance Journal 13 (3):185-188.

48. Eriksson KE, Kolar MC, Ljungquist P, Kringstad KP (1985) Studies on microbial and chemical conversions of chlorolignins. Environmental Science and Technology 19 (12):1219-1224.

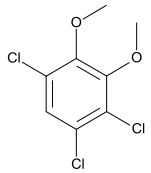
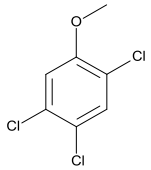
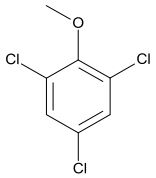
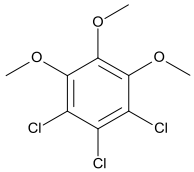
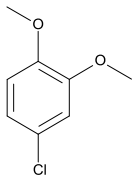
49. Neilson AH, Allard AS, Hynning PA, Remberger M, Landner L (1983) Bacterial methylation of chlorinated phenols and guaiacols: formation of veratroles from guaiacols and high-molecular-weight chlorinated lignin. Applied and Environmental Microbiology 45 (3):774-783.

4.7 Appendix

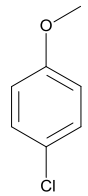
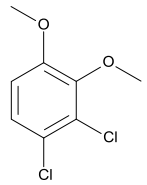
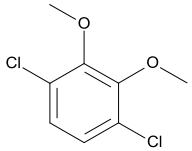
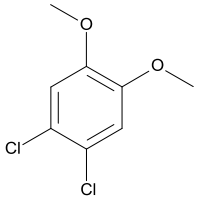
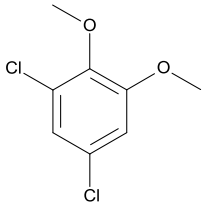
Table 4-2 Chromatographic, mass spectrometric and sensory (GC-O) data of synthesized *O*-methylated chlorophenolic compounds

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI ZB-5	LRI ZB-Wax	Odor description (GC-O)	*m/z* (intensity) of the 10 highest peaks
compounds synthesized using standard mixture EM-4181 [a]							
pentachloroanisole	1825-21-4		280.36	1751	2369	musty, TCA-like	265 (100), 280 (87), 237 (81), 263 (64), 267 (61), 278 (56), 235 (52), 282 (52), 239 (51), 165 (30)
3,4,5,6-tetrachloroveratrole	944-61-6		275.94	1740	2406	-	276 (100), 261 (93), 259 (82), 274 (80), 196 (47), 263 (46), 218 (45), 278 (44), 198 (41), 216 (36)
2,3,4,6-tetrachloroanisole	938-22-7		245.92	1546	2111	smoky, spicy, musty, TCA-like, musty	231 (100), 229 (76), 246 (75), 203 (63), 244 (57), 233 (50), 201 (49), 248 (36), 205 (31), 131 (29)
3,4,5-trichloroveratrole	16766-29-3		241.50	1657	2409	weakly musty, guaiacol-like, woody	225 (100), 242 (92), 240 (92), 227 (90), 162 (66), 164 (41), 244 (32), 229 (27), 147 (26), 133 (25)

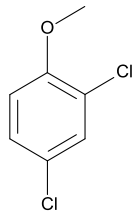
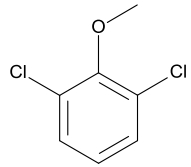
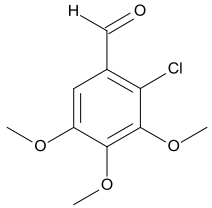
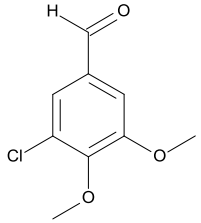
(continuation of Table 4-2)

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI ZB-5	LRI ZB-Wax	Odor description (GC-O)	*m/z* (intensity) of the 10 highest peaks
3,4,6-trichloroveratrole	85298-07-3		241.50	1527	2111	musty, eugenol-like, smoky, spicy, TCA-like	225 (100), 227 (97), 242 (95), 162 (92), 240 (91), 182 (41), 184 (38), 164 (35), 133 (35), 229 (34)
2,4,5-trichloroanisole	6130-75-2		211.47	1464	2125	-	212 (100), 210 (89), 197 (69), 167 (65), 195 (64), 169 (58), 214 (28), 199 (20), 97 (18), 171 (16)
2,4,6-trichloroanisole	87-40-1		211.47	1341	1833	TCA, musty, woody	195 (100), 197 (89), 210 (71), 212 (69), 169 (52), 167 (49), 199 (28), 214 (23), 97 (20), 171 (17)
compounds synthesized using standard mixture EM-4182 [a]							
4,5,6-trichloro-1,2,3-trimethoxy-benzene	77223-56-4		271.52	1733	2442	sweet, wax (weak)	270 (100), 272 (91), 255 (76), 257 (69), 212 (69), 214 (61), 227 (44), 229 (40), 274 (29), 259 (24)
compounds synthesized using standard mixture EM-4183 [a]							
4-chloroveratrole	16766-27-1		172.61	1332	2008	guaiacol-like, sweet	172 (100), 157 (64), 93 (36), 174 (30), 129 (28), 65 (22), 159 (20), 94 (16), 79 (15), 173 (11)

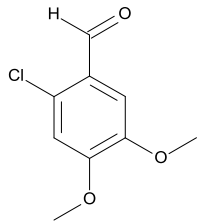
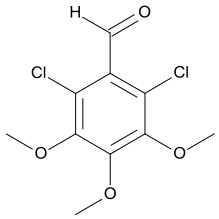
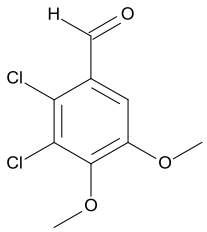
(continuation of Table 4-2)

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI ZB-5	LRI ZB-Wax	Odor description (GC-O)	*m/z* (intensity) of the 10 highest peaks
4-chloroanisole	623-12-1		142.58	1109	1649	wax, sweet	142 (100), 127 (49), 99 (37), 144 (33), 129 (14), 101 (13), 143 (10), 75 (8), 63 (8), 107 (6)
3,4-dichloroveratrole	90283-00-4 or 72361-17-2		207.05	1479	2193	smoky, sweet, spicy	206 (100), 191 (70), 208 (62), 193 (42), 128 (40), 127 (33), 113 (24), 99 (21), 163 (18), 148 (15)
3,6-dichloroveratrole	90283-02-6		207.05	1353	1913	very musty, dusty, TCA-like	206 (100), 191 (76), 208 (68), 128 (46), 193 (45), 127 (36), 148 (27), 150 (18), 99 (16), 163 (15)
4,5-dichloroveratrole	2772-46-5		207.05	1522	2275	smoky, medicinal, sweet, guaiacol-like, spicy, clove, cinnamon	206 (100), 191 (66), 208 (63), 128 (40), 193 (39), 127 (30), 99 (27), 163 (23), 113 (18), 165 (14)
3,5-dichloroveratrole	90283-01-5		207.05	1436	2075	woody, musty, TCA-like	191 (100), 206 (99), 208 (61), 193 (58), 128 (40), 127 (37), 163 (27), 99 (23), 165 (18), 113 (14)

(continuation of Table 4-2)

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI ZB-5	LRI ZB-Wax	Odor description (GC-O)	*m/z* (intensity) of the 10 highest peaks
2,4-dichloroanisole	553-82-2		177.03	1294	1920	phenolic, sweet, smoky, slightly musty,	161 (100), 176 (99), 178 (66), 163 (61), 133 (60), 135 (38), 63 (16), 75 (12), 180 (10), 162 (10)
2,6-dichloroanisole	1984-65-2		177.03	1204	1728	TCA-like, musty	176 (100), 161 (98), 133 (66), 178 (64), 163 (60), 135 (36), 63 (15), 75 (13), 177 (13), 73 (11)
compounds synthesized using standard mixture EM-4184 [a]							
2-chloro-3,4,5-trimethoxybenzaldehyde	164660-56-4		230.64	1709	2574	-	230 (100), 232 (34), 215 (33), 144 (30), 229 (21), 159 (19), 231 (17), 127 (14), 187 (10), 129 (10)
3-chloro-4,5-dimethoxybenzaldehyde	18268-68-3		200.62	1557 (A) or 1639 (B) [b]	2433 or 2555 [b]	sweet, musty, woody (A) or rubber, wax, musty (B) [b]	200 (100), 185 (48), 74 (47), 199 (46), 202 (31), 87 (31), 129 (27), 143 (24), 201 (20), 93 (18) (A) or 200 (100), 199 (73), 202 (31), 201 (31), 185 (24), 129 (17), 93 (13), 65 (11), 113 (10), 187 (7) (B) [b]

(continuation of Table 4-2)

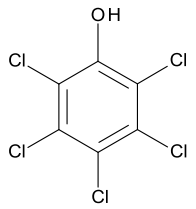
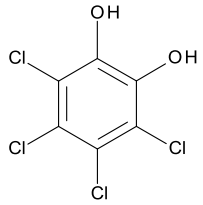
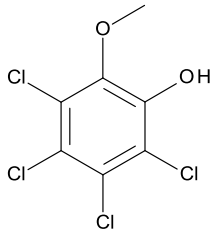
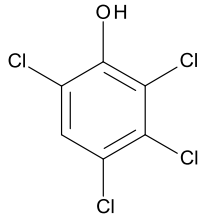
Compound	CAS no.	Chemical structure	MW (g/mol)	LRI ZB-5	LRI ZB-Wax	Odor description (GC-O)	*m/z* (intensity) of the 10 highest peaks
2-chloro-4,5-dimethoxybenzaldehyde	18093-05-5		200.62	1557 (A) or 1639 (B) [b]	2433 or 2555 [b]	sweet, musty, woody (A) or rubber, wax, musty (B) [b]	200 (100), 185 (48), 74 (47), 199 (46), 202 (31), 87 (31), 129 (27), 143 (24), 201 (20), 93 (18) (A) or 200 (100), 199 (73), 202 (31), 201 (31), 185 (24), 129 (17), 93 (13), 65 (11), 113 (10), 187 (7) (B) [b]
2,6-dichloro-3,4,5-trimethoxybenzaldehyde	75315-53-6		265.09	1856	2800	phenolic, woody	264 (100), 266 (61), 178 (48), 249 (42), 265 (31), 263 (29), 180 (28), 251 (22), 193 (22), 221 (19)
2,3-dichloro-4,5-dimethoxybenzaldehyde	125000-96-6		235.06	1729	2595	smoky, sweet	234 (100), 236 (61), 233 (47), 235 (35), 219 (31), 221 (26), 128 (22), 127 (20), 163 (18), 147 (18)

LRI, linear retention index. MW, molecular weight

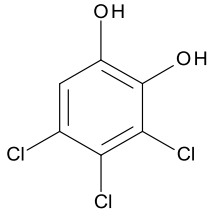
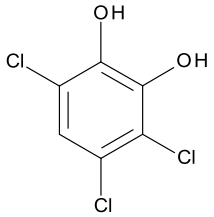
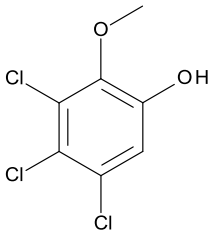
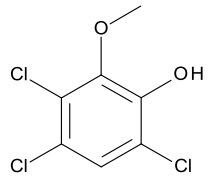
[a] compounds contained in standard mixtures EM-4181, EM-4182, EM-4183 and EM-4184 see Materials and Method section

[b] no assignment of correct isomer

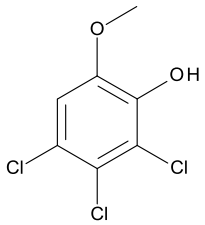
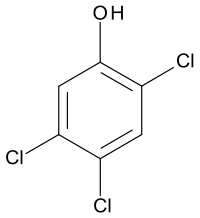
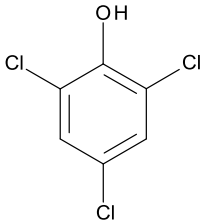
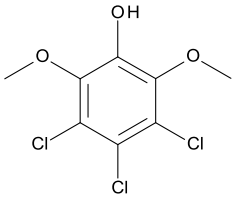
Table 4-3 Chromatographic and mass spectrometric data of chlorophenolic compounds contained in the standard mixtures EM-4181, EM-4182, EM-4183, EM-4184

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI Rxi-5SilMS	LRI ZB-Wax	*m/z* (intensity) of the 10 highest peaks
standard mixture EM-4181						
pentachlorophenol	87-86-5 or 131-52-2		266.35	1756	[a]	266 (100), 268 (70), 264 (65), 167 (35), 165 (34), 230 (23), 228 (22), 270 (21), 202 (21), 200 (18)
3,4,5,6-tetrachlorocatechol	1198-55-6		247.89	1805	[a]	248 (100), 246 (81), 250 (52), 147 (37), 154 (30), 182 (26), 212 (25), 149 (25), 249 (25), 247 (24)
3,4,5,6-tetrachloroguaiacol	2539-17-5		261.91	1775	[a]	247 (100), 245 (74), 262 (64), 260 (55), 249 (41), 219 (36), 264 (34), 217 (30), 221 (20), 183 (19)
2,3,4,6-tetrachlorophenol	58-90-2		231.89	1556	>2800	232 (100), 230 (79), 234 (45), 131 (41), 166 (33), 133 (30), 168 (30), 194 (28), 196 (26), 96 (16)

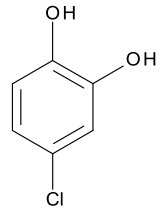
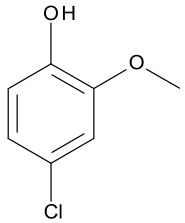
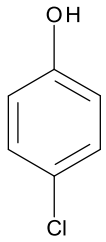
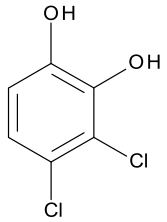
(continuation of Table 4-3)

