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GC-MS characterization of alkylated phenol isomers from exocrine secretions of arthropods

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Abstract

Title: GC-MS characterization of alkylated phenol isomers from exocrine secretions of arthropods

Author: Christoph Walcher, BSc.

1sr keyword: Dimethyl-ethylphenol

2nd keyword: Retention Index

3rd keyword: Opiliones

The identification of chemical compounds in complex mixtures by GC-MS is an important topic in various research fields. For fast and reliable identification the use of sophisticated databases, linking retention data to mass spectral data is advantageous. This connection is of particular interest when structural isomers should be differentiated. In this work five structural isomers of dimethyl-ethylphenol (4,5-dimethyl-2-ethylphenol, 2,3-dimethyl-5-ethylphenol, 2,3-dimethyl-6-ethylphenol, 2,5-dimethyl-3-ethylphenol, 2,5-dimethyl-6-ethylphenol) were synthesized and characterized by GC-MS. A reliable differentiation of these compounds on the basis of their mass spectra is not possible. When using the Van den Dool retention index as additional information, a significant distinction between 4,5-dimethyl-2-ethylphenol, 2,3-dimethyl-5-ethylphenol and 2,3-dimethyl-6-ethylphenol can be achieved with RI values of 1348, 1366 and 1315 (on a ZB-5-MS column) respectively. The compounds 2,5-dimethyl-3-ethylphenol and 2,5-dimethyl-6-ethylphenol co-elute on the ZB-5-MS as well as on a ZB-624 column and can therefore not be distinguished by their RI values of 1351 and 1354 (ZB-5-MS). Although it is not possible to distinguish between all isomers, this linkage of analytical data limits the number of possible compounds after a mass spectrometric identification from five to two or even a single isomer.

Zusammenfassung

Titel: GC-MS Charakterisierung alkylierter Phenolisomere aus exokrinen Sekreten von Arthropoden

Author: Christoph Walcher, BSc.

1. Stichwort: Dimethyl-ethylphenol

2. Stichwort: Retentions Index

3. Stichwort: Opiliones

Die Identifikation von chemischen Verbindungen in komplexen Mischungen mittels GC-MS ist ein wichtiges Thema in vielen Forschungsgebieten. Für eine schnelle und zuverlässige Identifikation ist die Nutzung von ausgereiften Datenbanken, in welchen massenspektrometrische Daten und Retentionsdaten der Verbindungen verlinkt sind, äußerst hilfreich. Diese Verbindung ist von besonderem Nutzen, wenn zwischen Strukturisomeren unterschieden werden soll. In der vorliegenden Arbeit wurden fünf Strukturisomere von Dimethyl-Ethylphenol (4,5-Dimethyl-2-Ethylphenol, 2,3-Dimethyl-5-Ethylphenol, 2,3-Dimethyl-6-Ethylphenol, 2,5-Dimethyl-3-Ethylphenol, 2,5-Dimethyl-6-Ethylphenol) synthetisiert und mit GC-MS charakterisiert. Eine sichere Unterscheidung dieser Isomere auf Grund ihrer Massenspektren ist nicht möglich. Die zusätzliche Verwendung von Van den Dool Retentionsindizes ermöglicht eine Differenzierung zwischen 4,5-Dimethyl-2-Ethylphenol, 2,3-Dimethyl-5-Ethylphenol und 2,3-Dimethyl-6-Ethylphenol mit den jeweiligen RI Werten von 1348, 1366 and 1315 (auf ZB-5-MS). Die Verbindungen 2,5-Dimethyl-3-Ethylphenol und 2,5-Dimethyl-6-Ethylphenol ko-eluieren sowohl auf der ZB-5-MS als auch auf der ZB-624 Säule und können daher nicht auf Basis ihrer RI Werte von 1351 und 1354 (auf ZB-5-MS) unterschieden werden. Obwohl eine Unterscheidung aller Isomere nicht möglich ist, kann die Anzahl der möglichen Verbindungen nach massenspektrometrischer Identifikation von fünf durch die Verwendung der RI Werte auf zwei oder sogar ein einziges Isomer reduziert werden.

Acknowledgements

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Table of Content

1 Introduction and Aim of Work

1.1 Chemical identification of (bioactive) natural products

In this work the term “bioactive natural products” names chemical compounds that are produced by a living organism and show a biological effect on other organisms. (Colegate & Molyneux 2008) These compounds may be produced as secondary metabolites used for protection or in a wide sense be part of the everyday metabolism of the organism with different effects in other organisms. Some of these effects render these substances useful for their therapeutic applications in humans and animals or as biodegradable toxins that could be applied as pesticides (Colegate & Molyneux 2008). Another reason for the identification of chemical compounds produced by organisms is the possibility of a classification of those organisms due to the composition of the compounds produced. This technique is called chemosystematics and will be described later on in detail. For the identification of a compound in a complex mixture, a reliable determination of the structure is required. Therefore structural data from various sources have to be put together to find a structure which is consistently and logically confirmed by all the information available. Modern structural analysis is based on spectroscopic methods like UV/VIS and IR spectroscopy NMR spectroscopy and mass spectrometry (MS). Various coupling possibilities of those techniques, called “hyphenated techniques” (e.g. HPLC-NMR, HPLC-DAD-MS-NMR, etc.) increased the speed and sensitivity of these analyses. Further X-ray diffraction studies are a possibility for structural elucidation of unknown compounds. Unfortunately this type of analysis requires the crystallisability of the examined compound. If the structural information obtained from spectroscopic methods is ambiguous and the X-ray diffraction is not possible, the structure has to be verified by the comparison with authentic standards. As these standards are very expensive or for many compounds not commercially available, it is necessary to limit the number of possible compounds to a minimum. For volatile compounds like the phenols of the opilionid secretions examined in this work, the combination of gas chromatographic and mass spectrometric data compared to literature values seems to be a suitable method to get a good idea about the structure of the component in order to restrict the number of standards needed.

1.2 Opiliones

Opiliones (or also called harvestmen) represent an order of arachnids. They can be divided in the suborders Cyphophthalmi, Eupnoi, Dyspnoi and Laniatores. The number of described species meanwhile reaches 6.500 (Kury 2011), but it is assumed to exceed 10.000 in total (Machado et al. 2007). This order is of particular interest, because all described species have a pair of prosomal exocrine glands called scent glands. While the presence of these glands is uniform in this order, the shape, organisation and the substances excreted by these glands as well as the mechanisms used to direct the effects of the chemical defense are very diverse (Machado et al. 2007). Up to the year 2012 more than 70 compounds of different chemical classes such as nitrogen containing compounds, phenols or benzoquinones have been identified. The composition of the substances excreted is highly specific and therefore useable like a fingerprint to characterize phyletic groups as well as to differentiate between closely related species. The number of components in the secretions varies from 20 different compounds in the secretion of Cyphophthalmi to a few, or even just one detectable compound in some Insidiatores and Grassatores (Raspotnig 2012). The Grassatores form the most diverse infraorder within the suborder of Laniatores with more than 3.500 described species (Kury 2003). The excretions of Grassatores are typically rich in phenols and benzoquinones (Raspotnig 2012).

1.3 Phenols

Phenols, or sometimes also called phenolics, are a class of substances with one or more hydroxyl groups directly attached to an aromatic ring as basic structural element. The other ring positions can be substituted in many different ways. Common substituents are alkyl-, aryl-, amino-, carboxyl-, carbonyl-, nitro-, sulfo- or halogenid-groups. The basic structure of phenols is shown in Figure 1.

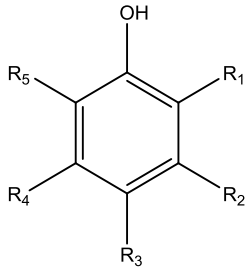

Figure 1 The basic structure of phenols

In human cells some phenols can have positive effects, such as antioxidant functions (Jin et al. 2010; Wijngaard et al. 2009; Hoye et al. 2008), lower the risk of cancer (Sawadongo et al. 2012; Pieme et al. 2010) or diabetes (Kusirisin et al. 2009; Scalbert et al. 2005). It was also reported that phenolic compounds could reduce inflammation (Mohanlal et al. 2012; Zhang et al. 2011; Jin et al. 2006) and rates of mutagenesis in mammalian cells (Sawadongo et al. 2012; Pedreschi et al. 2006; Gomez et al. 2001).

The commercial use of phenolic compounds is diverse. They are used as pesticides, explosives, developers, surfactants, pharmaceuticals or other health products (Licha 2002).

Alkyl substituted phenols are divided into three groups accounting their differences in solubility, toxicity and usage. The short chained alkyl phenols (SCAP) with up to three carbon atoms outside the ring have a good solubility in water and their toxicity and environmental behavior is controversial and speculative (Licha 2002, Fischer & Licha 2013). Compounds with 4- 8 carbon atoms are used as plasticizers or preservatives and therefore assumed to be non toxic. The compounds investigated in this work also belong to this group. Alkylphenols with 9 to 12 carbon atoms outside the ring are mainly used as tensides in the form of ethoxylates and are known to act as endocrine disruptors (Fischer & Licha 2013).

In nature phenols occur in many different forms and are especially known as phytochemicals that can be found in plants. Some phenols are formed as response to physical stress or wounding (Diaz Napal et.al 2010; Kennedy et.al 2011; Zulak et.al 2006; Chung et.at 2003), others occur as flavonoids (Ferriera et.al 2012; Rong et.al 2010) or phenolic acids (Periera et.al 2009). But not only plants produce phenols. Phenols and their derivatives can also be found in a number of arthropod taxa as exocrine compounds with the scent gland secretions of grassatorean

harvestmen representing one prominent example (Eisner et al. 1977; Roach et al. 1980; Duffield et al. 1981 Acosta et al. 1993; Hara et al. 2005; Shear et al. 2010, 2010b; Pomini et al. 2010).

With an increasing number of carbon atoms in the substituents, also the number of possible isomers increases. Four carbon atoms in the substituents result in 57 possible phenol isomers. A list of all possible isomers in the case of four Cs outside the ring, including structures is shown in the appendix. Because the dimethyl-ethylphenols were of main interest in this work, a list of the possible isomers is shown in Table **1**.

Table 1 List of all possible dimethyl-ethylphenol isomers with CAS number

Name	CAS #	Name	CAS #
2,3-dimethyl-4-ethylphenol	66142-72-1	**2,5-dimethyl-6-ethylphenol**	**---**
2,3-dimethyl-5-ethylphenol	**---**	2,6-dimethyl-3-ethylphenol	---
2,3-dimethyl-6-ethylphenol	**18441-55-9**	2,6-dimethyl-4-ethylphenol	10570-69-1
2,4-dimethyl-3-ethylphenol	62126-74-3	3,4-dimethyl-2-ethylphenol	---
2,4-dimethyl–5-ethylphenol	---	3,4-dimethyl-5-ethylphenol	62126-75-4
2,4-dimethyl-6-ethylphenol	2219-79-6	**3,4-dimethyl-6-ethylphenol**	**2219-78-5**
2,5-dimethyl-3-ethylphenol	**---**	3,5-dimethyl-2-ethylphenol	62126-76-5
2,5-dimethyl-4-ethylphenol	**32018-76-1**	3,5-dimethyl-4-ethylphenol	62126-77-6

From these 16 isomers, just one, the 3,4-dimethyl-6-ethylphenol is commercially available as standard. The others have to be synthesized alone or ordered costly by custom synthesis. The bald written compounds in Table 1 were synthesized and analyzed in the course of the present work. Although these compounds are relatively small and not very complex molecules there are not even CAS registry numbers available for all the isomers.