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI Rxi-5SilMS	LRI ZB-Wax	*m/z* (intensity) of the 10 highest peaks
3,4,5-trichlorocatechol	56961-20-7		213.44	1585 (A) or 1593 (B) [b]	[a]	214 (100), 212 (95), 216 (34), 176 (28), 120 (23), 148 (22), 178 (20), 140 (19), 113 (18), 122 (16) (A) or 212 (100), 214 (92), 176 (42), 120 (34), 178 (30), 216 (29), 113 (25), 122 (23), 148 (16), 213 (15) (B) [b]
3,4,6-trichlorocatechol	32139-72-3		213.44	1585 (A) or 1593 (B) [b]	[a]	214 (100), 212 (95), 216 (34), 176 (28), 120 (23), 148 (22), 178 (20), 140 (19), 113 (18), 122 (16) (A) or 212 (100), 214 (92), 176 (42), 120 (34), 178 (30), 216 (29), 113 (25), 122 (23), 148 (16), 213 (15) (B) [b]
3,4,5-trichloroguaiacol	57057-83-7		227.47	1569 (A) or 1581 (B) or 1696 (C) [b]	2796 or >2800 [b]	211 (100), 213 (92), 226 (76), 228 (74), 183 (42), 185 (38), 215 (29), 147 (24), 230 (22), 119 (18) (A) or 211 (100), 213 (88), 226 (53), 228 (49), 183 (43), 185 (38), 147 (29), 215 (28), 149 (18), 230 (14) (B) or 211 (100), 213 (91), 226 (77), 228 (73), 183 (44), 185 (42), 147 (32), 215 (29), 230 (22), 149 (19) (C) [b]
3,4,6-trichloroguaiacol	61966-36-7		227.47	1569 (A) or 1581 (B) or 1696 (C) [b]	2796 or >2800 [b]	211 (100), 213 (92), 226 (76), 228 (74), 183 (42), 185 (38), 215 (29), 147 (24), 230 (22), 119 (18) (A) or 211 (100), 213 (88), 226 (53), 228 (49), 183 (43), 185 (38), 147 (29), 215 (28), 149 (18), 230 (14) (B) or 211 (100), 213 (91), 226 (77), 228 (73), 183 (44), 185 (42), 147 (32), 215 (29), 230 (22), 149 (19) (C) [b]

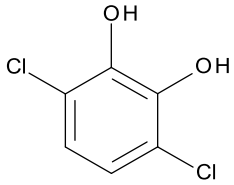
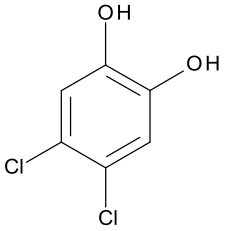
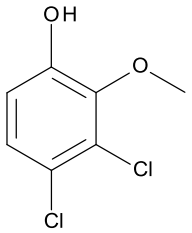
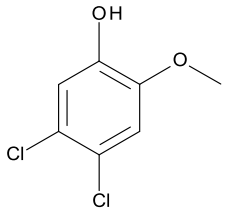
(continuation of Table 4-3)

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI Rxi-5SilMS	LRI ZB-Wax	*m/z* (intensity) of the 10 highest peaks
4,5,6-trichloroguaiacol	2668-24-8		227.47	1569 (A) or 1581 (B) or 1696 (C) [b]	2796 or >2800 [b]	211 (100), 213 (92), 226 (76), 228 (74), 183 (42), 185 (38), 215 (29), 147 (24), 230 (22), 119 (18) (A) or 211 (100), 213 (88), 226 (53), 228 (49), 183 (43), 185 (38), 147 (29), 215 (28), 149 (18), 230 (14) (B) or 211 (100), 213 (91), 226 (77), 228 (73), 183 (44), 185 (42), 147 (32), 215 (29), 230 (22), 149 (19) (C) [b]
2,4,5-trichlorophenol	95-95-4		197.45	1354 (A) or 1359 (B) [b]	2377 or >2557 [b]	196 (100), 198 (85), 132 (42), 97 (39), 200 (28), 134 (25), 160 (23), 99 (15), 162 (13), 133 (10) (A) or 196 (100), 198 (92), 97 (33), 200 (29), 132 (26), 134 (17), 133 (16), 99 (13), 135 (10), 197 (9) (B) [b]
2,4,6-trichlorophenol	88-06-2		197.45	1354 (A) or 1359 (B) [b]	2377 or >2557 [b]	196 (100), 198 (85), 132 (42), 97 (39), 200 (28), 134 (25), 160 (23), 99 (15), 162 (13), 133 (10) (A) or 196 (100), 198 (92), 97 (33), 200 (29), 132 (26), 134 (17), 133 (16), 99 (13), 135 (10), 197 (9) (B) [b]
standard mixture EM-4182						
3,4,5-trichlorosyringol	2539-26-6		257.50	1781	[a]	256 (100), 258 (100), 243 (72), 241 (71), 200 (43), 195 (41), 198 (41), 197 (39), 260 (31), 213 (29)

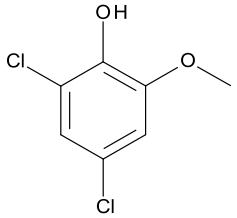
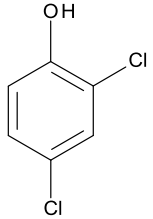
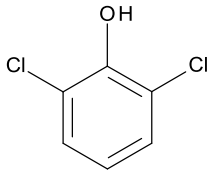
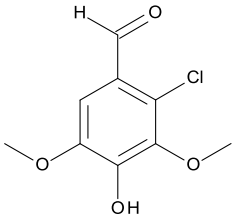
(continuation of Table 4-3)

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI Rxi-5SilMS	LRI ZB-Wax	*m/z* (intensity) of the 10 highest peaks
standard mixture EM-4183						
4-chlorocatechol	2138-22-9		144.56	1425	[a]	144 (100), 146 (33), 63 (23), 145 (9), 98 (9), 62 (6), 126 (6), 81 (6), 51 (6), 115 (5)
4-chloroguaiacol	16766-30-6		158.58	1275	2223	143 (100), 158 (97), 115 (56), 145 (31), 160 (29), 117 (18), 51 (11), 159 (10), 144 (9), 87 (7)
4-chlorophenol	106-48-9		128.56	1192	2461	128 (100), 130 (32), 65 (24), 100 (17), 64 (10), 129 (8), 63 (8), 99 (6), 102 (6), 73 (5)
3,4-dichlorocatechol	3978-67-4		179.00	1382 (A) or 1393 (B) or 1668 (C) [b]	[a]	178 (100), 180 (61), 142 (20), 79 (17), 114 (16), 86 (14), 51 (11), 182 (10), 179 (8), 144 (8) (A) or 178 (100), 180 (66), 106 (20), 142 (20), 182 (10), 78 (8), 86 (7), 144 (7), 50 (6), 51 (6) (B) or 178 (100), 180 (61), 97 (21), 115 (13), 182 (12), 179 (11), 143 (8), 132 (8), 99 (7), 149 (6) (C) [b]

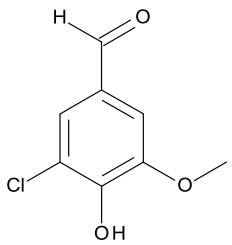
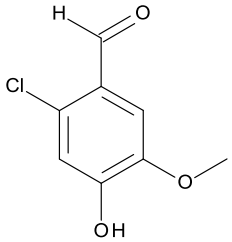
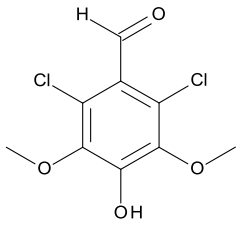
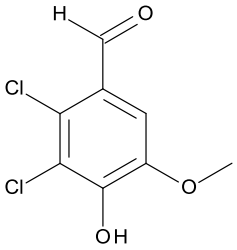
(continuation of Table 4-3)

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI Rxi-5SilMS	LRI ZB-Wax	*m/z* (intensity) of the 10 highest peaks
3,6-dichlorocatechol	3938-16-7		179.00	1382 (A) or 1393 (B) or 1668 (C) [b]	[a]	178 (100), 180 (61), 142 (20), 79 (17), 114 (16), 86 (14), 51 (11), 182 (10), 179 (8), 144 (8) (A) or 178 (100), 180 (66), 106 (20), 142 (20), 182 (10), 78 (8), 86 (7), 144 (7), 50 (6), 51 (6) (B) or 178 (100), 180 (61), 97 (21), 115 (13), 182 (12), 179 (11), 143 (8), 132 (8), 99 (7), 149 (6) (C) [b]
4,5-dichlorocatechol	3428-24-8		179.00	1382 (A) or 1393 (B) or 1668 (C) [b]	[a]	178 (100), 180 (61), 142 (20), 79 (17), 114 (16), 86 (14), 51 (11), 182 (10), 179 (8), 144 (8) (A) or 178 (100), 180 (66), 106 (20), 142 (20), 182 (10), 78 (8), 86 (7), 144 (7), 50 (6), 51 (6) (B) or 178 (100), 180 (61), 97 (21), 115 (13), 182 (12), 179 (11), 143 (8), 132 (8), 99 (7), 149 (6) (C) [b]
3,4-dichloroguaiacol	65724-16-5		193.03	1389 (A) or 1478 (B,C) [b]	2524 or 2541 or 2793 [b]	177 (100), 192 (73), 179 (64), 194 (47), 149 (42), 113 (32), 151 (27), 85 (14), 181 (11), 115 (10) (A) or 192 (100), 177 (90), 194 (61), 179 (55), 149 (52), 151 (31), 85 (11), 113 (11), 196 (10), 193 (10) (B, C) [b]
4,5-dichloroguaiacol	2460-49-3		193.03	1389 (A) or 1478 (B,C) [b]	2524 or 2541 or 2793 [b]	177 (100), 192 (73), 179 (64), 194 (47), 149 (42), 113 (32), 151 (27), 85 (14), 181 (11), 115 (10) (A) or 192 (100), 177 (90), 194 (61), 179 (55), 149 (52), 151 (31), 85 (11), 113 (11), 196 (10), 193 (10) (B, C) [b]

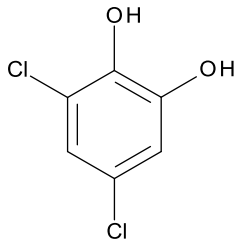
(continuation of Table 4-3)

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI Rxi-5SilMS	LRI ZB-Wax	*m/z* (intensity) of the 10 highest peaks
4,6-dichloroguaiacol	16766-31-7		193.03	1389 (A) or 1478 (B,C) [b]	2524 or 2541 or 2793 [b]	177 (100), 192 (73), 179 (64), 194 (47), 149 (42), 113 (32), 151 (27), 85 (14), 181 (11), 115 (10) (A) or 192 (100), 177 (90), 194 (61), 179 (55), 149 (52), 151 (31), 85 (11), 113 (11), 196 (10), 193 (10) (B, C) [b]
2,4-dichlorophenol	120-83-2		163.00	1169 (A) or 1202 (B) [b]	2132 or 2198 [b]	162 (100), 164 (60), 98 (29), 63 (23), 126 (16), 99 (12), 163 (10), 100 (9), 166 (9), 73 (6) (A) or 162 (100), 164 (64), 63 (26), 126 (23), 98 (23), 166 (10), 163 (10), 99 (9), 100 (8), 128 (7) (B) [b]
2,6-dichlorophenol	87-65-0		163.00	1169 (A) or 1202 (B) [b]	2132 or 2198 [b]	162 (100), 164 (60), 98 (29), 63 (23), 126 (16), 99 (12), 163 (10), 100 (9), 166 (9), 73 (6) (A) or 162 (100), 164 (64), 63 (26), 126 (23), 98 (23), 166 (10), 163 (10), 99 (9), 100 (8), 128 (7) (B) [b]
standard mixture EM-4184						
2-chlorosyringaldehyde	76341-69-0		216.62	1760	[a]	216 (100), 215 (66), 217 (33), 218 (32), 127 (15), 173 (13), 201 (10), 99 (9), 130 (8), 129 (8)

(continuation of Table 4-3)

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI Rxi-5SilMS	LRI ZB-Wax	*m/z* (intensity) of the 10 highest peaks
5-chlorovanillin	19463-48-0		186.59	1549 (A) or 1598 (B) [b]	>2800 [b]	185 (100), 186 (90), 187 (38), 188 (29), 115 (25), 143 (20), 171 (11), 51 (10), 157 (9), 117 (8) (A) or 185 (100), 186 (86), 187 (38), 188 (28), 143 (17), 115 (15), 157 (13), 51 (10), 79 (9), 65 (7) (B) [b]
6-chlorovanillin	18268-76-3		186.59	1549 (A) or 1598 (B) [b]	>2800 [b]	185 (100), 186 (90), 187 (38), 188 (29), 115 (25), 143 (20), 171 (11), 51 (10), 157 (9), 117 (8) (A) or 185 (100), 186 (86), 187 (38), 188 (28), 143 (17), 115 (15), 157 (13), 51 (10), 79 (9), 65 (7) (B) [b]
2,6-dichlorosyringaldehyde	76330-06-8		251.06	1909	[a]	250 (100), 249 (84), 252 (60), 251 (59), 161 (23), 164 (22), 163 (17), 253 (16), 235 (15), 207 (15)
5,6-dichlorovanillin	18268-69-4		221.04	1771	[a]	219 (100), 220 (85), 221 (71), 222 (50), 177 (21), 223 (16), 149 (15), 179 (14), 151 (11), 113 (11)

(continuation of Table 4-3)

Compound	CAS no.	Chemical structure	MW (g/mol)	LRI Rxi-5SilMS	LRI ZB-Wax	*m/z* (intensity) of the 10 highest peaks
other						
3,5-dichlorocatechol	13673-92-2		179.00	1459	[a]	178 (100), 180 (70), 86 (20), 114 (17), 142 (15), 51 (14), 97 (14), 182 (13), 79 (10), 50 (9)

LRI, linear retention index. MW, molecular weight

[a] no elution within temperature program

[b] no assignment of correct isomer

5 Quantification of cork off-flavor compounds in natural cork stoppers and wine by multidimensional gas chromatography mass spectrometry

Adapted with permission from P. Slabizki, C. Legrum, P. Wegmann-Herr, C. Fischer, H.-G. Schmarr, Quantification of cork off-flavor compounds in natural cork stoppers and wine by multidimensional gas chromatography mass spectrometr, *European Food Research and Technology*, **2016**, Accepted manuscript, DOI: 10.1007/s00217-015-2604-x, Copyright (2015) Springer-Verlag.

5.1 Abstract

Trace level analysis of cork off-flavor compounds considered as responsible for typical (2,4,6-trichloroanisole, TCA) and atypical cork taint (geosmin, GSM; 2-methylisoborneol, MIB; 3-isopropyl-2-methoxypyrazine, IPMP; 3-isobutyl-2-methoxypyrazine, IBMP; 3,5-dimethyl-2-methoxypyrazine, MDMP) was achieved for cork soaks and wines for concentrations below odor threshold (MIB only in cork soaks). The analytical approach was based on headspace solid phase microextraction and heart-cut multidimensional gas chromatography with tandem mass spectrometric detection. Quantification was done using a stable isotope dilution assay (SIDA). Individual cork stoppers with varying sensory off-odor descriptions were analyzed. In particular, IPMP and IBMP correlated with the cork stoppers described with green attributes. MDMP was found in samples described as dusty-musty or nutty-like. In a migration study transport of off-flavor compounds from affected cork stoppers into the corresponding wine could be observed after a storage period of 13 months. Multivariate statistics on the wines' sensory analysis and chemical data showed a good correlation of the individual off-flavor compound concentration, its sensory description and the off-flavor perceived in the wine.

5.2 Introduction

Cork taint with the primary responsible compound 2,4,6-trichloroanisole (TCA) [1] is often described as the best known off-flavor in wine. Over the years, the importance of this typical corky off-flavor decreased due to the reduction of microbiological growth on cork during the production process, the avoidance of hypochlorite as bleaching agent and rigorous quality

management in the production of natural cork stoppers. However, some experts in the wine industry report about a sensory alteration of wines different from the typical cork taint that is described with a reduced fruitiness often combined with moldy or musty notes. This *atypical* cork taint is often associated by customers to originate from the wine and not from the cork stopper. This assignment can then pose a problem to the winery due to a bad reputation. For the lack of a clear correlation of affected wines to the atypical corky off-flavor, resulting financial losses for the wine industry cannot even be estimated. Therefore, it is important to understand the chemical background and monitor the responsible compounds during quality control of cork stoppers as well as in rejected wines. Among the most important compounds discussed in previous studies associated with atypical cork off-flavors differing from the typical TCA taint are geosmin (GSM), 2-methylisoborneol (MIB), 3,5-dimethyl-2-methoxypyrazine (MDMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-isobutyl-2-methoxypyrazine (IBMP) [2-6]. These compounds were also confirmed in a comprehensive study on cork samples that had been considered as conspicuous in sensory trials [7].

GSM and MIB are well-known earthy off-odor substances in water supplies and marine foods [8,9], possibly originating from microorganisms [10,11]. Microorganisms isolated from cork were able to produce GSM and MIB as well as TCA and MDMP [3]. Sensory properties of MIB are described as “earthy”, “musty”, “muddy”, and in higher concentrations as “camphoraceous”. GSM is described with earthy attributes similar to MIB reminding of garden soil and table beet. In white wine the odor thresholds of GSM and MIB are 25 ng/l and 30 ng/l, respectively [2]. However, the role of GSM in wine was questioned as it was rapidly converted into the odorless argosmin under acidic conditions in model systems [10,12]. On the other hand, Darriet et al. found high concentrations of GSM in red and rosé wines and stated GSM to be relatively stable in acidic wines [13].

MDMP is described with the unpleasant sensory attributes “wet cardboard”, “musty”, “moldy”, “dusty”, “earthy” and was determined in cork for the first time by Simpson et al. causing a “fungal must” taint in wine [14]. It has also been found as a malodorous compound in water supplies [15] and seems to be generated by bacteria [16,17]. It is an extremely potent aroma compound with an odor threshold of about 2 ng/l in white wine [14]. In literature, MDMP is seen as the most important substance affecting cork stoppers and thus wine next to TCA [14,17]. The other alkyl methoxypyrazines, IPMP and IBMP, with a vegetative, green odor are flavor relevant compounds with very low odor thresholds in many vegetables [18] and also in *V. vinifera* varieties with IBMP as the major methoxypyrazine [19] (odor threshold in white wine 1-2 ng/l [20]). However, they can also contribute to green, earthy, potato-like off-flavors in wine, as, for example, IPMP being the major component associated with the so-called “ladybug taint” [21]. A possible contamination of cork stoppers with IPMP and its

migration in wine was early hypothesized in a study conducted by Allen et al. [22] in which individual IPMP concentrations were found for different bottles of the same wine.

Based on the previous studies, GSM, MIB, MDMP, IPMP, IBMP and TCA can be considered as the most important compounds affecting the odor of cork stoppers putting up the demand to quantify these compounds in both cork and wine samples. For this purpose, in the current study an analytical approach for multicomponent analysis should be developed using automated headspace solid phase microextraction (HS-SPME) and heart-cut multidimensional gas chromatography with tandem mass spectrometric detection (H/C MDGC-MS-MS). Reliable quantification on the low ng/l or even sub-ng/l level should be assured by a stable isotope dilution assay (SIDA). Furthermore, the migration of off-flavor compounds from cork stoppers into wine should be studied by sealing unaffected wines with off-odorous cork stoppers, followed by an appropriate storage period and finally evaluation by chemical and sensory analysis.

5.3 Materials and Methods

5.3.1 Chemicals

2,4,6-Trichloroanisole (CAS no. 87-40-1), geosmin (100 µg/ml in methanol, CAS no. 23333-91-7), 2-methylisoborneol (10 mg/ml in methanol, CAS no. 2371-42-8), 3-isopropyl-2-methoxypyrazine (CAS no. 25773-40-4) and 3-isobutyl-2-methoxypyrazine (CAS no. 24683-00-9) were from Sigma-Aldrich (Steinheim, Germany), and 3,5-dimethyl-2-methoxypyrazine (CAS no. 92508-08-2) was from Bellen Chemistry Co. Ltd. (Beijing, China). Deuterated internal standards used were 2-[$^2H_3$]-methylisoborneol (MIB-d_3, CAS no. 135441-89-3) from EQ Laboratories/CDN Isotopes (Augsburg, Germany) and 2β,6α-Dimethyl-[$^2H_5$]-bicyclo[4.4.0]decan-1β-ol (geosmin-d_5, GSM-d_5, CAS no. 216166-83-5) from Azur Isotopes (Marseille, France); 3-isopropyl-2-[$^2H_3$]-methoxypyrazine (IPMP-d_3, CAS no. 588732-60-9), 3-isobutyl-2-[$^2H_3$]-methoxypyrazine (IBMP-d_3, CAS no. 588732-63-2), 3,5-dimethyl-2-[$^2H_3$]-methoxypyrazine (MDMP-d_3, CAS no. 1335402-04-4) and [$^2H_5$]-2,4,6-trichloroanisole (TCA-d_5, CAS no. 352439-08-8) were synthesized in-house as described earlier [23-25]. Commercial chemicals were usually of analytical grade and used as such.

5.3.2 Cork and wine samples

Natural cork stoppers (before industrial processing, e.g. coating, imprinting) were sensory evaluated in a quality control process by three panelists experienced in assessing the odor of cork stoppers. For this purpose, the cork stoppers were moisturized by dipping in purified water and put in screw top jars. After keeping them overnight at room temperature, the

supernatant air was sniffed and the cork stoppers with an intensive deviant odor were chosen for further analysis. For the analysis of individual cork stoppers, their off-odor was described in detail. Cork stoppers described as inconspicuous were used as control sample.

For the migration study, wines were sealed with off-odorous cork stoppers. The stoppers were sorted into the groups musty (M), moldy/earthy (ME), green (GR), bell pepper (BP), and typical TCA (T). A white wine (Riesling, dry, 2013, 11.5 %vol., pH 3.2) was filled into 1-l glass bottles and sealed with the off-odorous cork stoppers of the cork groups or inconspicuous cork stoppers as control. The wine used was fermented and stored in a stainless steel tank at the Dienstleistungszentrum Ländlicher Raum Rheinpfalz and tested by five sensory experts prior bottling, rendering it inconspicuous with respect to a taint. Three bottles per cork group were stored horizontally in a dark cellar room. After storage of 13 months, the wine samples and their corresponding cork stoppers were analyzed by HS-SPME-H/C MDGC-MS-MS. These wines were also used for descriptive sensory analysis.

In the course of this study, wine samples (Chardonnay) from a situation in which the routine laboratory was confronted with a customer complaint for cork tainted wines were also included in the analysis as TCA concentrations had been found to be not relevant (< 0.4 ng/l).

5.3.3 Sample preparation and headspace solid phase microextraction

Cork stoppers were soaked individually in 90 ml deionized water for 24 h at room temperature in a 100-ml wide mouth Erlenmeyer flask, enough to fully cover the cork stopper. Wine samples were used as such. A sample volume of 5 ml was diluted with 5 ml of 2 % sodium hydroxide solution and mixed with 3 g of sodium chloride (previously conditioned at 180 °C) for the trace level analysis of alkyl methoxypyrazines (MDMP, IPMP, IBMP), considering the basicity of these compounds. A sample volume of 10 ml was used for analysis of MIB, TCA and GSM after adding 2 g of sodium chloride. For SIDA-based quantification, internal standards were added in concentrations of 5 ng/l (MDMP-d_3), 5 ng/l (IPMP-d_3), 5 ng/l (IBMP-d_3), 21 ng/l (MIB-d_3), 2 ng/l (TCA-d_5), 17 ng/l (GSM-d_5), each in an ethanolic solution. Automated HS-SPME (TriPlusRSH, ThermoFisher Scientific, Dreieich, Germany) was done using a 2 cm divinylbenzene/carboxen/polydimethylsiloxane fiber (DVB/CAR/PDMS, 50/30 µm; Supelco, Sigma-Aldrich). After an incubation time of 2 min, extraction was done at 50 °C for 20 min (MIB, TCA, GSM) or for 30 min (MDMP, IPMP, IBMP). Thermodesorption of the SPME fiber was done in a programmed temperature vaporizing (PTV) injector at 250 °C with a splitless time of 2 min. Fiber conditioning of 15 min at 270 °C was done prior and after analysis to avoid memory effects, utilizing a dedicated fiber conditioning station of the autosampler with N_2 as purge gas.

5.3.4 Quantitative analysis by H/C MDGC-MS-MS

Analysis of MDMP, IPMP, IBMP, MIB, TCA and GSM was done with the H/C enantio-MDGC-MS-MS system basically described previously [26]. A TraceGC ultra (ThermoFisher Scientific) was equipped with a Deans' switching device (SGE Analytical Science, Victoria, Australia) for H/C and a dual-jet modulator (ThermoFisher Scientific) using liquid CO_2 for cryo-trapping of the transferred fraction. This GC was coupled to a triple quadrupole mass spectrometer (Quantum Ultra; ThermoFisher Scientific).

The first dimension (^{1}D) analytical column was a 30 m × 0.25 mm i.d. fused silica capillary column coated with 0.25 µm of Stabilwax-MS (Restek, Bad Homburg, Germany). The analytical column in the second dimension (^{2}D) consisted of a 25 m × 0.25 mm i.d. fused silica capillary coated with a derivatized cyclodextrin stationary phase (Lipodex G®, Macherey-Nagel, Düren, Germany). Detection after the ^{1}D separation was via a flame ionization detector (FID) set to 250 °C. Carrier gas used was helium with an inlet pressure of 197 kPa and a midpoint pressure of 121 kPa (Deans' Switch). In addition to the earlier described system, a ^{1}D column backflush was incorporated thus releasing the effluent through the PTV in a high split mode. This was initiated after the last H/C simply by lowering the PTV inlet pressure to 15 kPa.

For the analysis of the methoxypyrazines, two oven temperature programs were applied. Temperature was programmed from 40 °C (2 min isothermal) at 5 °/min to 100 °C (1 min hold) and at 1.5 °/min to 109 °C (MDMP, IPMP) or to 115 °C (IBMP). Then the temperature was lowered to 60 °C at 30 °/min (5 min isothermal) before the ^{2}D-separation started by raising the temperature to 69 °C (MDMP, IPMP) or to 75 °C (IBMP) at 1 °/min and at 50 °/min to 190 °C (10 min hold). Two oven temperature programs were applied for the analysis of MIB, TCA and GSM. Temperature was programmed from 40 °C (2 min isothermal) at 10 °/min to 130 °C and at 5 °/min to 155 °C (MIB) or to 179 °C (TCA, GSM). Then the temperature was lowered to 80 °C (MIB) or to 100 °C (TCA, GSM) at 30 °/min (2 min isothermal) before the ^{2}D-GC separation started by raising the temperature to 150 °C at 5 °/min and at 30 °/min to 190 °C (5 min hold). Cut-windows were set according to peak width with an offset on both sides ensuring full transfer also for higher concentrated samples. Negligible isotope effects were observed on the polar ^{1}D stationary phase supporting the cut-window minimization strategy for SIDA-based H/C MDGC analysis as described in [27]. The cryo jet was actuated ca. 1 min before the first H/C and ca. 1 min after the oven temperature reached the corresponding ^{2}D initial temperature.