1.4 Gas chromatography - mass spectrometry (GC - MS)

For the identification and quantification of organic substances in complex mixtures the coupling of gas chromatography and mass spectrometry (GC-MS) is the most common used analytical technique. Among others this technique is used in the fields of forensics, medicinal and biological research, food safety and environmental science. The combination of gas chromatography and mass spectrometry has many advantages (Abian 1999). The components of a mixture can be separated by the gas chromatograph in time to obtain structural information of the single compounds in the mass spectra recorded. The detection limits of this technique are in the lower femtogram range (Sparkman et al. 2011). Nowadays the use of capillary columns with a length of 25 – 60 m is common in GC and makes this type of chromatography very powerful. The principals of gas chromatography as well as the different column types and stationary phases are described elsewhere (e.g. Sparkman et al. 2011). The retention data obtained by GC can be used to calculate retention indices (RI) to make the data comparable among databases. The use of a mass spectrometer as detector can provide good information about the structure of the examined compound. When using electron ionization (EI) very reproducible spectra according to the ionization energy (commonly 70 eV) can be obtained. Therefore it is again possible to compare the mass spectrum of an unknown compound with spectra of a database. Most databases available belong to a special topic or chemical classes like terpenes, pesticides, flavors and fragrances or drugs and contain between 300 and 30.000 mass spectra and RI values. The two largest databases available are the NIST/EPA/NIH Mass Spectral Database (NIST 11) and the Wiley/ NIST Registry of MS Data (Wiley Registry 10th edition). The NIST11 contains 212,961 mass spectra of 192,262 compounds. This database also includes 346,757 RI values for 70,835 compounds with complete literature citation (www.nist.gov). It has to be mentioned that only for about one third of the compounds the corresponding mass spectral data is given. In addition a tandem MS/MS library (nist_msms2) containing 121,586 spectra of 15,180 ions is available. The Wiley registry contains 870,000 mass spectra of about 736,000 compounds. Further the complete NIST EI and MSMS databases are implemented (eu.wiley.com). As this is just a MS database, no RI values are implemented. A full list with a detailed description of available EI-MS databases can be found in Sparkman et al. (2011).

1.5 Retention indices

With the establishment of gas chromatography there was a need to bring the chromatographic data like retention times into an easily reproducible standard form. This standard form should be as independent as possible from varying operating conditions and just depend on the interaction mobile phase – sample – stationary phase. In GC the mobile phase should have less influence, so the characteristics of the stationary phase have the highest influence on the chromatographic behavior of the analyte. In the year 1958, E. Kovats proposed, based on the column model of Martin and Synge (1941) a factor called retention index I derived from the retention volume. This index should be independent to the chromatographic system used. Therefore he used a set of saturated n-alkanes with an even number of carbon atoms from butane to dodecane as a standard. He defined the retention indices (RI) of the n-alkanes for every temperature as 100 * number of carbon atoms. Hence the RI for ethane would be 200, for butane 400 and so on. When using isothermal conditions, a linear coherence is given for the number of carbon atoms in the alkane and the log of the retention time. This coherence is shown in Figure 2.

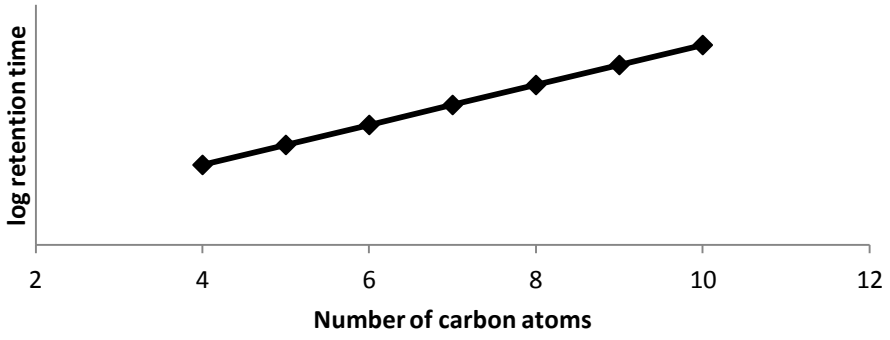

Figure 2 Log of retention time vs. number of carbon atoms of an n-alkane standard (isothermal)

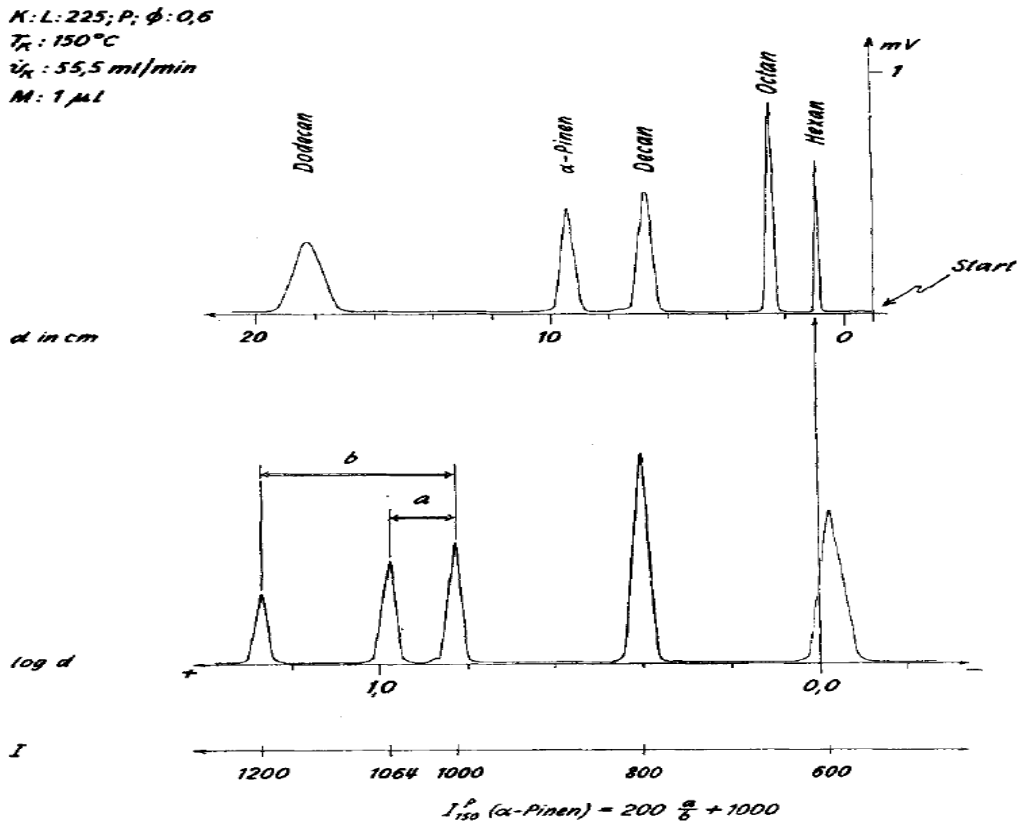

Figure 3 Determination of the retention index Figure from Kovats (1958)

To determine the RI of a compound, the RI values of the neighboring n-alkanes are logarithmically interpolated. The equation for the calculation of the so called Kovats RI is defined as

$$I_X = 100\,n + 100\,i\,\frac{log\,V_x - log\,V_n}{log\,V_{n+i} - log\,V_n}$$ (Kovats 1958)

where n is the number of C atoms of the n-alkane eluting before the unknown substance and i is the difference between the numbers of C atoms of the neighboring n-alkanes. V_X is the retention volume of the compound of interest while V_n and V_{n+I} are the retention volumes of the alkanes eluting before and after this compound. Kovats assessed the accuracy of the RIs within a range of ± 3 RI values. The major drawback of this equation is, that it can only be used if the column temperature is kept constant during the whole run (Kovats 1958). Since isothermal working conditions lead to a peak broadening in increasing retention time, the use of temperature ramps or custom temperature programs is preferred. Figure 4 shows the

peak broadening when running a chromatography isothermal versus temperature programmed (Van den Dool & Kratz 1963).

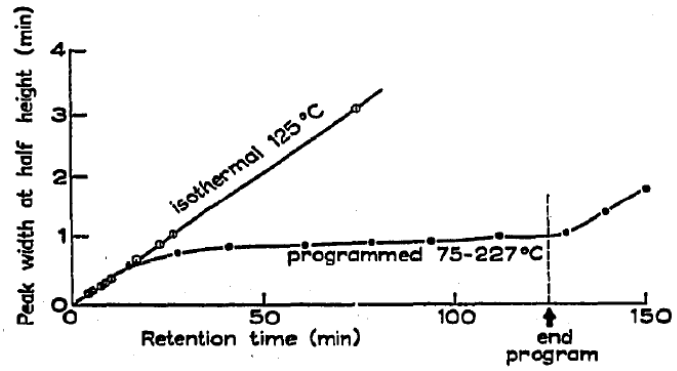

Figure 4 Relation of retention time and peak width in isothermal and programmed chromatography. Figure from Van den Dool & Kratz (1963)

When using non isothermal conditions, the linear dependence of the log of the retention time t and the number of carbon atoms as it can be done with isothermal conditions is not given. Thus, it is not possible to directly use the retention index system proposed by Kovats. To apply the RI system also to temperature programmed chromatography, Van den Dool and Kratz formulated a more general form of the RI equation. The general form of this equation is defined as

$$I = 100n + 100\,i\ * \frac{X - M_n}{M_{n+1} - M_n}$$ (Van Den Dool and Kratz 1962)

For isothermal conditions X and M are replaced by the logarithms of the retention volumes and the equation of Kovats shown above is reached again. When using a linear temperature program, X and M are replaced with either the retention temperature or the retention times. The substitution of X and M with the retention times leads to the known equation for the Van den Dool and Kratz retention index, which is defined as:

$$I = 100n + 100\,i\ * \frac{t_x - t_n}{t_{n+1} - t_n}$$ (Van Den Dool and Kratz 1962)

While retention indices calculated from isothermal conditions are independent of column properties and carrier gas flow, indices from temperature programmed chromatography are not. Column length or phase composition, increased temperature gradient or decreased carrier gas flow lead to the same effect as an

increased temperature in isothermal chromatography. Because of that, retention indices calculated from temperature programmed runs are less reproducible than indices from isothermal ones (Gonzalez and Nardillo 1999). The retention index of some compounds, like the 4 - isopropyl - 3 – methylphenol increases about 5 RI values when increasing the heating rate from 1 to 4°C/min, while other compounds like the 2-tert.butylphenol just increase 2 RI values at the same conditions (Mjøs, Meier, et al., 2006). Generally the RI value increases when the heating rate is increased. Another reason for RI value variations can be the overloading of the column (Angelini 1992). Although the column length influences the separation its influence on the RI is very small, whereas the stationary film thickness distinctly affects the retention behavior of the analyte and therefore the RI value (Shibamoto et.al 1980). Thus it is essential to exactly know the conditions under which the measurements were done.

1.6 Chemosystematics

Initially, chemosystematics was used to differentiate whether plants and other organisms are suitable as food in a non written form and on the principal of trial and error. By increasing knowledge, compounds were classed into useful, harmful and inactive chemicals. Components of interest were examined and recorded. This led to an insight in the taxonomy and a better understanding of the examined organisms. Meanwhile the progress in instrumentation has opened up access to fields like metabolic profiling (metabolomics), the study of the DNA (genomics) and proteins (proteomics). This is the scope of molecular biology where the phenotype of a taxon is related to its genome. However in most cases the characterization of organisms does not consider the chemical compounds produced for communication or defense. Chemosystematics has found entrance to the classification of plants (Raynolds 2007), it is still rarely used in animals. Harvestmen for example excrete specific mixtures of compounds for defense when they are threatened (Holmberg 1986). According to the composition of their scent gland secretions, it is possible to reconstruct the evolution of characteristic compounds in a given phylogeny.

Actual studies on lower grassatoreans show that some of the excretions contain a phenolic substance with a mass of 150 amu. Based on mass spectrometric data this

substance was tentatively identified as dimethyl-ethylphenol. In the literature 2,3-dimethyl-5-ethylphenol is the only substance of this class reported as content of opilionid secretions (Duffield et al. 1981; Acosta et al. 1993). According to the analysis of an authentic standard, the substance found in the actual studies is definitely not the 2,3-dimethyl-5-ethylphenol (Raspotnig, in progress). This fact leads to the assumption that the substance reported in literature could also be a different dimethyl-ethylphenol isomer. Due to the fact that the quantity of the secretions is way too small for an extraction and direct identification of the substance by NMR, a comparison to authentic standards is the only promising way for certain identification.

2 Results and Discussion

2.1 Synthesis of the phenol isomers

4,5-dimethyl-2-ethylphenol, 2,3-dimethyl-5-ethylphenol, 2,3-dimethyl-6-ethylphenol

Nitration

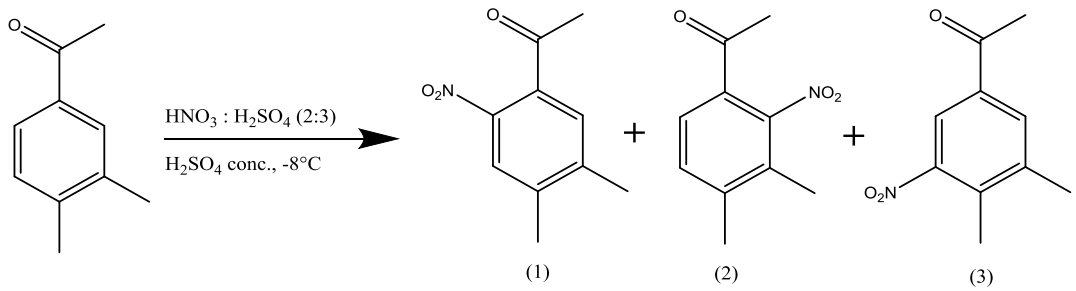

Figure 5 Nitration of the 3,4-dimethylacetophenone

By the classical nitration, using H_2SO_4 : HNO_3 (2:3) as nitrating reagent, it was possible to obtain a mixture of all three mononitrated nitroacetophenone isomers. The selectivity of the nitration could be controlled by the reaction time. In detail it was possible to obtain the 4,5–dimethyl–2-nitroacetophenone (1) as pure compound by recrystallization and to identify it unambiguously by NMR. The melting point of the colorless needles was determined at 120° C. Within the standard GC-MS analysis it was only possible to completely separate compound 1, while the compounds 2 and 3

coelute in one peak. Therefore it is only possible to give the ratio of this compound (1) compared to the other two ((2) & (3)) together.
The ratios of the isomers obtained when trying different reaction times are shown in Table 2.

Table 2 Results of different reaction times used for the nitration of 3,4-dimethylacetophenone

Reaction time [min]	Isomer ratio [%] (1)	(2) / (3)	Theoretical yield [%]
1	50	50	50
15	20	80	50
60	65	35*	60

The reaction time is the time between the complete addition of the nitrating reagent and the time the reaction mixture was poured on ice and water to stop the reaction. In both cases the addition of the nitrating reagent was done dropwise in about 30 minutes in order to not exceed a temperature of about -5° C. Higher temperatures led to a dramatically decreased yield. Longer reaction times led to just a little higher yields and also one isomer (2) or (3) was not found anymore. Therefore the 60 minutes-method was used to produce the crystalline 4,5–dimethyl–2-nitroacetophenone. With a three times repeated recrystallization using water and washing with ice cold EtOH it was possible to obtain 95 % pure isomer (1) as colorless needles. The ratios after each recrystallization step are shown in Table 3.

Table 3 Results of the recrystallization of the 4,5–dimethyl–2-nitroacetophenone

Step	Isomer ratio [%] (1)	(2), (3)
1	75	25
2	93	7
3	95	5

As seen in Table 3, only the first step led to a 10 % shift of the ratio, whereas the loss was about one third of the crystalline material. The second recrystallization had the largest effect, whereas the third step would not have been necessary.
For the production of the other isomers, the 15 minutes method was applied due to the favorable isomer ratio produced. Subsequently it was tried to eliminate isomer (1) by recrystallization and filtration, followed by an extraction of the filtrate with ethyl acetate. The ethyl acetate was evaporated, the residue again solved in a lower amount of water and the recrystallization step repeated. By this procedure it was possible to obtain 95 % isomers (2) and (3), with the ratio of about 70 : 30 %.

Reduction to the amine

The reduction of the nitro group to the amine under conventional conditions was done with iron in dilute HCl. The reaction is shown in Figure 6.

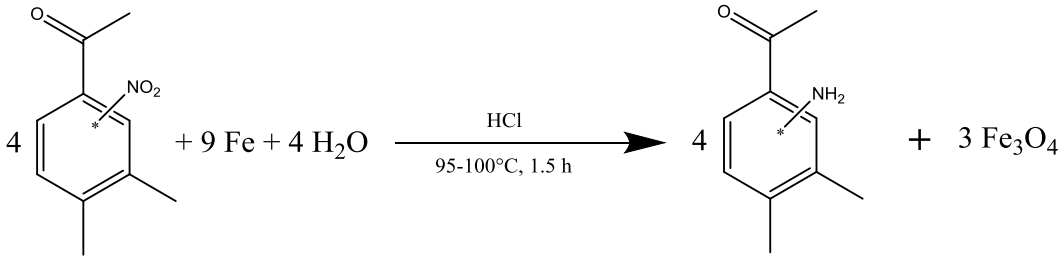

Figure 6 Reduction of the nitroacetophenones to the amines with Iron fillings in dilute acid

The big disadvantage of this reaction is that the iron powder used for the reduction has to be added in very small amounts during the whole reaction time. As soon as the iron enters the reaction mixture, it is attracted by the magnetic stir bar. The top layer of the iron is oxidized to Fe_3O_4 and forms a film which excludes the iron below from the reaction. Therefore a more modern method to reduce the nitro compound was searched for.
The method of choice was a microwave supported reduction with palladium on carbon as catalyst. The formula of the reaction is shown in Figure 7.

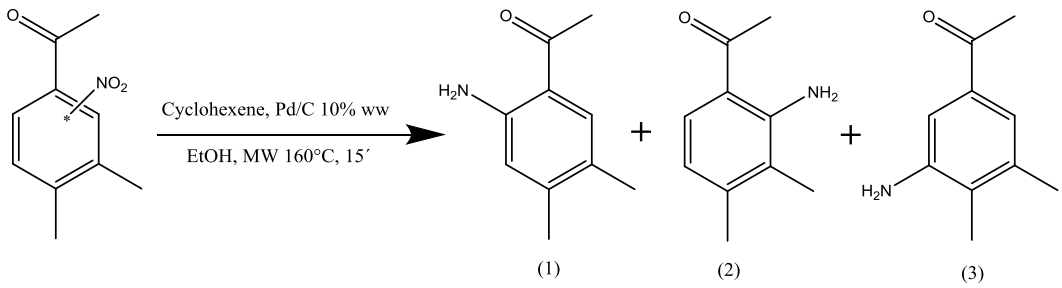

Figure 7 Palladium catalyzed reduction of the 3,4-dimethyl-nitroacetophenones to the amines with microwave heating

The palladium catalyzed reduction of the nitro group to an amine group was done in two steps. In the first step 70 % conversion was the maximum. The conversion rate did not change with the amount of catalyst, reactant or extended reaction time. After the addition of some more Pd/C and cyclohexene and heating the mixture again in the microwave for 5 minutes at 160°C, full conversion was achieved. The course temperature, pressure and microwave power during the reaction are shown in Figure 8.

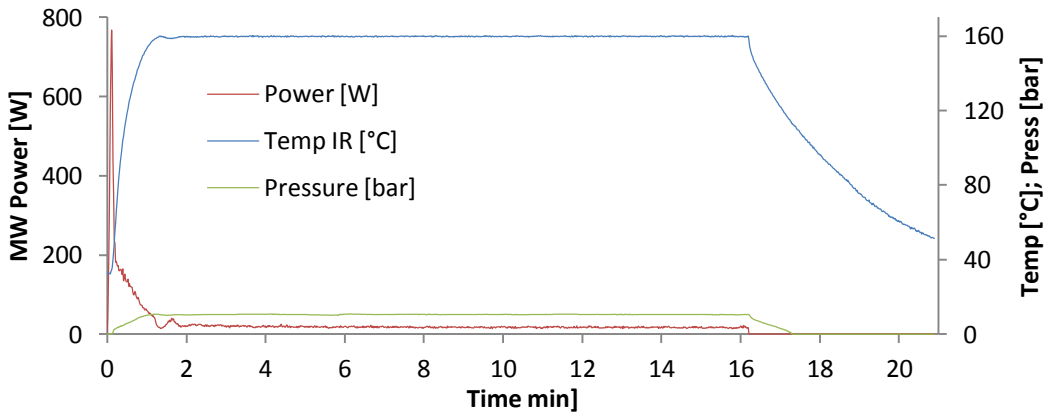

Figure 8 Heating profile of the microwave assisted reduction over the total reaction time

The microwave heating in closed pyrex vials allowed to reach a higher reaction temperature of 160°C due to the increased pressure of about 11 bar in the vial. As can be seen in Figure 8 the desired reaction temperature of 160°C could be reached within 1 minute. Also the cooling process with pressurized air was done in 5 minutes.

This leads to an overall process time of 21 minutes. The second heating process lasted 10 minutes. Compared to the conventional heating in the oil-bath, this reaction including heating and cooling requires at least 2 hours in which an operator has to be present all the time, the microwave assisted reaction can be done in about ¼ of the time. Yields and the workup steps are comparable for both reactions.

Conversion to the hydroxy-acetophenone

The introduction of the hydroxyl group was done in two steps. The first step was the formation of a diazonium compound with nitrous acid, followed by a Sandmeyer reaction. The reaction is shown in Figure 9.

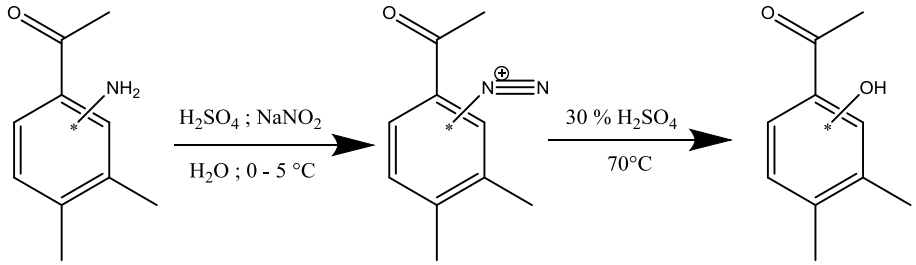

Figure 9 Diazotisation and introduction of the hydroxyl group

The nitrous acid, needed for the reaction, was formed in situ from $NaNO_2$ and diluted H_2SO_4 (~ 2%). Due to the fact that diazonium compounds are not stable above 5°C, the temperature had to be kept low during the diazotization step for about 1 hour. The originally slightly yellow solution turned to dark brown during this time. The reaction product was not isolated, but directly converted to the hydroxyl compound by a dramatic increase of acid concentration and temperature. The increased temperature led to the release of N_2 from the compound and the formation of the aryl cation. The increased acid concentration was necessary to stabilize the cation and allow a nucleophilic addition of H_2O. After about 15 minutes the reaction was completed and the reaction mixture was cooled to room temperature. The solution contained carbon black impurities which were removed by filtration. The chromatogram of the reaction products is shown in Figure 10.