Mass spectrometric detection was performed in positive EI mode at 70 eV. Transferline and ion source temperatures were set to 190 °C and 230 °C, respectively. For selected reaction monitoring (SRM) argon (99.999% purity) was used as collision gas with a collision cell

pressure of 1.1 mTorr. Mass resolution in Q1 and Q3 was set to 0.7 amu. The total cycle time was 300 ms. The optimized MS-MS transfers and collision energies were (quantifiers are highlighted in bold) 137 → 107 (10 V), 138 → 109 (12 V), **138 → 120** (8 V) for **MDMP**, 137 → 109 (8 V), 152 → 124 (8 V), **152 → 137** (8 V) for **IPMP**, 151 → 123 (8 V), 124 → 95 (10 V), **124 → 94** (10 V) for **IBMP**, 107 → 65 (20 V), 107 → 91 (12 V), 135 → 91 (15 V), 135 → 107 (8 V), **150 → 107** (12 V) for **MIB**, 209.9 → 166.9 (20 V), 211.9 → 168.9 (20 V), **211.9 → 196.9** (15 V) for **TCA** and 182 → 112 (12 V), 182 → 97 (12 V), **112 → 97** (12 V) for **GSM**. The corresponding MS-MS transfers for the deuterated internal standards were 141.1 → 111.1 (12 V), 141.1 → 121.1 (10 V), **141.1 → 122.1** (8 V) for **MDMP-d_3**, 127.1 → 95 (8 V), 140 → 112.1 (8 V), **155.1 → 140** (8 V) for **IPMP-d_3**, 154 → 126 (8 V), 127 → 83 (8 V), **127 → 95** (10 V) for **IBMP-d_3**, 138 → 110 (10 V), 153 → 138 (10 V), **153 → 110** (12 V) for **MIB-d_3**, 217 → 170.9 (20 V), 217 → 198.9 (15 V), **215 → 168.9** (20 V) for **TCA-d_5**, 114 → 99 (10 V), 129 → 114 (10 V), **115 → 100** (10 V) for **GSM-d_5**. Instrument control and data acquisition was performed via Xcalibur software (version 2.2; Thermo Fisher Scientific).

5.3.5 Calibration and validation

Calibration for cork soak samples was carried out in deionized water. Calibration standards for wine samples were prepared in a white wine that was previously “disaromatized” by solid phase extraction using LiChrolut EN® (Merck, Darmstadt, Germany) according to standard protocols described for wine aroma analysis. Quantification was done via SIDA using the corresponding deuterated isotopologues as internal standards. Data processing was done using the TraceFinder software (version 2.1; ThermoFisher Scientific). Calibration functions were calculated with 1/X weighting. Validation data were achieved by spiking a cork soak and a white wine (Weißburgunder, Grauburgunder) with known amounts of analytes. Limits of detection (LOD) and limits of quantification (LOQ) were calculated according to DIN 32645 [28].

5.3.6 Sensory analysis

The wines sealed with off-odorous cork stoppers described before were analyzed in regard of musty off-flavors by a descriptive sensory analysis. The sensory panel consisted of 20 panelists (eleven female, nine male) experienced in sensory evaluation of wine. The wine samples were randomized and labeled with three-digit random numbers. They were served in DIN 10960 glasses (SENSUS, Schott-Zwiesel, Zwiesel, Germany) and stored at 12 °C until tasting. The tasting was performed in individual booths at a room temperature of 22 °C. The descriptive analysis was focused on the following olfactory attributes: musty,

green/vegetative, moldy/earthy and typical cork taint. Therefore, standard solutions were prepared by spiking a Riesling of the same vintage (2013, sealed with screw caps) with MDMP, IPMP, GSM and TCA, respectively. In a training session, the panelists were familiarized with the sensory attributes by rating varying intensities of the standard solutions. Information about the intensity was given after each task. The descriptive analysis was based on an unstructured line scale labeled with “not noticeable” on the left (representing a score of 0) and “strong noticeable” on the right (representing a score of 10). Data acquisition was done using FIZZ software (version 2.40, Biosystems, Courtenon, France). Analysis of variance (ANOVA) on sensory data and partial least squares (PLS) regression (correlation of sensory and analytical data) were done using XLSTAT, version 2011.1.02 (Addinsoft, Andernach, Germany) and Microsoft Excel 2003.

5.4 Results and Discussion

5.4.1 Development of analytical methods

The analysis of MDMP, IPMP, IBMP, MIB, TCA and GSM was done with HS-SPME-H/C MDGC-MS-MS methods . The calibration and validation data are depicted in Table 5-1. Calibration graphs express good linearity in the targeted concentration ranges with LODs and LOQs that allow the quantification at concentration levels in the range of the odor thresholds and even below. Validation data were about ±15 % of targeted values, besides the MDMP determination in wine matrix that was about 75 % of the targeted value. Repeatabilities for IPMP, MIB, TCA and GSM are good (RSD below 10 %) whereas MDMP and IBMP showed higher RSDs. One should note the low concentration levels used for the determination of the validation data that were adjusted to the trace level target analysis. Additional validation data with higher concentrations showed tentatively better values (data not shown). The proposed analytical approach is not ideal for high-throughput analysis as it had to be split in four separate methods (MDMP and IPMP, IBMP, MIB, GSM and TCA, respectively). A major drawback of the instrumental setup was that a one oven system had to be used. When trying to apply multiple H/Cs breakthroughs of initially cryo-trapped compounds were realized. Obviously, when an excessive time period was between the first and last H/C, with the continually rising oven temperature the cryogen was insufficient to fully trap compounds from the first cut. Therefore, this time period had to be reduced by separating into individual methods. On the other side, this reduced the risk for transferring potentially co-eluting compounds in ^{2}D that might even interfere with MS-MS detection. In a future system, either a dual oven GC or a more efficient cryo-trap could overcome this problem.

Table 5-1 Calibration and validation data of the HS-SPME-H/C MDGC-MS-MS methods

Analyte	Matrix	Calibration function (n=2)	R^2	Calibration range (ng/l)	LOD (ng/l)	LOQ (ng/l)	Validation[a] (%) (n=3)	Repeatability[a] RSD (%) (n=3)
MDMP	CS	y=0.215x-0.021	0.9851	0.2 - 10	0.3	1.1	97 ± 4	3.0
	W	y=0.223x-0.036	0.9719		0.3	1.1	73 ± 17	21
IPMP	CS	y=0.205x-0.002	0.9984	0.1 - 10	0.1	0.2	87 ± 3	3.0
	W	y=0.221x-0.019	0.9981		0.2	0.6	112 ± 1	1.2
IBMP	CS	y=0.154x+0.017	0.9737	0.1 - 10	0.1	0.4	118 ± 15	16
	W	y=0.194x-0.007	0.9700		0.2	0.6	89 ± 6	5.7
MIB	CS	y=0.062x+0.015	0.9977	0.5 - 100	0.3	1.2	106 ± 3	1.9
TCA	CS	y=0.353x+0.010	0.9980	0.05 - 10	0.05	0.2	113 ± 3	2.5
	W	y=0.357x+0.008	0.9976		0.06	0.2	94 ± 4	4.2
GSM	CS	y=0.154x+0.002	0.9982	0.25 - 50	0.2	0.7	89 ± 2	1.6
	W	y=0.143x+0.001	0.9980		0.3	0.8	86 ± 4	3.2

RSD, relative standard deviation; CS, cork soak; W, wine

[a] concentration levels were 1 – 5 ng/l

Following the standard quality control procedure applied in the cork industry, cork soaks are usually made with an aqueous solution of 10 %vol. ethanol. In the case of the methoxypyrazines and MIB analyses, the ethanol content resulted in inadequate sensitivity and a poor reproducibility. Therefore, cork soaks were made using deionized water. Wine samples were diluted (1:1) to reduce the ethanol content. However, dilution of wine samples had only a beneficial effect for the methoxypyrazines but not for MIB. The analysis of MIB by MS-MS with EI was demanding since the optimization of the MS-MS transfers was difficult due to the very common masses being mainly in the lower m/z range (<110). Furthermore, there are overlapping MS-MS transfers with MIB-d_3 due to incorporation of the deuteriums in the methyl group that is a common fragment in both isotopologues. Five suitable (but not optimal) MS-MS transfers were found and all were applied. In cases of interferences, this allowed a higher flexibility. In the analysis of wine samples a working range below 50 ng/l could not be achieved. Therefore, data for MIB analysis in wine is not listed in Table 5-1. In order to achieve a lower working range, the analysis of MIB in wine would have to be further optimized, e.g. by using another SPME fiber or chemical ionization as proposed by McCallum et al. [29]. Exemplary chromatograms with quantifier MS-MS traces are illustrated in Figure 5-1.

Compared to previous studies, e.g. Prat et al. [30] that analyzed IPMP, MIB, GSM and TCA in water-based cork soaks with a HS-SPME-GC-SIM-MS method, the results particularly for the corresponding LODs and LOQs are well below, allowing trace level analysis. A

multicomponent analysis targeting main odorants causing off-flavors in wine, again using HS-SPME-GC-SIM-MS, revealed in one case a better LOD (MIB) as in the here described study [31]. On the other hand, a better performance was achieved with the here presented methods for the remaining compounds. Considering a dedicated method for haloanisoles in wine that was based on a comparable HS-SPME-GC-MS-MS method, the LOD for TCA in the work presented here was about a factor of ten lower, possibly explainable by the additional H/C MDGC approach [32]. Additionally, in a recent study in both aqueous and alcoholic matrices (grape and wine), LOD results published by Sadoughi et al. are in the range of the data presented in this study [33].

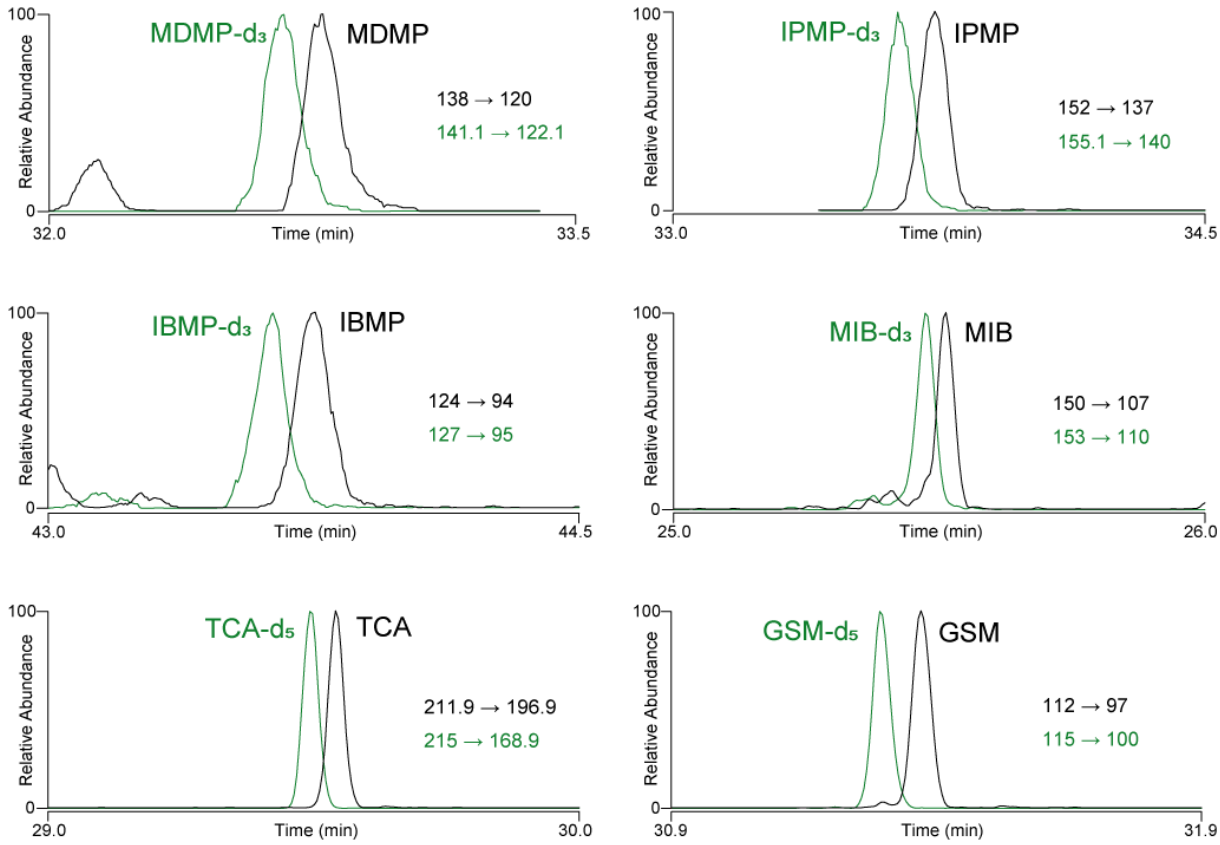


Figure 5-1 Quantifier MS-MS traces of cork off-flavor compounds after HS-SPME-H/C MDGC-MS-MS analysis of calibration samples. Concentrations were 5 ng/l (MDMP, IPMP, IBMP, GSM) and 2 ng/l (TCA) in wine, and 5 ng/l (MIB) in water. Experimental conditions and concentrations of deuterated internal standards were as described in chapter 5.3

With regard to previous studies on HS-SPME-MDGC methods [34] on trace level analysis of wine aroma compounds, the newly added ^{1}D column backflush can be seen as a considerable improvement. The long-term system stability was greatly enhanced as deduced from stable calibration curves and quality control samples monitored for an extended analysis period. This is an important aspect for routine analysis particularly in complex matrices (wine). Technically, a ^{1}D column backflush can be easily achieved when using any

midpoint pressure based H/C MDGC system with an electronic pressure control for the ^{1}D inlet.