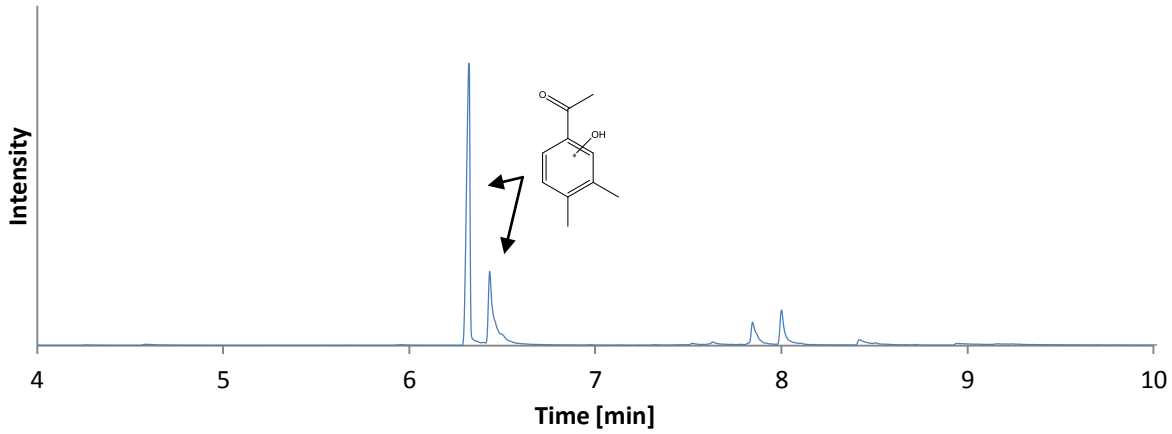

Figure 10 GC-MS results of the conversion to the hydroxyl compound

As shown in the chromatogram, it was not possible to separate all three isomers by the method used herein. Although the conditions were kept exactly the same in several attempts of the reaction, the conversion rates ranged between 60 and 90% for no reason accountable. Sometimes not specified byproducts were formed in varying quantities as well.

Reduction to the Phenol

The final reduction of the carbonyl group was achieved by an iron(III) catalyzed reaction with polymethylhydrosiloxane (PMHS) as mild and stable reducing agent. The reaction is shown in Figure 11

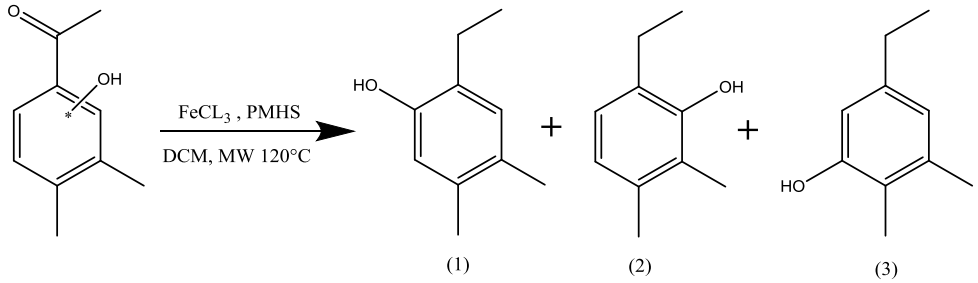

Figure 11 $FeCl_3$ catalyzed reduction of the hydroxy- 3,4-dimthylacetophenone with microwave supported heating

Like the first reduction step, also this reduction had to be done twice. After 1 hour reaction time at 120°C, about 50% conversion was achieved. A longer reaction time,

or higher quantities of catalyst and reducing agent did not lead to higher conversion rates. When some more catalyst and reducing agent were added after the reaction time of 1 hour and by repeating the heating step, almost full conversion could be achieved (> 94%). After the filtration through a plug of celite and evaporation of the solvent, a dirty orange, oily mixture of phenol isomers was achieved. In Figure 12 a gas chromatogram of the final product is shown.

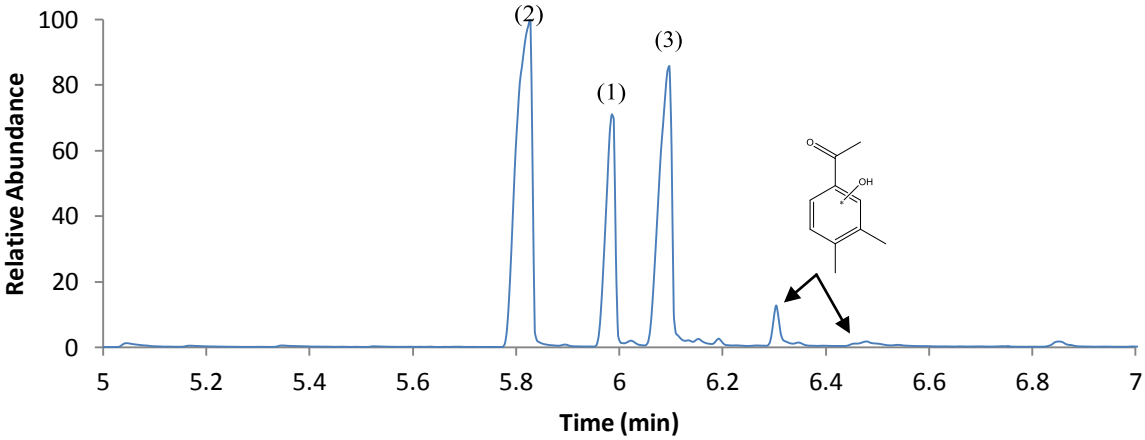

Figure 12 GC-MS results of the final reduction

As pictured in the chromatogram in Figure 12, almost full conversion was achieved and according to GC-MS almost no detectable impurities were present in the mixture. The ratio of the isomers was 50 % for isomer (2), 20 % for isomer (1) and 30 % for isomer (3).

In order to obtain pure compounds for a structural elucidation with NMR the compounds were separated using different methods like crystallization and flash chromatography. For the 2,3-dimethyl-6-ethylphenol crystallization worked fine and the 4,5-dimethyl-2-ethylphenol was commercially available, so it was possible to identify the third isomer using GC-MS and the method of elimination. To obtain the third isomer in a pure form, a preparative HPLC separation seemed to be a suitable method. Therefore a HPLC method, using a reversed phase (C18) column was developed.

Method development for a RP-HPLC separation of the synthesis products

The systematic method development was started with several runs using the combination of acetonitrile and MilliQ. The ratios tested were 40, 50, 55, 60 and 65 % acetonitrile. The chromatograms of the runs are shown in Figure 13.

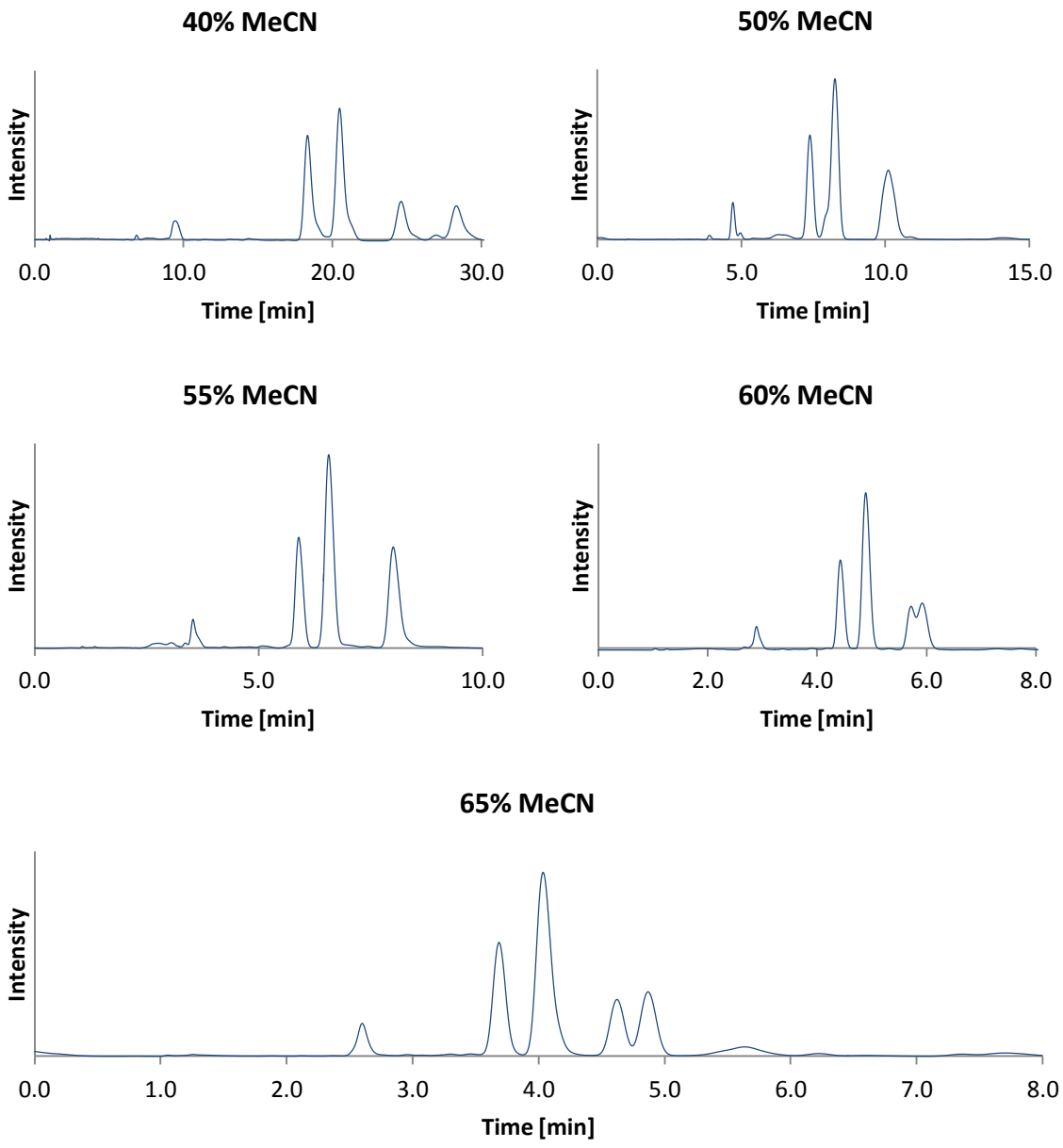

Figure 13 Chromatograms of the 3,4- dimethyl isomers; ZORBAX SB–C 18 150 x 4.6 mm 5 µm; MeCN : MilliQ 40- 65% MeCN; Flow 1 ml/min, 25°C; Inj. Vol.: 5 µl Detection wavelength 210 nm

Obviously the two big peaks which represent two of the isomers are well separated in all shown chromatograms. The third isomer was represented by the small peak to the

right of the big peak in the 40 % MeCN chromatogram. This peak was well separated with 40 % MeCN, co-elutes with an unknown substance from 50 to 60 % MeCN and starts to separate again with 65 % MeCN. When exceeding 65 % MeCN, the two big peaks were not separated anymore. When using 40 % and 50 % MeCN it became obvious that the two isomers represented by the big peaks coelute with one or more unknown substances. Because of these reasons, the 65 % MeCN method appeared to be the best for a preparative separation. Although the third isomer was not completely separated, this method was a good compromise of separation and time.

When testing the same ratios with methanol instead of acetonitril, completely different results due to the elution behavior were obtained. Because the separation in all approaches was worse than with acetonitril, only the 60 % MeOH chromatogram of the synthesis with just two isomers is shown overlaid with the 60 % MeCN chromatogram in Figure 14.

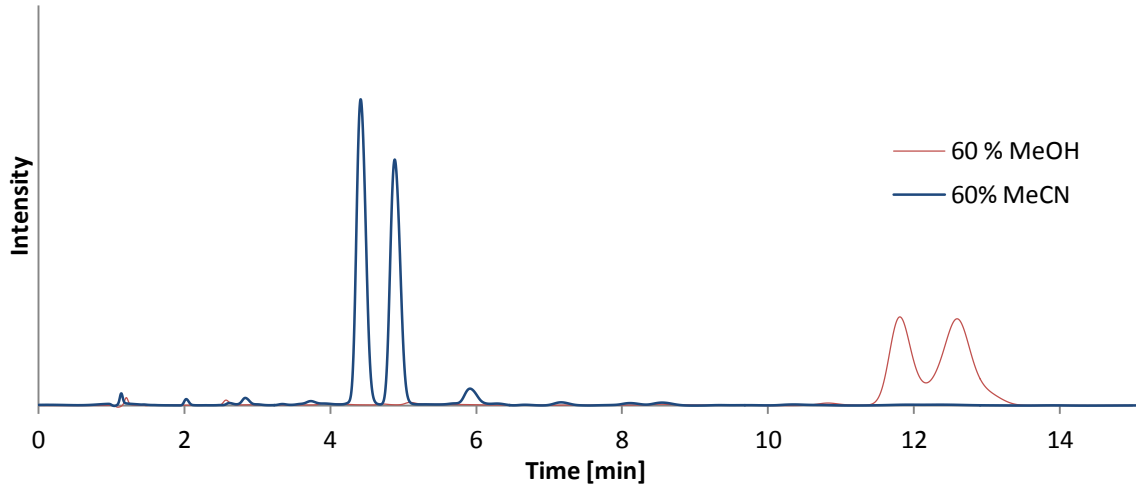

Figure 14 Chromatogram overlay of the 3,4- dimethyl isomers MeCN vs MeOH; ZORBAX SB–C 18 150 x 4.6 mm 5 µm; MeOH : MilliQ; 60% MeOH and MeCN : MilliQ; 60% MeCN; Flow 1 ml/min, 25°C; Inj. Vol.: 5 µl Detection wavelength 210 nm

The different sample was chosen because it was much cleaner and therefore a clearer illustration is possible. The overlay shows that the use of methanol leads to no change in selectivity. Furthermore the retention time increased almost threefold with the same concentration of organic solvent and the separation became worse

because of the peak broadening. When using higher amounts of MeOH, the retention time decreased, but the peaks stayed much broader than with MeCN. Also attempts using THF and Isopropanol as organic solvents led to no improvement in compared to acetonitril.

2,5-dimethyl-3-ethylphenol, 2,5-dimethyl-6-ethylphenol

Basically, this synthesis is exactly the same as for the isomers described before, only the starting material was a 2,5-dimethylacetophenone instead of a 3,4-dimethylachetophenone. Interestingly this substrate showed a totally different behavior.

Nitration

Classical nitration with a mixture of HNO3 and H2SO4 led to the formation of two of the three possible isomers in detectable quantities. The reaction is shown in Figure 15.

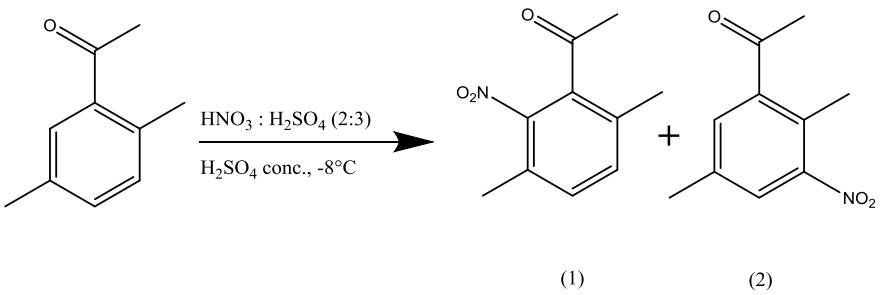

Figure 15 Nitration of 2,5-dimethylacetophenone with H_2SO_4/HNO_3

During the addition of the nitrosulfuric acid, the reaction mixture became very viscous and showed an intense orange color. Because of the high viscosity, it was not possible to use a magnetic stir bar anymore. A overhead mechanical stirrer had to be used instead. After pouring the mixture on ice, about 3 g of a pasty white precipitate with an isomer ratio of 1:1 was achieved. This is equivalent to 77 % of the theoretical yield. For this substrate changes in reaction time led to nearly no change in isomer ratio or yield. It was not possible to crystallize any of the isomers.

In order to obtain all three isomers, the following additional possibilities for the nitration were tested. One method was a solvent free nitration with HNO_3 in the

presence of P_2O_5 on silica gel (Abdol et al. 2005). As second, a nitration with amonium cerium (IV) nitrate (CAN), following a paper from Mellor et al. (2000) was done. Further the nitration with $AgNO_3$ and N-bromosuccinimide (NBS) (Nowrouzi et al. 2012) was tried. The results of all tested methods are listed in Table 4

Table 4 Overview about the different methods used for the nitration of 2,5-dimethylacetophenone

Method	RT [min]	Conversion [%]	Yield [%]	Reference
HNO_3 / H_2SO_4	60	100	77	Morgan and Pettet (1934)
P_2O_5 on SiO_2	2	100	< 58	Abdol et al. (2005)
CAN	1080	0	0	Mellor et al. (2000)
$AgNO_3$ / NBS	240	0	0	Nowrouzi et al. (2012)

The nitration attempts with CAN and $AgNO_3$ / NBS led to no conversion of the substrate to the desired product. While the substrate remained completely unchanged when using $AgNO_3$/NBS, the total substrate was converted into a lot of unknown compounds by using CAN. The classical nitration with HNO_3/H_2SO_4 and the nitration with P_2O_5 on SiO_2 led both to 100 % conversion of the substrate. Both nitrations led to the formation of the same two isomers with a similar isomer ratio of about 1:1. The reaction with P_2O_5 on SiO_2 was very vigorous and a lot of yellow fume was generated. The results were also not very reproducible due to the byproducts formed and the maximum yield was 58 %. For those reasons the classical nitration was used for all the further work.

Reduction to the amine

This reduction was done using microwave heating with Pd/C and cyclohexene. The conditions were the same as for the reduction of the 3,4-dimethylnitroacetophenone. The reaction is shown in Figure 16.

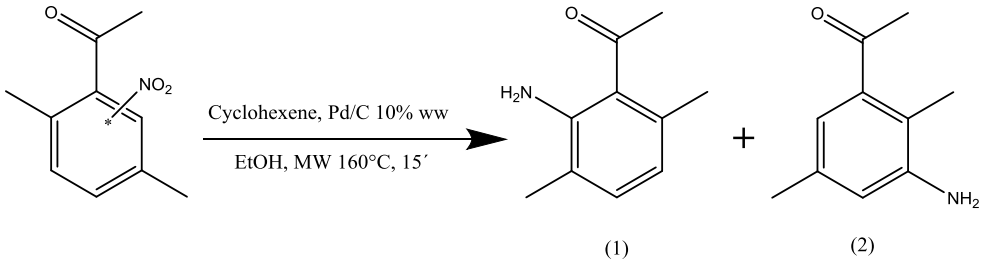

Figure 16 Palladium catalyzed reduction of the 2,5-dimethyl-nitroacetophenones to the amines with microwave supported heating

Although the analysis of this step shows three peaks, only the two identified isomers are shown in the reaction. The third peak, possibly representing a further isomer, could only be observed in this step. Also the final product did not show three peaks. The gas chromatogram of this reduction is shown as an overlay of substrate, first reduction step and final reduction step in Figure 17.

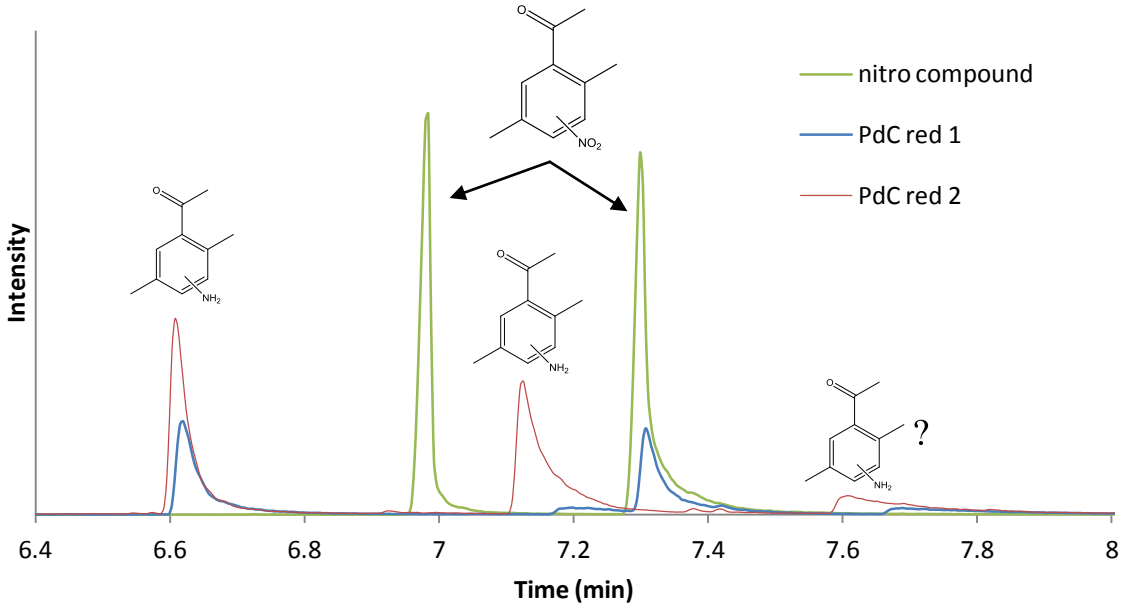

Figure 17 GC-MS analysis of the Pd catalyzed reduction step

In this overlay the green colored chromatogram represents the sample before the reduction. The blue colored chromatogram “PdC red1” shows this sample after the first reduction attempt. As can be seen only one isomer is reduced. The different peak areas result from different concentrations of the GC-MS samples. The

chromatogram of the final reduction product is colored in red and shows a complete conversion of the nitro compound to the amine. The third peak at 7.6 minutes, represents an unknown substance which shows a mass spectrum very similar to the other amine components. Therefore it could be possible that the third isomer was formed and could not be separated in the nitro stage. Due to the very small area of this peak compared to the others (~4%), this compound is formed in very small quantities.

Conversion to the hydroxy-acetophenone

Diazotization and subsequent heating of this product was done in the same way as described above. The reaction is shown in Figure 18.

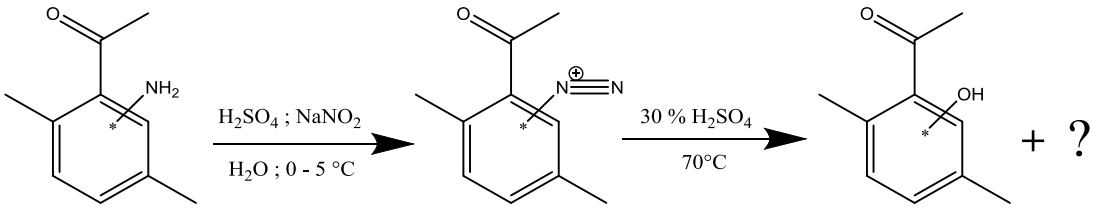

Figure 18 Conversion from the amino-2,5-dimethylacetophenone to the hydroxy-2,5-dimethylacetophenone

This reaction was characterized by a very low yield (<20% of the theoretical) and the formation of many byproducts. A chromatogram of the extracted reaction mixture is shown in Figure 19.

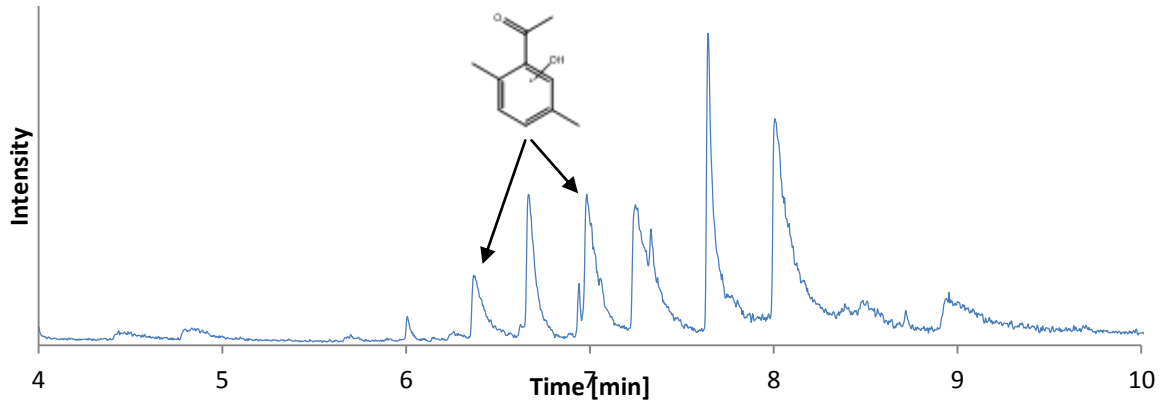

Figure 19 GC-MS analysis of the introduction of the hydroxyl group

The desired hydroxy compounds were not the main products of this reaction. The numbers and the quantity of byproducts varied with every reaction done for no reason accountable. Because of the byproducts formed, the following reduction did not work at all. Therefore the crude mixture was cleaned by flash chromatography. A chromatogram of the products after the cleanup step is shown in Figure 20.