5.4.2 Analysis of cork and wine samples

Individual cork stoppers described with intensive off-odors were analyzed by HS-SPME-H/C MDGC-MS-MS to determine the presence and concentration of the targeted off-flavor compounds. The results are presented in Table 5-2. The compounds detected correspond well with the odorous description of the cork stoppers. For instance, IBMP and IPMP, characteristic flavor compounds of bell peppers and peas, were detected in cork stoppers described with such green notes. GSM and MIB appeared often together in cork samples with moldy, cellar-like, camphoraceous attributes but each in a concentration range below the odor threshold in wine. MDMP was found mainly in dusty-musty, nutty-like cork stoppers with elevated concentrations up to 40 ng/l in the corresponding soaks. Cork stoppers described with a typical TCA taint among their sensory attributes contained high concentrations of TCA (up to about 300 ng/l) whereas the others showed TCA in a non-

Table 5-2 Concentrations of corky off-flavor compounds in aqueous cork soaks made of individual cork stoppers with an intensive deviant odor (n=1)

Odor description of cork stopper	MDMP (ng/l)	IPMP (ng/l)	IBMP (ng/l)	TCA (ng/l)	GSM (ng/l)	MIB (ng/l)
Dusty-musty, moldy	22	<LOD	<LOD	<LOQ	<LOD	<LOD
Dusty-musty, moldy	40	<LOD	<LOD	<LOD	<LOD	<LOD
Woody, earthy, musty, green-nutty-like	21	<LOD	<LOD	<LOQ	<LOQ	<LOD
Musty, earthy, wet-cardboard, nutty (MDMP-like)	2.0	<LOD	<LOD	0.22	<LOD	<LOD
Musty, earthy, wet-cardboard, nutty (MDMP-like)	5.8	<LOD	<LOD	<LOQ	<LOD	<LOD
Earthy, cellar-like, moldy, camphoraceous (MIB-like)	<LOQ	<LOD	<LOD	<LOQ	<LOQ	4.8
Earthy, cellar-like, moldy, MIB-/GSM-like, wet-cellar	<LOD	<LOD	<LOD	<LOD	4.1	7.2
Earthy, wet-cellar, moldy, damp, mineral, moldy	<LOD	<LOD	<LOD	<LOQ	1.0	15
Mildewed, musty, cabinet, cellar-like, TCA-like	<LOD	<LOD	<LOD	69	<LOD	<LOD
Typical TCA	<LOD	<LOD	<LOD	38	<LOD	<LOD
Typical TCA	<LOQ	<LOD	<LOD	56	<LOD	<LOD
Typical TCA	<LOQ	<LOD	<LOD	298	<LOQ	<LOD
Green, vegetative, bell pepper, pea-like	<LOD	<LOD	6.7	<LOQ	<LOD	<LOD
Green, vegetative, bell pepper, pea, earthy	<LOD	1.2	<LOD	<LOQ	<LOD	<LOD
Green, vegetative, bell pepper, pea, earthy	<LOD	3.2	<LOD	<LOQ	<LOD	<LOD
Sensorially inconspicuous	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOD
Sensorially inconspicuous	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Sensorially inconspicuous	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

relevant concentration range (<0.2 ng/l). Control samples without sensory peculiarities had for all target compounds concentrations below LOD or LOQ. Compared to the olfactometric (GC-O) results from previous work [7] where TCA and MDMP were found in all group types of off-odorous cork stoppers, here, often only one target compound was detected per individual cork stopper. The fact that TCA is not detectable in every off-odorous cork stopper confirms that TCA is not the only marker for such off-odors. Such cork stoppers would not be considered conspicuous by a quality control monitoring TCA alone.

Table 5-3 Mean concentrations (n=3) of corky off-flavor compounds in wines and in cork soaks made of the corresponding cork stoppers. Cork stoppers were described with an off-odor and grouped before bottling. Three wine bottles per cork group were stored for 13 month before HS-SPME-H/C MDGC-MS-MS analysis (concentration range of the three bottles is given in brackets)

Cork group	Sample type	MDMP (ng/l)	IPMP (ng/l)	IBMP (ng/l)	TCA (ng/l)	GSM (ng/l)	MIB (ng/l)
M	W	<LOD	<LOD	<LOD	62 (2.9–156)	<LOQ	-
	CS	<LOD	<LOD	<LOD	37 (13–81)	<LOD	<LOD
ME	W	2.2 (<LOQ–5.2)	<LOD	<LOD	4.3 (<LOQ–12)	0.9 (<LOQ–1.0)	-
	CS	17 (4.4–40)	<LOD	<LOD	3.3 (<LOQ–9.6)	0.8 (<LOQ–1.2)	<LOD
T	W	<LOD	<LOD	<LOD	24 (8.8–46)	<LOQ	-
	CS	<LOD	<LOD	<LOD	32 (13–62)	<LOD	<LOD
GR	W	<LOD	1.6 (<LOD–2.6)	<LOD	0.3 (<LOQ–0.8)	<LOQ	-
	CS	2.4 (<LOD–6.6)	3.2 (<LOD–5.1)	<LOD	0.2 (<LOQ–0.4)	<LOQ	<LOD
BP	W	<LOD	<LOD	1.5 (<LOQ–4.0)	<LOD	<LOQ	-
	CS	<LOD	<LOD	5.3 (3.8–7.6)	<LOD	<LOD	<LOD
control	W	<LOD	<LOD	<LOQ	0.7 (<LOD–1.8)	0.8 (<LOQ–1.1)	-
	CS	<LOD	<LOD	<LOD	1.1 (<LOD–3.2)	<LOD	<LOD

CS, cork soak; W, wine

After a storage period of 13 month, the wines from the migration study of atypical off-flavor compounds from cork stopper into the wine were analyzed by HS-SPME-H/C MDGC-MS-MS. The results of the chemical analysis are presented in Table 5-3. The wines sealed with cork stoppers of the groups musty (M) and typical TCA (T) showed high concentrations of

TCA as well as the cork soaks made of the corresponding cork stoppers. Similar results could be observed for IPMP and IBMP in wines sealed with cork stoppers of the groups green (GR) and bell pepper (BP). Wines sealed with moldy/earthy (ME) cork stoppers showed detectable amounts of MDMP and TCA in concentrations below LOQ and up to 12 ng/l. Since three individual cork stoppers were used for the migration study such a variance of the analytical data for one off-odorous group (ME) clearly indicates the uniqueness of each individual cork stopper. The aforementioned analytes were also found in the corresponding cork stopper soaks. MIB was never detected in any cork soaks. The concentrations of MDMP, IPMP, IBMP and TCA in the analyzed wines were often in the range of their odor thresholds, indicating a possible variation of the sensory perception of such a wine. GSM concentrations were always below its odor threshold in wine. Considering the low GSM concentrations in individual cork stoppers (Table 5-2), this indicates an only minor role of GSM for the atypical cork taint.

In addition to the model studies, real-life samples with a customer complaint were analyzed. Several bottles of a 2012 Chardonnay wine sealed with natural cork stoppers were rejected because of cork taint. The TCA concentrations were found to be negligible, thus below its odor threshold value of 2-5 ng/l [35,36]. However, three bottles had MDMP concentrations of 2.6, 3.8 and 128 ng/l MDMP. The cork soak of the corresponding cork stopper of the latter bottle also showed a high concentration of 99 ng/l MDMP. With an odor threshold of MDMP in white wine of 2 ng/l [14] a customer rejection for such bottles can easily be understood and traced back to the presence of MDMP as off-flavor compound; however, sensory description being deviant from the typical TCA cork taint.

In conclusion, a migration of the methoxypyrazines MDMP, IPMP and IBMP could be demonstrated based on the presented results. Whereas migration of TCA from cork stoppers into wine has been studied in detail [37-39], little work has been published on methoxypyrazines. Capone et al. found a low affinity of natural cork stoppers for IBMP in contrast to their high affinity for TCA [40].

5.4.3 Sensory analysis

The results of the descriptive analysis and ANOVA calculation are presented in Figure 5-2. In general, a sensory deviation of the wines by using off-odorous cork stoppers was observed since the four descriptors varied significantly among the cork groups. In particular, the cork stoppers of the groups T, ME and M had an effect on the attribute typical cork taint (with the highest score of almost 4 for T) compared to the control that was normalized to one. The cork stopper of the group GR had a high effect on the green attribute (almost 2.5). The attributes cellar/earthy and musty were affected significantly by the cork groups M and T

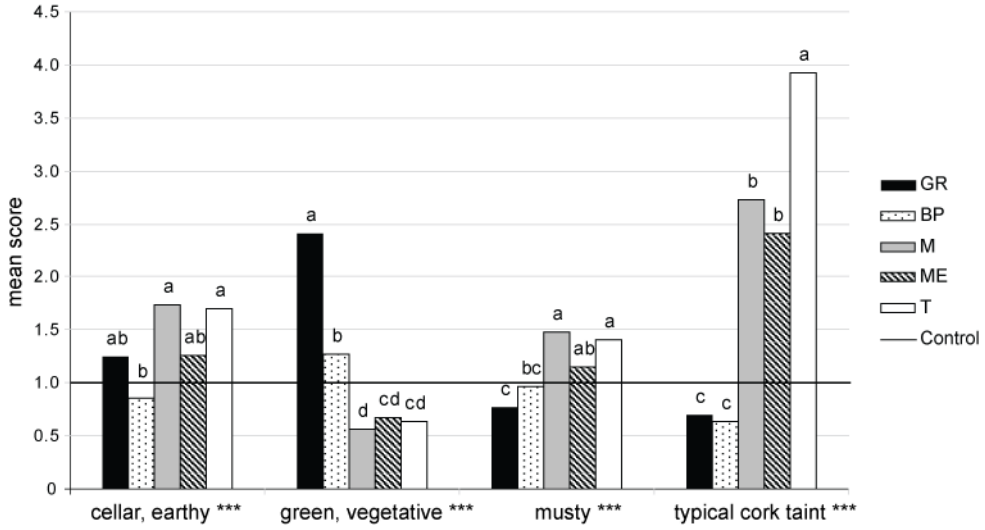


Figure 5-2 Sensory attribute mean scores from descriptive analysis of wines sealed with off-odorous cork stoppers. Groupings of the categories after pairwise comparisons (Fisher's LSD test) are indicated with letters (Post hoc results of control: cellar/earthy *b*, green/vegetative *bc*, musty *bc*, typical cork taint *c*). The difference between categories with the same letter is not significant (levels of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

compared to the control. Since the wines of the groups T and M contained high amounts of TCA it is not remarkable that a considerably typical cork taint was perceived and they were furthermore described with the similar attributes cellar/earthy and musty. Interestingly, the wines of the group GR contained IPMP in a concentration range of its odor threshold; thus the green perception of the wine originates probably from the migration of IPMP from the cork stopper into the wine. A migration of IBMP into the wines of the group BP can be deduced from the chemical analysis data as described in the previous section. On the other hand, an alteration of the sensory perception in comparison with the control could not be observed. Considering the three wines of group BP individually (Table 5-4), one wine was clearly described with green attributes and this was also the one with the highest concentration of IBMP at 4 ng/l. The wines of the group ME were in the mean described with a typical cork taint, but only one of the three wines contained TCA in a relevant concentration range above odor threshold (Table 5-4) whereas the others contained TCA below odor threshold. However, in one of these wines MDMP was detected in a concentration of about 5 ng/l, possibly explaining the perceived corkiness. In one wine, neither of the analyzed compounds was detected in a concentration range above the odor thresholds and also sensory description was as not with any of the off-flavor attributes. This emphasizes the uniqueness of each individual cork stopper. Thus, a high variation of chemical composition and sensory description has to be expected even when cork stoppers were categorized in sensory groups.

The before-discussed correlations of sensory data and chemical analysis data on the migration study is further supported by a PLS regression (Figure 5-3). In general, the

Table 5-4 Analytical data for individual bottles from migration experiment (groups BP and ME)

	Bottle 1	**Bottle 2**	**Bottle 3**
Group BP			
IBMP	<LOQ	<LOQ	4.0 ng/l
Group ME			
MDMP	5.2 ng/l	<LOQ	<LOQ
TCA	<LOQ	12.2 ng/l	0.3 ng/l

concentrations of the targeted off-flavor compounds in the wines (W) and the cork soaks (CS) of their corresponding cork stoppers correlated well with each other. Since the loadings of the observation GR and the analytical data IPMP (W) and IPMP (CS) match perfectly, the wine of the group GR showed a very high correlation with the IPMP concentration. They also correlated well with the sensory attribute green/vegetative. The wines of the groups M and T correlated with the TCA concentrations and the sensory attributes typical cork taint, cellar/earthy and musty. A notable fact was that the loadings of these three attributes are very close together and in the opposite direction of the variable green/vegetative. This could be explained as the sensory standards of the attributes cellar/earthy and musty as well as typical cork taint were very similar in their overall odorous perception. Thus, they were difficult to differentiate in the samples by the panelists. The observation ME could be mostly explained by the MDMP concentrations in wine and cork soak. The samples of the group BP showed a high correlation to the IBMP concentrations but could not be differentiated from the control. Also, both show a highly negative correlation with all the sensory attributes, especially to the musty, earthy, cork taint notes meaning these observations could not be explained by any of the sensory attributes chosen. The variables GSM and MDMP appear to contribute to a minor extend to the explanation of the observations due to their vector length being short. This is also supported by the concentrations of GSM being below its odor threshold value. However, the role of MDMP seems to be more important than estimated from vector length as the additional samples from a customer complaint showed high correlation of MDMP concentration with perceived cork taint.