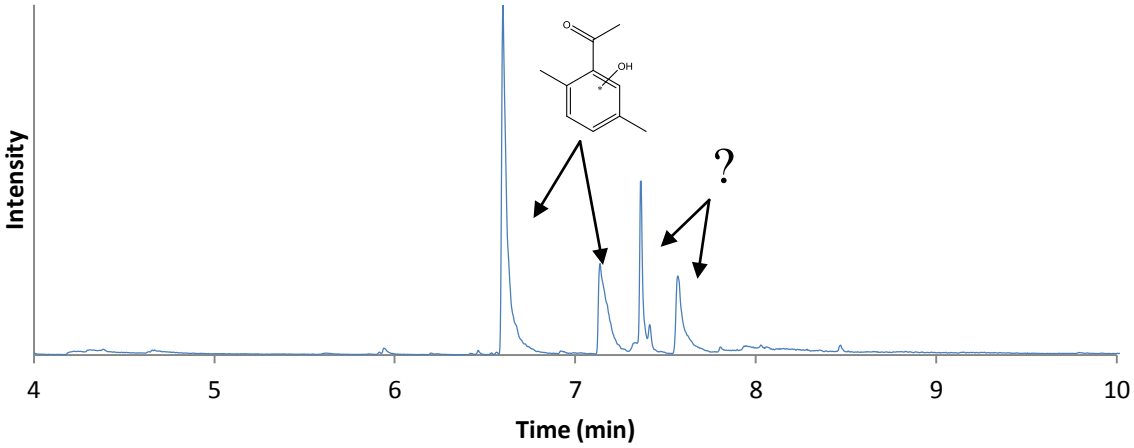

Figure 20 GC-MS analysis of the reaction mixture after the cleanup with flash chromatography

It was possible to reduce the number of impurities dramatically by flash chromatography. Although it was not possible to obtain completely clean hydroxyacetophenone, the impurities that prevented a successful reduction were removed by this cleanup step. The low yield of < 8% could not be assigned to the reaction itself or to losses caused by chromatography.

Reduction of the keto function

The reduction of the carbonyl group was done following the same procedure as for the 3,4-dimethyl isomers. The reaction is shown in Figure 21.

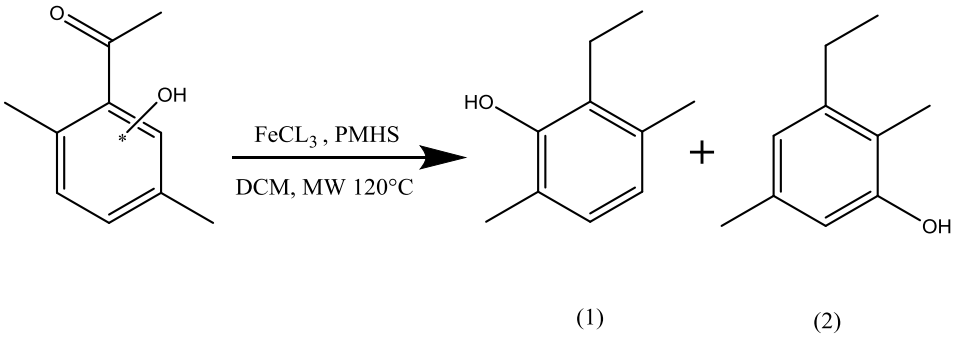

Figure 21 Final reduction of the 2,5-dimethyl-hydroxyacetophenones under microwave supported heating

As for the reaction with the 3,4-dimethyl isomers, this reduction had to be done twice to reach maximum yield. As opposed to the other substrate, here it was not possible to reach more than 50 % conversion. Also a lot of byproducts were formed in this reaction. A chromatogram of the crude reduction product is shown in Figure 22.

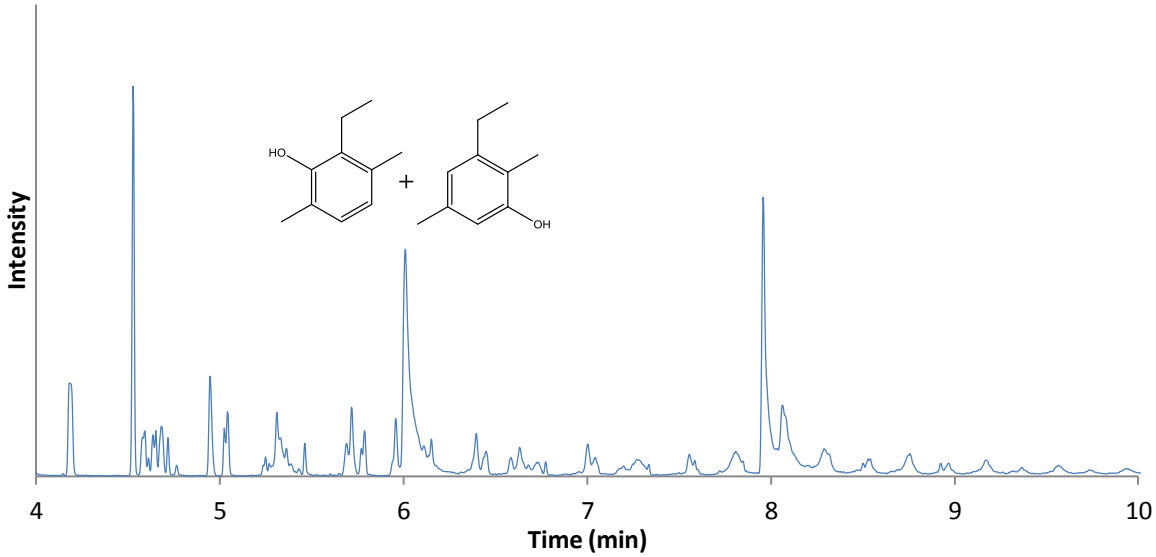

Figure 22 GC-MS analysis of the final reduction step

Due to the large number of unknown byproducts and the very low solubility of the mixture in aqueous solvents, a preparative separation of the isomers with HPLC was not possible in an acceptable quantity. For this reason the crude mixture was precleaned by flash chromatography to eliminate some impurities. A chromatogram of the cleaned sample is shown in Figure 23.

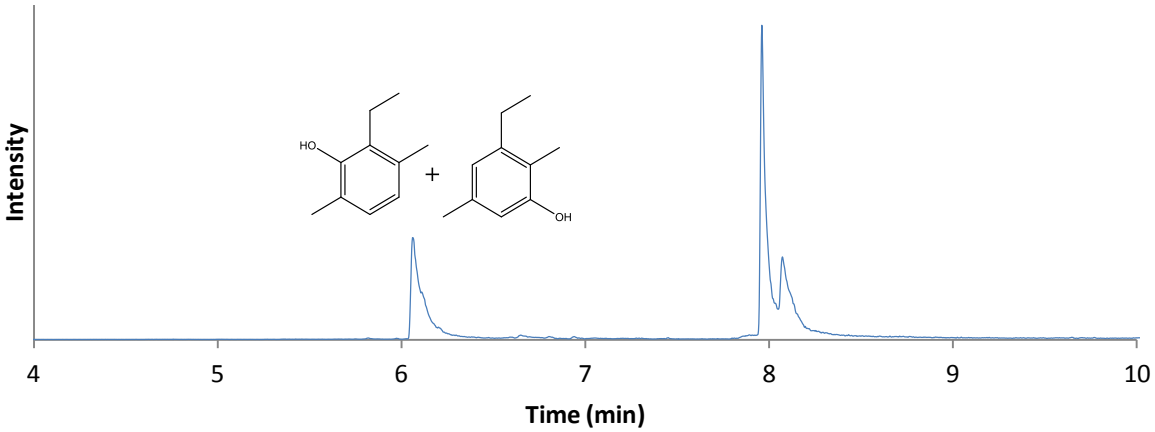

Figure 23 GC-MS analysis of the cleaning step with flash chromatography

As can be seen, most of the impurities were eliminated by this step. Compared to the area of the peaks at circa 8 minutes about 20% more of the phenol isomers than of the impurity were lost due to the chromatography. Since it was not possible to obtain cleaner products by flash chromatography and with the purpose of a separation of the isomers a HPLC method was developed.

Separation of the 2,5 – dimethyl isomers with RP-HPLC

As these isomers were suspected to show very similar chromatographic behavior as the 3,4- dimethyl isomers, the same conditions as above were tested. Chromatograms of the runs performed with acetonitril and water with the ratios of 35 – 60 % MeCN are shown in Figure 24.

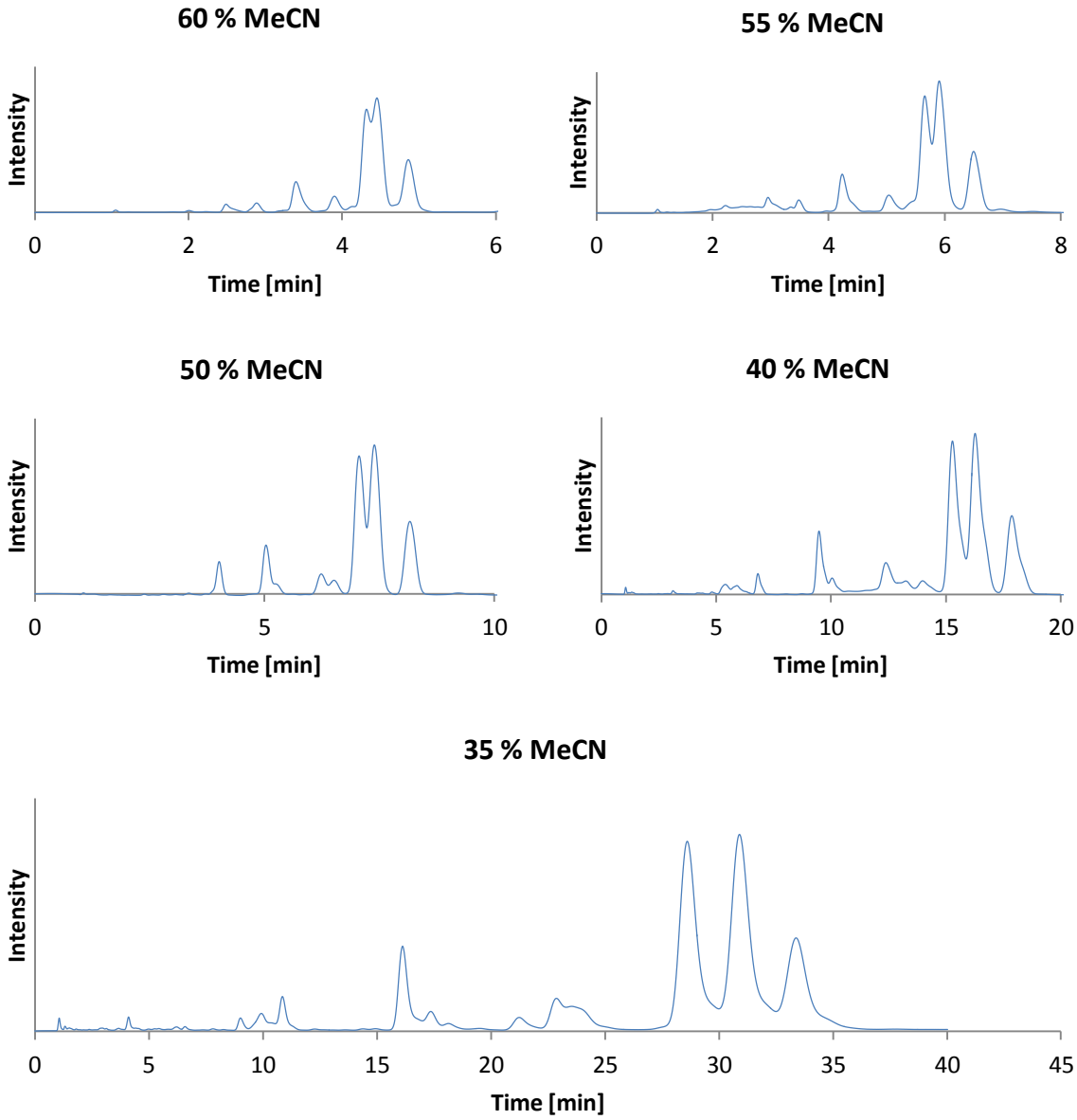

Figure 24 Chromatograms of the 2,5- dimethyl isomers; ZORBAX SB–C 18 150 x 4.6 mm 5 µm; MeCN : MilliQ 35- 60 % MeCN; Flow 1 ml/min, 25° C; Inj. Vol.: 5 µl Detection wavelength 210 nm

The two phenol isomers are represented by the two largest peaks in the chromatograms. The third largest peak represents residues of the educts of the synthesis due to incomplete conversion in the last step. In contrast to the 3,4-dimethyl isomers, it was not possible to obtain an adequate separation with a high amount of organic solvent. When using higher amounts of MeCN than 60 %, all substances co-eluted and showed just one broad peak. On the other hand, when lowering the MeCN below 35 %, the length of the chromatographic run increased to about 60 minutes and the peaks became extremely broad. The best separation was achieved with 35 % MeCN. Because the separation at 40 % MeCN was good enough

to collect fractions with a different ratio of isomers and the time was just half as long as when using 35 % MeCN, these conditions were used for a fractionation on an analytical column. For a separation with preparative HPLC, this method appeared to be not suitable because of the length of the chromatography needed to obtain not even baseline separated peaks. Another problem was the poor solubility of the synthesis mixture is in aqueous solvent mixtures. Therefore the concentrations of the samples had to be kept low in order to avoid problems like extreme broad peaks and not reproducible results.

2.2 GC-MS Analysis

The GC analysis was performed on two columns of different polarity, in order to obtain two different retention indices for each compound. The non polar ZB 5 MS column is a widely used standard column. Therefore the retention indices, obtained from measurements with this column should be comparable to a lot of literature references. Secondly, a medium polar ZB 624 column was used. For this column no RI values were found in the literature.

Because the analysis was only of qualitative nature and exclusively the retention times were of interest, the phenolic compounds were measured directly without any derivatization. Ten compounds with an exact mass of 150.22 g/mol were investigated in this work. To highlight the similarity in the mass spectra of these compounds, the spectra are shown in Figure 25.

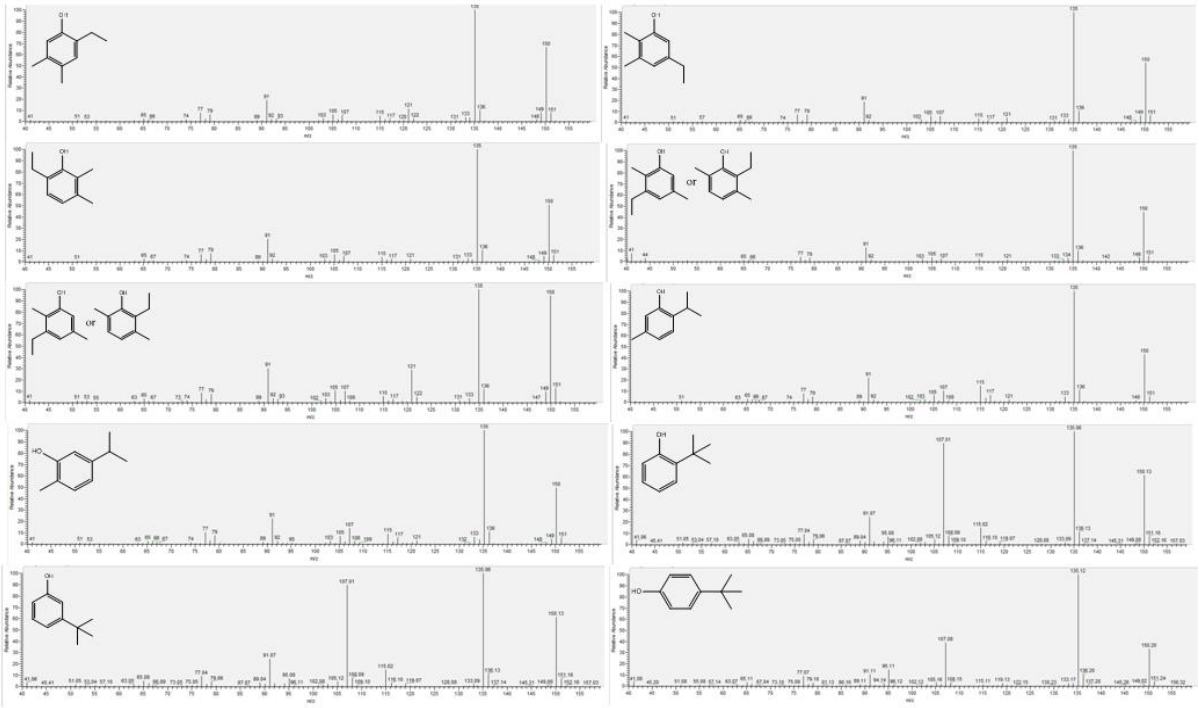

Figure 25 Mass spectra of the examined phenolic compounds with mass 150. (EI 70eV)

The spectra of compounds containing similar structural elements just differ in the intensity of molecular ion peak or some fragment peaks. Therefore a differentiation is only possible based on a valuable database. Additional three trimethylphenol isomers (136.09 g/mol) and the 2-tert.butyl-4-methylphenol (164.12 g/mol) were measured. These compounds could be clearly differentiated from the compounds with the mass 150 by their mass spectra. The pre-identification of the principal structure was done with a library search implemented in the QualBrowser of the Xcalibur software, using the “MAINLIB” from the NIST library. The accuracy of the library search is strongly dependent on the number of library entries of one compound. Some of the investigated compounds were not listed at all. Thus for the commercially available compounds as the tert.-butylphenol isomers, thymol and carvacrol the library search worked much better than for other, more exotic compounds. The library search results for the tert.-butylphenol isomers are listed in Table 5.

Table 5 Results from the automatic library search (Integration: average spectrum over total peakwidth): Xcalibur, QualBrowser V 2.0.7, MAINLIB

Hit	Probability	Compound
o-tert.-butylphenol		
1	**65.20**	**o-tert.-butylphenol**
2	10.92	p-tert.-butylphenol
3	9.23	m-tert.-butylphenol
m-tert.-butylphenol		
1	**37.18**	**m-tert.-butylphenol**
2	35.74	p-tert.-butylphenol
3	9.55	o-tert.-butylphenol
p-tert.-butylphenol		
1	**61.48**	**p-tert.-butylphenol**
2	20.60	m-tert.-butylphenol
3	4.78	o-tert.-butylphenol

All isomers were correctly identified within the first hit. Especially for the ortho and the para substituted isomer with 65.2 % and 61.48 % probability respectively, the identification via the implemented library search worked very good and gave a good hint to the correct structure. For the meta isomer the identification was correct with the first hit, but as second the para isomer with almost the same probability is listed. As a second example, the hit list of the two isopropyl-methylphenol isomers thymol and carvacrol is shown in Table 6.

Table 6 Results from the automatic library search (Integration: average spectrum over total peakwidth): Xcalibur, QualBrowser V 2.0.7, MAINLIB

Hit #	Probability [%]	Compound
Carvacrol		
1	**55.60**	**carvacrol**
2	15.75	4-isopropyl-3-methylphenol
3	10.49	4,5-dimethyl-2-ethylphenol
thymol		
1	24.60	carvacrol
2	**17.36**	**thymol**
3	9.55	4-isopropyl-3-methylphenol

Here the identification of carvacrol led to a good result with 55.8 % probability. For thymol the automatic search listed the wrong compound on the first place, but the basic structural elements for both hits are one isopropyl and one methyl group attached to a phenolic ring. From the dimethyl-ethylphenol isomers, only 4,5-dimethyl-2-ethylphenol is commercially available and only this compound is listed in the library as well. Whenever an automatic library search with one of the dimethyl-ethylphenol isomers was performed, the first hit was 4,5-dimethyl-2- ethylphenol. The probabilities ranged between 20 and 40 %. The highest probability was not achieved with the 4,5-dimethyl-2-ethylphenol sample. Also the trimethylphenol isomers and the 2-tert.butyl-4-methylphenol were pre-identified in this way. A pre identification regarding the basic structural elements of the residues attached to the phenol can be done very reliably with the automatic library search. For further information about the compound, the gas chromatographic data or more specifically the retention indices are useful values. For the calculation of the retention indices an n-alkane standard (C9 – C30) was measured under the same conditions as the samples. The chromatogram of the standard, measured with the ZB5-MS column, is shown in Figure 26.

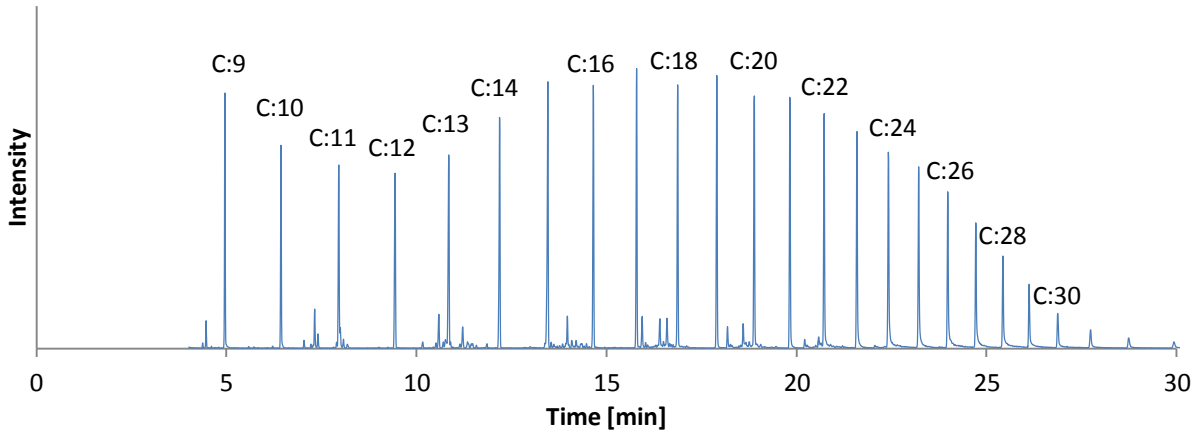

Figure 26 Chromatogram of the n-alkane standard (C : 9 – C : 30) used for the calculation of the retention indices; ZB-5-MS

In this chromatogram, some small peaks show up between the peaks representing the alkanes. In order to get cleaner chromatograms, these impurity peaks are smoothed out in the following figures where the alkane standard is used. Due to the

structural similarity of the investigated compounds, their retention behavior does not differ significantly as well. With the setup used, all investigated compounds elute within one minute. An overlay of the investigated isomers and the alkane standard are shown in Figure 27.