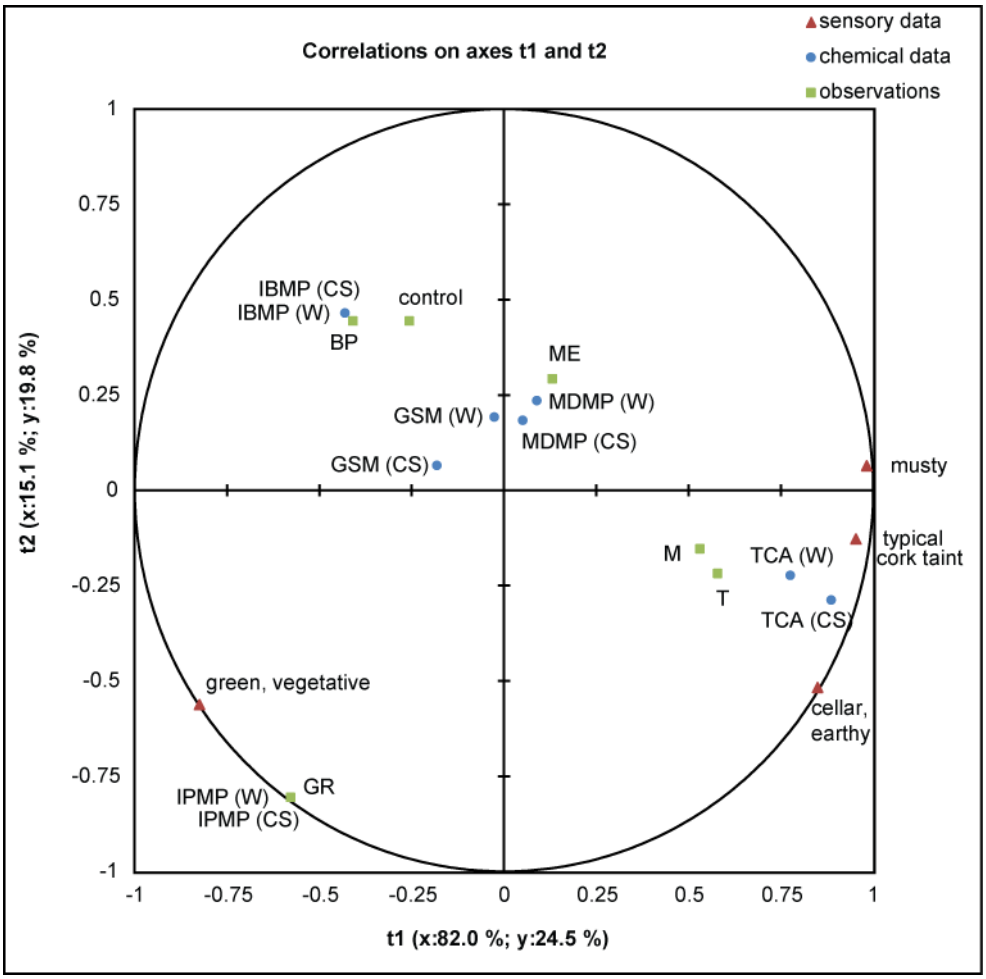


Figure 5-3 PLS regression of sensory and chemical analysis data (*CS*: cork soak, *W*: wine) of wines sealed with off-odorous cork stoppers (observations)

5.5 Conclusion

A HS-SPME-H/C MDGC-MS-MS approach was developed for trace level analysis of MDMP, IPMP, IBMP, TCA and GSM in wine and cork soaks below odor thresholds. Detection limits for MIB were reasonable for cork soaks but not sufficient in wine matrix. System stability was greatly enhanced by implementing a ^{1}D column backflush utilizing midpoint pressure of the Deans' switch device and pressure programming of the PTV inlet. A drawback of the described analytical system has to be seen in the splitting into individual sub-methods. Since this was due to the insufficient cryo-trapping, future improvements could either incorporate a more efficient cryo-trapping device or a dual oven system allowing individual temperature programming.

Analyzing individual cork stoppers which differed in their sensory off-odor description revealed elevated concentrations of off-odor compounds correlating with the corresponding sensory description. Still, individual cork stoppers have to be considered as unique with respect to the combination and concentration of cork off-flavor compounds. The additional

migration study supported the transport of off-flavor compounds from affected cork stoppers into the corresponding wine after an appropriate storage period. The corresponding concentrations in wine and cork soak and the sensory data could be correlated by multivariate statistics. In contrast to some previous studies, in this study MIB and GSM seem to play only a minor role in atypical cork taint. Supplementary to the usually targeted haloanisoles for quality control of the typical cork taint, MDMP should also be monitored especially with respect to the atypical cork taint.

5.6 References

1. Buser HR, Zanier C, Tanner H (1982) Identification of 2,4,6-trichloroanisole as a potent compound causing cork taint in wine. Journal of Agricultural and Food Chemistry 30 (2):359-362.

2. Amon JM, Vandepeer JM, Simpson RF (1989) Compounds responsible for cork taint in wine. The Australian and New Zealand Wine Industry Journal 4 (1):62-69.

3. Prat C, Trias R, Cullere L, Escudero A, Antico E, Baneras L (2009) Off-Odor Compounds Produced in Cork by Isolated Bacteria and Fungi: A Gas Chromatography-Mass Spectrometry and Gas Chromatography-Olfactometry Study. Journal of Agricultural and Food Chemistry 57 (16):7473-7479.

4. Simpson RF (1990) Cork Taint in Wine: A Review of the Causes. The Australian and New Zealand Wine Industry Journal 5:286-296.

5. Chatonnet P, Guimberteau G, Dubourdieu D, Boidron J (1994) Nature et origine des odeurs de “moisi” dans les caves. Incidences sur la contamination des vins. Journal International des Sciences de la Vigne et du Vin 28:131-151.

6. Sefton MA, Simpson RF (2005) Compounds causing cork taint and the factors affecting their transfer from natural cork closures to wine – a review. Australian Journal of Grape and Wine Research 11 (2):226-240.

7. Slabizki P, Fischer C, Legrum C, Schmarr H-G (2015) Characterization of Atypical Off-Flavor Compounds in Natural Cork Stoppers by Multidimensional Gas Chromatographic Techniques. Journal of Agricultural and Food Chemistry 63 (35):7840-7848.

8. Lovell RT, Broce D (1985) Cause of musty flavor in pond-cultured penaeid shrimp. Aquaculture 50 (1-2):169-174.

9. Watson SB, Brownlee B, Satchwill T, Hargesheimer EE (2000) Quantitative analysis of trace levels of geosmin and MIB in source and drinking water using headspace SPME. Water Research 34 (10):2818-2828.

10. Gerber NN, Lechevalier HA (1965) Geosmin, a earthy-smelling substance isolated from actinomycetes. Applied Microbiology 13 (6):935-938.

11. Dickschat JS, Martens T, Brinkhoff T, Simon M, Schulz S (2005) Volatiles released by a Streptomyces species isolated from the North Sea. Chemistry & Biodiversity 2 (7):837-865.

12. Hsieh W-H, Hung W-N, Wang G-S, Hsieh S-T, Lin T-F (2012) Effect of pH on the analysis of 2-MIB and geosmin in Water. Water, Air, and Soil Pollution 223 (2):715-721.

13. Darriet P, Pons M, Lamy S, Dubourdieu D (2000) Identification and Quantification of Geosmin, an Earthy Odorant Contaminating Wines. Journal of Agricultural and Food Chemistry 48 (10):4835-4838.

14. Simpson RF, Capone DL, Sefton MA (2004) Isolation and Identification of 2-Methoxy-3,5-dimethylpyrazine, a Potent Musty Compound from Wine Corks. Journal of Agricultural and Food Chemistry 52 (17):5425-5430.

15. Ventura F, Quintana J, Gomez M, Velo-Cid M (2010) Identification of alkyl-methoxypyrazines as the malodorous compounds in water supplies from northwest spain. Bulletin of Environmental Contamination and Toxicology 85 (2):160-164.

16. Mottram DS, Patterson RLS, Warrilow E (1984) 2,6-Dimethyl-3-methoxypyrazine: a microbiologically-produced compound with an obnoxious musty odor. Chemistry & Industry (London, United Kingdom) (12):448-449.

17. Chatonnet P, Fleury A, Boutou S (2010) Origin and incidence of 2-methoxy-3,5-dimethylpyrazine, a compound with a “fungal” and “corky” aroma found in cork stoppers and oak chips in contact with wines. Journal of Agricultural and Food Chemistry 58 (23):12481-12490.

18. Murray KE, Whitfield FB (1975) Occurrence of 3-alkyl-2-methoxypyrazines in raw vegetables. Journal of the Science of Food and Agriculture 26 (7):973-986.

19. Lacey MJ, Allen MS, Harris RLN, Brown WV (1991) Methoxypyrazines in Sauvignon blanc grapes and wines. American Journal of Enology and Viticulture 42 (2):103-108.

20. Allen MS, Lacey MJ, Harris RLN, Brown WV (1991) Contribution of Methoxypyrazines to Sauvignon blanc Wine Aroma. American Journal of Enology and Viticulture 42 (2):109-112.

21. Pickering G, Lin J, Riesen R, Reynolds A, Brindle I, Soleas G (2004) Influence of *Harmonia axyridis* on the Sensory Properties of White and Red Wine. American Journal of Enology and Viticulture 55 (2):153-159.

22. Allen MS, Lacey MJ, Boyd SJ (1995) Methoxypyrazines in Red Wines: Occurrence of 2-Methoxy-3-(1-methylethyl)pyrazine. Journal of Agricultural and Food Chemistry 43 (3):769-772.

23. Schmarr HG, Sang W, Ganß S, Koschinski S, Meusinger R (2011) New insights into the synthesis and characterization of 2-methoxy-3-alkylpyrazines and their deuterated isotopologues. Journal of Labelled Compounds & Radiopharmaceuticals 54 (8):438-440.

24. Schmarr H-G, Koschinski S, Sang W, Slabizki P (2012) Trace level analysis of corky off-flavor compounds: Development of a new analytical method based on solid phase extraction and analysis by multidimensional gas chromatography with mass spectrometric detection. Journal of Chromatography, A 1226 (0):96-102.

25. Slabizki P, Legrum C, Meusinger R, Schmarr H-G (2014) Characterization and analysis of structural isomers of dimethyl methoxypyrazines in cork stoppers and ladybugs (*Harmonia axyridis* and *Coccinella septempunctata*). Analytical and Bioanalytical Chemistry 406 (25):6429-6439.

26. Legrum C, Slabizki P, Schmarr H-G (2015) Enantiodifferentiation of 3-*sec*-butyl-2-methoxypyrazine in different species using multidimensional and comprehensive two-dimensional gas chromatographic approaches. Analytical and Bioanalytical Chemistry 407 (1):253-263.

27. Schmarr H-G, Slabizki P, Legrum C (2013) Optimization in multidimensional gas chromatography applying quantitative analysis via a stable isotope dilution assay. Analytical and Bioanalytical Chemistry 405 (20):6589-6593.

28. DIN 32645 - Chemische Analytik - Nachweis-, Erfassungs- und Bestimmungsgrenze unter Wiederholbedingungen - Begriffe, Verfahren, Auswertung (2008). Beuth, Berlin.

29. McCallum R, Pendleton P, Schumann R, Trinh MU (1998) Determination of geosmin and 2-methylisoborneol in water using solid-phase microextraction and gas chromatography-chemical ionisation/electron impact ionisation-ion-trap mass spectrometry. Analyst 123 (10):2155-2160.

30. Prat C, Baneras L, Antico E (2008) Screening of musty-earthy compounds from tainted cork using water-based soaks followed by headspace solid-phase microextraction and gas chromatography-mass spectrometry. European Food Research and Technology 227 (4):1085-1090.

31. Boutou S, Chatonnet P (2007) Rapid headspace solid-phase microextraction/gas chromatographic/mass spectrometric assay for the quantitative determination of some

of the main odorants causing off-flavours in wine. Journal of Chromatography, A 1141 (1):1-9.

32. Hjelmeland AK, Collins TS, Miles JL, Wylie PL, Mitchell AE, Ebeler SE (2012) High-Throughput, Sub ng/L Analysis of Haloanisoles in Wines Using HS-SPME with GC-Triple Quadrupole MS. American Journal of Enology and Viticulture 63 (4):494-499.

33. Sadoughi N, Schmidtke LM, Antalick G, Blackman JW, Steel CC (2015) Gas Chromatography–Mass Spectrometry Method Optimized Using Response Surface Modeling for the Quantitation of Fungal Off-Flavors in Grapes and Wine. Journal of Agricultural and Food Chemistry 63 (11):2877-2885.

34. Legrum C, Gracia-Moreno E, Lopez R, Potouridis T, Langen J, Slabizki P, Weiand J, Schmarr H-G (2014) Quantitative analysis of 3-alkyl-2-methoxypyrazines in German Sauvignon blanc wines by MDGC–MS or MDGC–MS/MS for viticultural and enological studies. European Food Research and Technology 239 (4):549-558.

35. Fischer C (1997) Neue und kostengünstige Analyse. Dem Korkton auf der Spur. Das Deutsche Weinmagazin 16 (17):30-33.

36. Prescott J, Norris L, Kunst M, Kim S (2005) Estimating a “consumer rejection threshold” for cork taint in white wine. Food Quality and Preference 16 (4):345-349.

37. Capone DL, Skouroumounis GK, Barker DA, McLean HJ, Pollnitz AP, Sefton MA (1999) Absorption of chloroanisoles from wine by corks and by other materials. Australian Journal of Grape and Wine Research 5 (3):91-98.

38. Fischer C (2000) Die Migration von 2,4,6-Trichloranisol aus dem Korken in den Wein - Kinetik und sensorische Relevanz. Dissertation, University Kaiserslauern, Fachbereich Chemie, Kaiserslautern, Germany

39. Juanola R, Subira D, Salvado V, Garcia Regueiro JA, Antico E (2005) Migration of 2,4,6-trichloroanisole from cork stoppers to wine. European Food Research and Technology 220 (3-4):347-352.

40. Capone D, Sefton M, Pretorius I, Høj P (2003) Flavour scalping by wine bottle closures—the winemaking continues post vineyard and winery. The Australian and New Zealand Wine Industry Journal 18 (5):16-20.

6 Concluding remarks

Analysis of typical cork off-flavor compounds

In general, reliable trace level analysis of aroma compounds in a complex matrix like wine is a demanding task due to co-elution problems that are often encountered. Thus, the analysis of the haloanisoles responsible for the typical cork taint based on HS-SPME and ^{1}D-GC-ECD failed in wine at the low ng/l level. For this purpose, trace level analysis in wine benefits from the reduction of matrix through a proper sample preparation, an increased separation efficiency (e.g. using MDGC), and a specific detection. Hence, the application of a H/C MDGC-MS method and a preceding sample clean-up by SPE was finally able to overcome the co-elution problems. As this method was laborious and clearly not fitted for routine analysis, it was substituted with an automated HS-SPME method in combination with a H/C MDGC-ECD setup. The additional separation achieved by H/C MDGC and the halogen sensitive ECD allowed low LODs at the sub-ng/l level in wine and hence a quantification below the odor thresholds that may be crucial in customer conflict situations. Due to the automation of HS-SPME and the consequently minor sample preparation steps the analytical method proved to be more suitable for routine application. Furthermore, a reliable quantification benefitted from using highly deuterated isotopologues as internal standards. Though, the chromatographic conditions, particularly the stationary phase, have to be selected with care allowing good resolution of the isotopologues in a non-MS SIDA approach. Still, co-elutions in analysis of slightly deviant matrices are possible and would reveal the flaw of such a non-MS based detection, as ECD response and retention time are the only means for compound identification.

Characterization and analysis of structural isomers of dimethyl methoxypyrazines

In a study regarding the characterization of off-flavor compounds, dimethyl methoxypyrazines that have been described as off-odor compounds in wine related with cork stoppers (MDMP) and with ladybugs (DMMP) were analyzed. However, their unequivocal identification was critical since the two structural isomers showed non-distinguishable mass spectra and almost identical retention properties on common stationary phases used for GC. The unambiguous assignment could finally be achieved by homo- and heteronuclear NMR correlation experiments. In GC analysis the unambiguous differentiation of the dimethyl methoxypyrazines presupposed fully characterized reference substances and a sufficient chromatographic separation that could be finally achieved on a stationary phase based on octakis-(2,3-di-*O*-pentyl-6-*O*-methyl)-γ-cyclodextrin. By applying H/C MDGC-MS-MS the presence of MDMP could be confirmed as a musty off-odor compound in tainted cork stoppers. However, DMMP could not be identified in the analyzed ladybug species (*H. axyridis*, *C. septempunctata*). Instead, the structural isomer MDMP was identified in these

ladybug species that has not been described yet. This example of a critical identification of structurally similar compounds affirms an earlier statement of Molyneux and Schieberle on how compound identification should be conducted particularly in the complex field of aroma analysis [1]. A mass spectrometric identification based solely on comparison with commercial databases is not sufficient to depend upon. At least retention indices on stationary phases of different polarities have to be additionally considered including the possible structural isomers as well as the utilization of structurally verified reference substances for comparison.

In conclusion, the analysis of such structurally related compounds is a demonstrative example for the importance of chromatographic separation, as mass spectrometric data by itself could not guarantee the unequivocal identification. Even the application of high-resolution-MS cannot be of any use in the case of structural isomers with an identical molecular weight and a non-distinguishable MS-fragmentation pattern.

Characterization of atypical cork off-flavor compounds

Regarding the atypical cork taint, the responsible compounds were identified by comparing off-odorous cork stoppers with sensorially inconspicuous cork stoppers using several multidimensional GC methods including a H/C MDGC-O application. Here, the olfactometric approach benefitted from using H/C MDGC thus a more reliable detection of odor events was achieved due to the reduction of matrix and the higher resolution of odor events. Although the investigated cork stoppers had been described with an off-odor different from the typical cork taint, TCA was detected in all sub-groups of the off-odorous cork stoppers. Therefore, TCA appears to be still a good marker for cork taint in general. However, another compound perceived in all off-odorous cork samples was the hitherto not often described MDMP. This compound seems to be a good marker for the atypical cork taint and analytical monitoring should be extended by MDMP. Compounds like GSM and MIB were mainly perceived in the specific sensory sub-groups moldy and cellarlike, as well as IPMP and IBMP in the sensory sub-group described as green, probably contributing with their potent individual flavor notes. 1-Octen-3-one and guaiacol were present in all cork samples and showed no distinct difference to the control. Hence, their contribution to the atypical cork taint seems to play only a minor role. Nevertheless, potential synergism effects cannot be excluded. Besides TCA, other chlorinated compounds were detected in off-odorous cork stoppers, e.g. chloroveratroles (3,5-dichloroveratrole and 3,4,6-trichloroveratrole) that have not yet been described as a constituent of off-odorous cork stoppers. Since the occurrence of chlorinated compounds and their contribution to today's cork taint situation has been relativized due to the discontinuation of chlorine bleaching in the cork industry, the question about their origin arises. Such chlorinated compounds like chloroveratroles probably originate in the chlorination of lignin followed by microbial degradation [2]. However, the source of chlorine is

unknown. Further origins could be environmental influences due to the usage of chlorinated pesticides or biochemical synthesis by microorganisms.

In general, a mixture of different off-flavor compounds were detected in the off-odorous cork stoppers that are in good agreement with the identified compounds in earlier studies [3,4]. As the most off-flavor compounds on cork stoppers are probably of microbial origin, it is only to be expected that the diversity of microorganisms found on the surface of corks generate their individual mix of substances [4]. Depending on the composition of the mixture of compounds there are small deviations in the sensory perception. Furthermore, synergisms between several off-flavor compounds are possible and should be further investigated in future studies.

Analysis of atypical cork off-flavor compounds

In order to quantify the most important cork off-flavor compounds MDMP, IPMP, IBMP, GSM, MIB, and TCA, an analytical approach based on HS-SPME-H/C MDGC-MS-MS was developed for trace level analysis in wine and cork soaks. Detection limits were below the compounds' odor threshold and allowed reliable quantification in a relevant concentration range, except for the analysis of MIB in wine samples. Therefore, the analysis of MIB would have to be further optimized, e.g. by using chemical ionization. Previous studies about the analysis of musty off-flavor compounds in wine and cork soaks that were mostly based on HS-SPME-GC-SIM-MS revealed higher or in one case comparable detection limits [5-7]. Actually, in some cases they give better LODs for MIB in wine. A major drawback of the instrumental setup was the one oven system that had to be used. Therefore, the splitting into individual sub-methods was necessary due to insufficient cryo-trapping. Future improvements could either incorporate a more efficient cryo-trapping device or a dual oven system allowing individual temperature programming. On the other side reducing the number of heart-cuts reduced the risk of transferring potential co-eluting compounds. System stability was greatly enhanced by implementing a ^{1}D column backflush utilizing midpoint pressure of the Deans' switch device and pressure programming of the PTV inlet.

Analyzing individual cork stoppers which differed in their sensory off-odor description revealed elevated concentrations of off-odor compounds correlating with the corresponding sensory description and confirmed the observations in the olfactometric approach described above. For instance, IPMP and IBMP, characteristic flavor compounds of bell peppers and peas, were detected in cork stoppers with such green notes. Still, individual cork stoppers have to be considered as unique with respect to the combination and concentration of cork off-flavor compounds. Compared to the previous olfactometric results, MDMP and TCA were not detected in each type of the off-odorous cork stoppers. Therefore, TCA does not seem to

be the sole marker for such off-odors in cork stoppers and the present routine quality control in the cork industry, where only TCA is monitored, should be extended.

In an additional migration experiment, the transport of off-flavor compounds from affected cork stoppers into the corresponding wine and an associated sensory alteration of the wine could be shown after an appropriate storage period and was supported by multivariate statistics. In the case of alkyl methoxypyrazines there are only few publications about a possible migration from cork stoppers into wine as reported here. Particularly for IPMP and IBMP, the migration and thus a sensory alteration of the wine in a practice-oriented experiment has not been described yet. The off-flavor compounds GSM and MIB seem to play only a minor role for the atypical cork taint, since the concentrations found in wine and cork soaks were below their odor thresholds in wine. Supplementary analyses of rejected wines by customers due to cork taint deviant from the typical TCA cork taint showed a high correlation of MDMP concentration with the perceived cork taint in these wines. Furthermore, based on preliminary sensory tests, MDMP is rather difficult to describe in wine and seems to be associated with the perception of a reduced fruitiness in the wine that is reported to be characteristic for the atypical cork taint. This observation should be further investigated in future studies involving recombination experiments and descriptive sensory analysis considering the potentially altered perception of fruity attributes.

Outlook

Since MDMP appears to be the most important compound when it comes to the atypical cork taint, quality control in cork and wine industry that so far has been limited to the analysis of haloanisoles should be extended by MDMP. Therefore, in order to be able to correlate MDMP concentrations in cork soaks with the potential extraction in wine and also in order to be able to set a critical value for the evaluation of cork stoppers, further investigations should be made. Similarly to studies with TCA, issues concerning the distribution of MDMP on the surface of cork stoppers, the migration within cork stoppers, the suitability of extraction media, the affinity for cork stoppers and the equilibrium conditions should be elucidated.

Besides the analysis for quality control purposes, the origin of contamination with off-flavor compounds has to be elucidated and prevention strategies have to be considered. Since most compounds seem to be of microbial origin, microbial growth on cork stoppers should be avoided. In this respect, it is already standard procedure to closely monitor the moisture content in cork stoppers and prevent or sort out moldy cork barks. Furthermore, storage conditions have to be monitored with a focus on microbial contamination or the potential contamination of packaging material in e.g. transport situations. Storage near the ground should be minimized to avoid contamination with soil bacteria that are probably able to produce MDMP among other off-flavor compounds. Additionally, sterilization treatments that

actually have already been discussed and applied in some cases should be considered [8]. Cellar-derived cork taint could be prevented by avoiding wood preservatives and flame retardants on the basis of chlorine or bromine as well as cleaning products and sanitizers containing chlorine. Curative strategies include venting the affected rooms and periodical exchanging plastic parts in the cellar surroundings that are good sorbents for off-flavor compounds or up to an entire renovation of the cellar [9].

References

1. Molyneux RJ, Schieberle P (2007) Compound identification: A Journal of Agricultural and Food Chemistry perspective. Journal of Agricultural and Food Chemistry 55 (12):4625-4629.
2. Neilson AH, Allard AS, Hynning PA, Remberger M, Landner L (1983) Bacterial methylation of chlorinated phenols and guaiacols: formation of veratroles from guaiacols and high-molecular-weight chlorinated lignin. Applied and Environmental Microbiology 45 (3):774-783.
3. Amon JM, Vandepeer JM, Simpson RF (1989) Compounds responsible for cork taint in wine. The Australian and New Zealand Wine Industry Journal 4 (1):62-69.
4. Prat C, Trias R, Cullere L, Escudero A, Antico E, Baneras L (2009) Off-Odor Compounds Produced in Cork by Isolated Bacteria and Fungi: A Gas Chromatography-Mass Spectrometry and Gas Chromatography-Olfactometry Study. Journal of Agricultural and Food Chemistry 57 (16):7473-7479.
5. Boutou S, Chatonnet P (2007) Rapid headspace solid-phase microextraction/gas chromatographic/mass spectrometric assay for the quantitative determination of some of the main odorants causing off-flavours in wine. Journal of Chromatography, A 1141 (1):1-9.
6. Prat C, Baneras L, Antico E (2008) Screening of musty-earthy compounds from tainted cork using water-based soaks followed by headspace solid-phase microextraction and gas chromatography-mass spectrometry. European Food Research and Technology 227 (4):1085-1090.
7. Sadoughi N, Schmidtke LM, Antalick G, Blackman JW, Steel CC (2015) Gas Chromatography–Mass Spectrometry Method Optimized Using Response Surface Modeling for the Quantitation of Fungal Off-Flavors in Grapes and Wine. Journal of Agricultural and Food Chemistry 63 (11):2877-2885.

8. Vlachos P, Kampioti A, Kornaros M, Lyberatos G (2007) Development and evaluation of alternative processes for sterilization and deodorization of cork barks and natural cork stoppers. European Food Research and Technology 225 (5-6):653-663.

9. Schäfer V (2013) Untersuchungen zum Auftreten, der Herkunft, Behandlung und Vermeidung sensorisch wirksamer, dumpf-muffiger Fehltöne im Wein, die durch Trauben, Weinbereitung, Schönung, Weinbehandlung und Abfüllung verursacht werden können. Dissertation, Justus Liebig-University, Gießen

7 Appendix

7.1 List of Abbreviations

%vol.	% by volume
AC	analytical column
AEDA	aroma extraction dilution analysis
AMDIS	Automated Mass Spectral Deconvolution and Identification System
amu	atomic mass unit
ANOVA	analysis of variance
CAR	carboxen
CAS no.	registry number assigned by Chemical Abstracts Service
CHARM	combined hedonic response measurement
CIS	cold injection system
^{1}D	one-dimensional, first dimension
^{2}D	second dimension
Da	dalton
d_f	film thickness
DMMP	2,5-dimethyl-3-methoxypyrazine
DVB	divinylbenzene
ECD	electron capture detector
e.g.	for example (exempli gratia)
EI	electron ionization
EPC	electronic pressure control
FID	flame ionization detector
GC	gas chromatography
GC×GC	comprehensive multidimensional gas chromatography
(MD)GC-O	(multidimensional) gas chromatography olfactometry
GSM	geosmin
GSM-d_5	deuterated geosmin
H/C	heart-cut
HMBC	heteronuclear multiple bond correlation
HS	headspace
HSQC	heteronuclear single quantum coherence
i.d.	inner diameter
i.e.	meaning/ in other words (id est)
IBMP	3-isobutyl-2-methoxypyrazine
IBMP-d_3	deuterated 3-isobutyl-2-methoxypyrazine

IPMP	3-isopropyl-2-methoxypyrazine
IPMP-d_3	deuterated 3-isopropyl-2-methoxypyrazine
LOD	limit of detection
LOQ	limit of quantification
LRI	linear retention index
M^+	molecular ion
MCSS	moving capillary stream switching
MDGC	multidimensional gas chromatography
MDMP	3,5-dimethyl-2-methoxypyrazine
MDMP-d_3	deuterated 3,5-dimethyl-2-methoxypyrazine
MHz	megahertz
MIB	2-methylisoborneol
MIB-d_3	deuterated 2-methylisoborneol
MS	mass spectrometry
MS-MS	tandem mass spectrometry
MW	molecular weight
NIST	National Institute of Standards and Technology
NMR	nuclear magnetic resonance
OAV	odor activity value
PCA	pentachloroanisole
PCP	pentachlorophenol
PDMS	polydimethylsiloxane
PLS	partial least squares
PTFE	polytetrafluoroethylene
PTV	programmed temperature vaporizing
Q1	first quadrupole of a triple quadrupole mass spectrometer
Q3	third quadrupole of a triple quadrupole mass spectrometer
RC	restrictor capillary
rpm	rounds per minute
Rs	resolution
RSD	relative standard deviation
SAFE	solvent assisted flavor evaporation
SBSE	stir bar sorptive extraction
SDE	simultaneous distillation-extraction
SIDA	stable isotope dilution assay
SIM	selected ion monitoring
SPE	solid phase extraction

SPME	solid phase microextraction
SRM	selected reaction monitoring
SSL	split/splitless
TCA	2,4,6-trichloroanisole
TCA-d_5	deuterated 2,4,6-trichloroanisole
TCP	2,4,6-trichlorophenol
TBA	2,4,6-tribromoanisole
TBA-d_5	deuterated 2,4,6-tribromoanisole
TBP	2,4,6-tribromophenol
TD	thermodesorption
TDU	thermodesorption unit
TeCA	2,3,4,6-tetrachloroanisole
TeCP	2,3,4,6-tetrachlorophenol
TMO	trimethyloxonium tetrafluoroborate (Meerwein salt)
TMSD	trimethylsilyldiazomethane

7.2 List of figures

7.3 List of tables

7.4 List of publications

Peer-reviewed journal articles

1) Schmarr, H.-G., Koschinski, S., Sang, W., & **Slabizki, P.** (2012). Trace level analysis of corky off-flavor compounds: Development of a new analytical method based on solid phase extraction and analysis by multidimensional gas chromatography with mass spectrometric detection. *Journal of Chromatography A, 1226*, 96-102.

2) Schmarr, H.-G., **Slabizki, P.**, Müntnich, S., Metzger, C., & Gracia-Moreno, E. (2012). Ionic liquids as novel stationary phases in gas liquid chromatography: Inverse or normal isotope effect? *Journal of Chromatography A, 1270*, 310-317.

3) **Slabizki, P.**, & Schmarr, H.-G. (2013). Analysis of corky off-flavour compounds at ultra trace level with multidimensional gas chromatography-electron capture detection. *Journal of Chromatography A, 1271* (1), 181-184.

4) Schmarr, H.-G., **Slabizki, P.**, & Legrum, C. (2013). Optimization in multidimensional gas chromatography applying quantitative analysis via a stable isotope dilution assay. *Analytical and Bioanalytical Chemistry, 405* (20), 6589-6593.

5) Langen, J., Wang, C.-Y., **Slabizki, P.**, Wall, K., & Schmarr, H.-G. (2013). Quantitative analysis of γ-and δ-lactones in wines using gas chromatography with selective tandem mass spectrometric detection. *Rapid Communications in Mass Spectrometry, 27* (24), 2751-2759.

6) **Slabizki, P.**, Legrum, C., Meusinger, R., & Schmarr, H.-G. (2014). Characterization and analysis of structural isomers of dimethyl methoxypyrazines in cork stoppers and ladybugs (*Harmonia axyridis* and *Coccinella septempunctata*). *Analytical and Bioanalytical Chemistry, 406* (25), 6429-6439.

7) **Slabizki, P.**, Legrum, C., Meusinger, R., & Schmarr, H.-G. (2014). Erratum to: Characterization and analysis of structural isomers of dimethyl methoxypyrazines in cork stoppers and ladybugs (*Harmonia axyridis* and *Coccinella septempunctata*). *Analytical and Bioanalytical Chemistry, 406* (25), 7743-7744.

8) Legrum, C., Gracia-Moreno, E., Lopez, R., Potouridis, T., Langen, J., **Slabizki, P.**, Weiand, J., & Schmarr, H.-G. (2014). Quantitative analysis of 3-alkyl-2-methoxypyrazines in German Sauvignon blanc wines by MDGC–MS or MDGC–MS/MS for viticultural and enological studies. *European Food Research and Technology, 239* (4), 549-558.

9) Legrum, C., **Slabizki, P.**, & Schmarr, H.-G. (2015). Enantiodifferentiation of 3-*sec*-butyl-2-methoxypyrazine in different species using multidimensional and comprehensive two-

dimensional gas chromatographic approaches. *Analytical and Bioanalytical Chemistry, 407* (1), 253-263.

10) **Slabizki, P.**, Potouridis, T., & Schmarr, H.-G. (2014). Ion Trap Mass Spectrometric Effect Leading to Peak Suppression and Solvent-Free Peak Focusing due to Co-Chromatography. *Chromatographia, 77* (23-24), 1727-1730.

11) **Slabizki, P.**, Fischer, C., Legrum, C., & Schmarr, H.-G. (2015). Characterization of atypical off-flavor compounds in natural cork stoppers by multidimensional gas chromatographic techniques. *Journal of Agricultural and Food Chemistry*, 63 (35), 7840-7848.

12) **Slabizki, P.**, Legrum, C., Wegmann-Herr, P., Fischer, C., & Schmarr, H.-G. (2015). Quantification of cork off-flavor compounds in natural cork stoppers and wine by multidimensional gas chromatography mass spectrometry. *European Food Research and Technology*, Accepted manuscript, DOI: 10.1007/s00217-015-2604-x.

Other journal articles

1) Schmarr, H.-G., **Slabizki, P.**, Legrum, C., & Langen, J. (2014). Wenn Nanogramm das Aroma verändern. *Nachrichten aus der Chemie, 62* (12), 1192-1196.

Oral presentations

1) **Slabizki, P.** (2013). Herausforderungen der Korkanalytik: Spurenanalytik von Haloanisolen. *23. Doktorandenseminar des AK Separation Science der GDCh, Hohenroda.*

2) Rudy, H., & **Slabizki, P.** (2013). Mufftöne in Wein. *Fortbildungstagung für Lehr- und Beratungskräfte in Weinbau, Oenologie und Marktwirtschaft des Landes Rheinland-Pfalz, Neustadt an der Weinstraße.*

3) **Slabizki, P.** (2014). Identifizierung muffiger Fehlaromen in Naturkorken mittels multidimensionaler Gaschromatographie Olfaktometrie. *Arbeitstagung 2014, LChG, Regionalverband Südwest.*

Poster presentations

1) Schmarr, H.-G., Koschinski, S., Sang, W., & **Slabizki, P.** (2011). Trace level analysis of corky off-flavor compounds: Wine matrix problems and analytical method comparison of HS-SPME-ECD versus SPE//MDGC-MS. *35th International Symposium on Capillary Chromatography, San Diego, CA, USA.*

2) Schmarr, H.-G., Potouridis, T., Gracia-Moreno, E., Koschinski, S., & **Slabizki, P.** (2012). Trace Level Wine Aroma Compound Analysis: A Final Breakthrough using State-of-the-art Equipment and Stable Isotopes as Internal Standards? *36th International Symposium on Capillary Chromatography and 9th GC×GC Symposium, Riva del Garda, Italien.*

3) **Slabizki, P.**, & Schmarr, H.-G. (2012). Stable Isotope Dilution Analysis of Corky Off-Flavour Compounds at Ultra Trace Level with Multidimensional Gas Chromatography - Electron Capture Detection. *36th International Symposium on Capillary Chromatography and 9th GC×GC Symposium, Riva del Garda, Italien.*

4) **Slabizki, P.**, Koschinski, S., Fischer, U., & Schmarr, H.-G. (2012). Multidimensionale Gaschromatographie kombiniert mit ECD oder QqQMS zur Spurenanalytik von Kork *off-flavour* Verbindungen. *41. Deutscher Lebensmittelchemikertag, Münster, Deutschland.*

5) Langen, J., **Slabizki, P.**, Wang, C.-Y., & Schmarr, H.-G. (2013). Quantitative Bestimmung von gamma- und delta-Lactonen in Wein mit selektiver Tandem-Massenspektrometrie. 42. Deutscher Lebensmittelchemikertag, Braunschweig, Deutschland.

6) Langen, J., **Slabizki, P.**, & Schmarr, H.-G. (2014). Quantitative analysis of α- and β-ionone in wine using HS-SPME-enantio-MDGC-MS-MS. *38th International Symposium on Capillary Chromatography and 11th GC x GC symposium, Riva del Garda, Italy.*

7) **Slabizki, P.**, Potouridis, T., & Schmarr, H.-G. (2014). Mass spectrometric curiosity encountered during enantio-GC-IT-MS analysis of whisky lactones. *38th International Symposium on Capillary Chromatography and 11th GC x GC symposium, Riva del Garda, Italy.*

7.5 Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

7.6 Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

Identification and Quantification of Cork Off-flavor Compounds in Natural Cork Stoppers by Multidimensional Gas Chromatographic Methods

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Obernheim, im Januar 2016

7.7 Danksagung

An dieser Stelle möchte ich mich bei allen bedanken, die in irgendeiner Form zu dieser Arbeit beigetragen haben und mich in den letzten Jahre unterstützt haben.

Ein besonderer Dank geht an meinen Betreuer und Doktorvater PD Dr. habil. Hans-Georg Schmarr, der mir stets mit Rat und Tat zur Seite stand und von dem ich außergewöhnlich viel lernen durfte. Vielen Dank für die Unterstützung, die zahlreichen hilfreichen Diskussionen, das Zuhören bei Problemen und die aufbauenden Worte.

Prof. Dr. Torsten Schmidt von der Universität Duisburg-Essen möchte ich für die freundliche Übernahme des Zweitgutachtens danken.

Ganz herzlich möchte ich mich bei Dr. Claus Fischer bedanken für die vielen guten Ideen und Ratschläge bei diesem Projekt, das ihm sehr am Herzen lag, sowie die hilfreichen Diskussionen und das Teilen seiner Erfahrungen über Wein und Flaschenverschlüsse und vor allem Korken.

Weiterhin möchte ich der Firma Rudolf Ohlinger GmbH & Co. KG für die materielle und finanzielle Unterstützung, sowie den Mitarbeitern für die geruchliche Beurteilung und Sortierung der Korken danken. Vielen Dank auch für die Möglichkeit einer Kurzreise nach Portugal, um mir die Korkproduktion aus der Nähe ansehen zu können.

PD Dr. Reinhard Meusinger von der TU Darmstadt danke ich ganz herzlich für die Messung der NMR Spektren und die freundliche Hilfe bei der Interpretation der Spektren, die sich als nicht ganz trivial herausstellten.

Dem Abteilungsleiter Prof. Dr. Ulrich Fischer möchte ich danken für die Freigabe der Gelder und damit die Förderung des Projektes durch das Ministerium für Umwelt, Landwirtschaft, Ernährung, Weinbau und Forsten.

Bei Prof. Juha Knuutinen (University of Jyväskylä, Finnland) bedanke ich mich sehr für die freundliche Überlassung von Referenzsubstanzen.

Außerdem möchte ich mich bei allen Mitarbeitern der Abteilung Weinbau und Oenologie bedanken mit denen ich in den letzten Jahren zusammenarbeiten durfte. Vor allem danke ich den Kollegen Charlotte Legrum, Doreen Schober, Engela Kritzinger, Hai-Linh Trieu, Johannes Langen, Michael Wacker, Patrick Nickolaus und Stefan Koschinski für die guten Diskussionen und das gegenseitige Zuhören, aber ganz besonders für den gemeinsamen Spaß. Ein ganz besonderes Dankeschön geht an Charlotte Legrum für die sehr gute Teamarbeit, die vielen guten hilfreichen Diskussionen und das regelmäßige und detailreiche Austauschen von Erkenntnissen, Problemen und Erfahrungen, das irgendwann zur Selbstverständlichkeit wurde. Besonders möchte ich auch dem ehemaligen Mitarbeiter

Theodoros Potouridis danken für das was ich von ihm über Organisation im Labor, Wartung von Geräten, Troubleshooting und Datenauswertung lernen durfte. Danke den Labormitarbeitern Jutta Keiser, Susann Krautwald und Maximilian Mathes für die Übernahme der Korkanalytik in der Routine. Pascal Wegmann-Herr, Sandra Klink und Benedict Grein möchte ich für die Hilfe bei der Planung, Durchführung und Auswertung der sensorischen Arbeiten danken.

Letztendlich möchte ich mich ganz herzlich bei meiner Familie und meinen Freunden bedanken, die mich immer unterstützten und für die nötige Ablenkung sorgten.