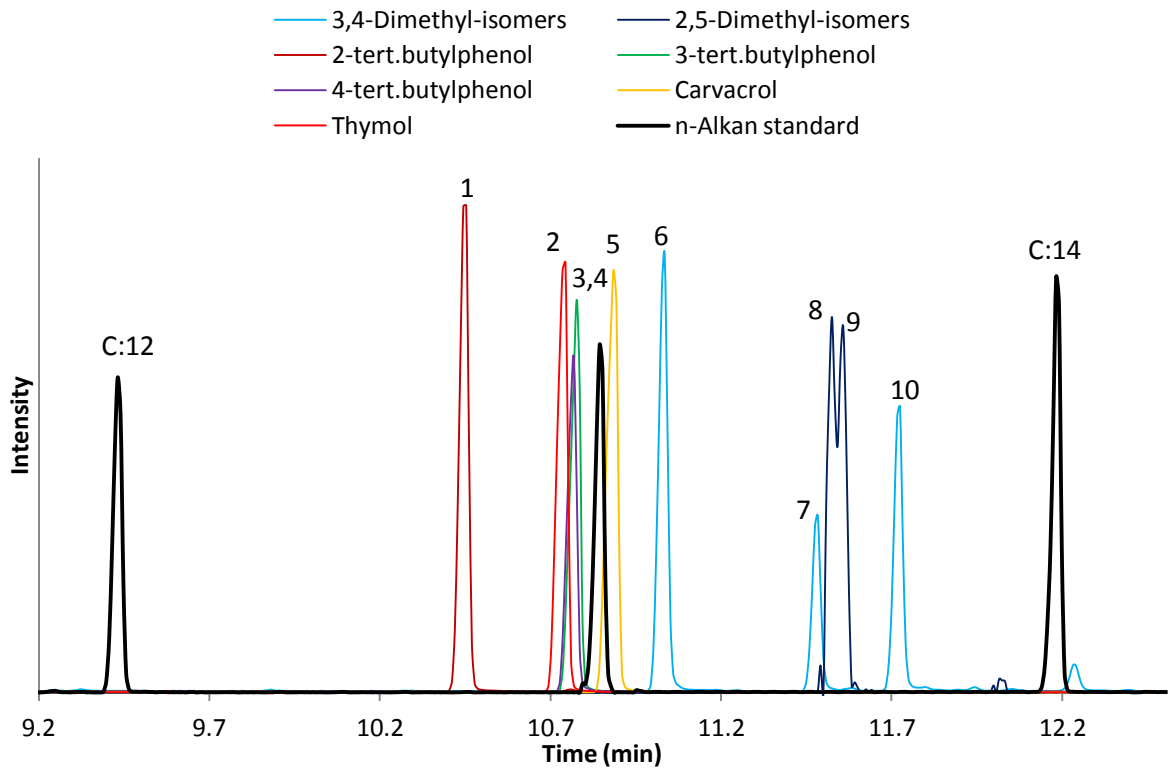

Figure 27 Overlay of the investigated components and the alkane standard, measured with the ZB 5 column; 1: 2 - tert-butylphenol; 2: Thymol; 3: 4 - tert-butylphenol; 4: 3 - tert-butylphenol; 5: Carvacrol; 6: 2,3-dimethyl-6-ethylphenol; 7: 3,4-dimethyl-6-ethylphenol; 8: 2,5-dime-1; 9: 2,5-dime-2; 10: 2,3-dimethyl-5-ethylphenol

The numbering of the compounds was done according to the order of elution on the ZB-5 column. This numbering was used for the measurement with the ZB-624 too. All peaks are located between the C:12 and C:14 alkanes. Most of them group around the C:13 peak. For a clearer illustration of the peaks aggregated around 10.75 minutes, this area is shown in a smaller scale in Figure 28.

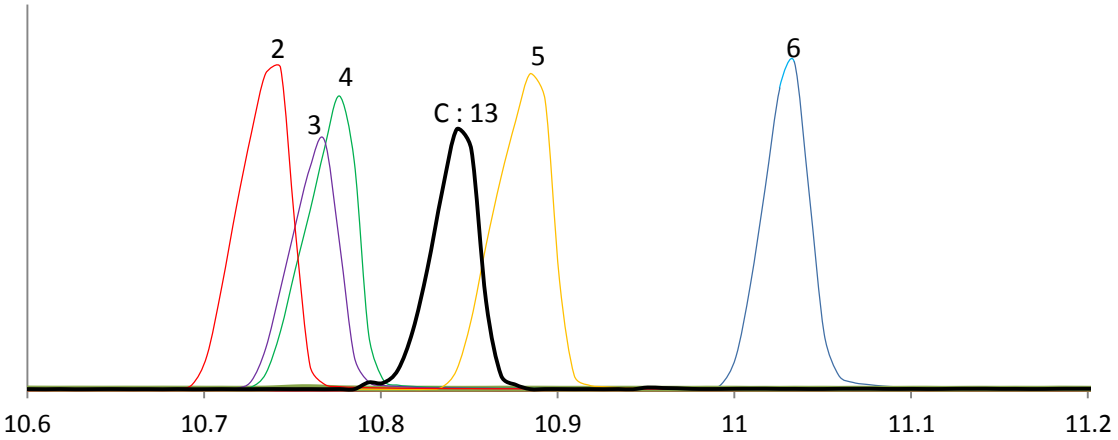

Figure 28 Overlay of the chromatograms: 2 - tert-butylphenol; 2: Thymol; 3: 4 - tert-butylphenol; 4: 3 - tert-butylphenol; 5: Carvacrol; 6: 2,3-dimethyl-6-ethylphenol

As the retention times of the peaks do not differ very much, also the RI values are very close together. The components 3 and 4 (o- and p-tert.-butylphenol) could not be separated at all. Although this overlay did not rule out the possibility that a differentiation could be possible, both substances eluted in one symmetric peak when injected together. The retention indices calculated with the equation of Van den Dool and Kratz are shown in Table 7.

Table 7 Overview of the Retention times (RT) and retention indices (I) with the ZB-5 column Literature: 1: Mjøs, Meier, et al., 2006; 2 Salido, Valenzuela, et al., 2004; 3 Zenkevich, Makarov A.A., et al., 2009; 4 Javidnia, Miri, et al., 2005

#	Compound	CAS #	RT	I	I (lit)
1	2 – tert.-butylphenol	88-18-6	10.45	1272	1273[1]
2	Thymol	89-83-8	10.76	1294	1294[2]
3	4 – tert.-butylphenol	98-54-4	10.77	1295	1292[3]
4	3 – tert.-butylphenol	585-34-2	10.78	1296	1296[1]
5	Carvacrol	499-75-2	10.9	1304	1304[4]
6	2,3-dimethyl-6-ethylphenol	18441-55-9	11.04	1315	--
7	3,4-dimethyl-6-ethylphenol	2219-78-5	11.48	1348	--
8	2,5-dime-1	---	11.52	1351	--
9	2,5-dime-2	---	11.56	1354	--
10	2,3-dimethyl-5-ethylphenol	---	11.72	1366	--

The calculated RI values well correspond to the values found in literature, but just for the half of the compounds such literature values were available. Also the chosen heating rate with 10°C/minute is much higher than most of the heating rates used in literature. According to the NIST database, the common heating rates in the literature range between 2 and 5°C/minute. Furthermore it could be shown that according to the ± 3 values accuracy of the RI´s it is not possible to clearly distinguish between all the examined compounds. Especially the m- and p-tert.-butylphenol isomers could not be separated at all and also their mass spectra are almost identically. Therefore these compounds cannot be differentiated with this method. Although thymol also has a very similar RI, its mass spectrum is different enough to allow a differentiation from the tert.-butylphenol isomers. Also the compounds number 7, 8 and 9, all of which with very similar mass spectra are not distinguishable with the method used. The compounds 8 and 9 could not even be baseline separated with the temperature program used. In order to obtain a second RI from a column of different polarity, the n-alkane and the component standards were measured with a ZB-624 column of medium polarity. The chromatogram of the n-alkane standard is shown in Figure 29.

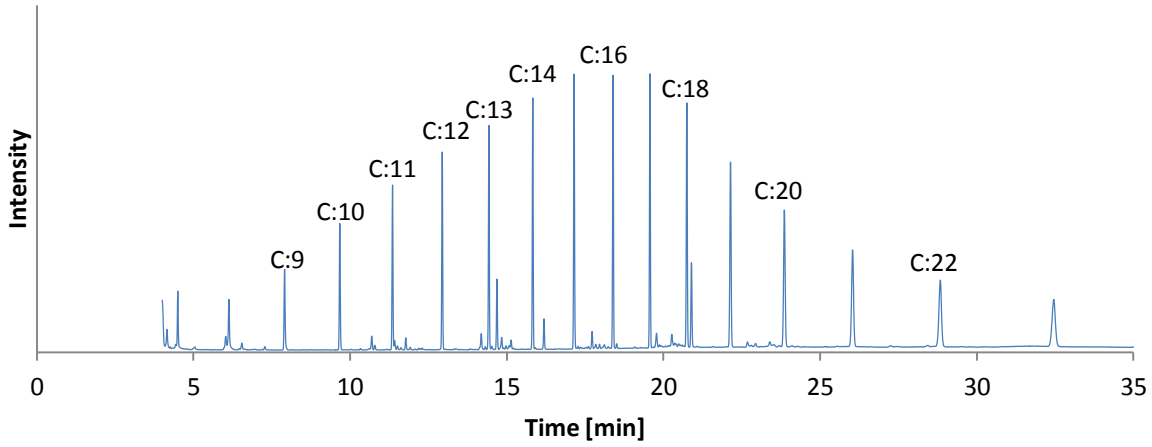

Figure 29 Chromatogram of the n-alkan standard (C : 9 – C : 22) used for the calculation of the retention indices; ZB-624

When measuring the n-alkane standard with the ZB-624 column almost the same situation, according to the distance of the neighboring alkane peaks in the range of interest was achieved as with the ZB-5 column. The behavior of the peak distances for both columns is shown in Figure 30.

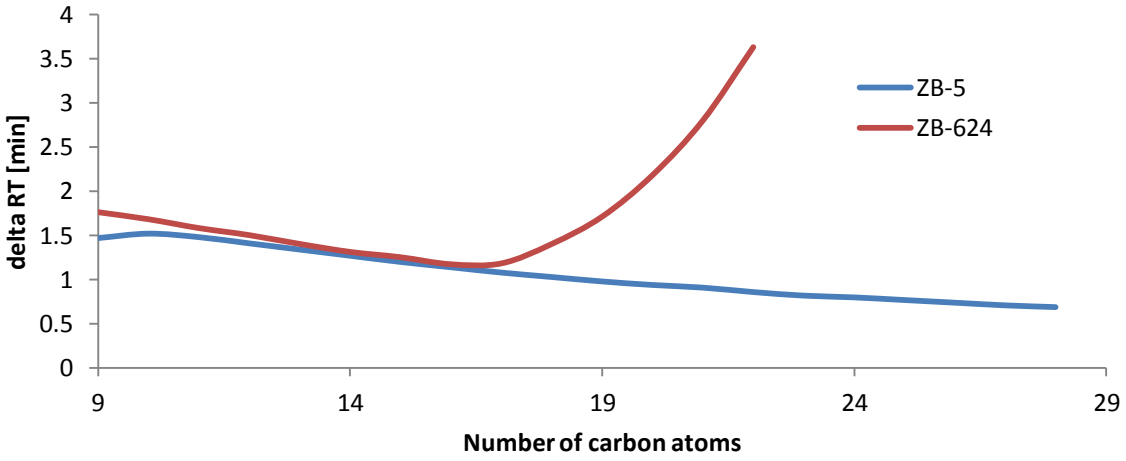

Figure 30 Distances between the n-alkane peaks ZB-5 vs. ZB-624

Both columns started with almost the same distance of the first peaks. From C:9 to C:16 both columns also showed the same trend of almost linear decrease of the peak distances. From C:17 on, the distance between the alkane peak exhibited an exponential increase. Due to this behavior, it was just possible to record 23 alkane peaks in a time of 35 minutes.

In this case again the impurity peaks between the alkane peaks are smoothed out in the following figures to get clearer chromatograms. An overlay of the sample chromatograms with the alkane standard measured with the ZB- 624 column is shown in Figure 31.

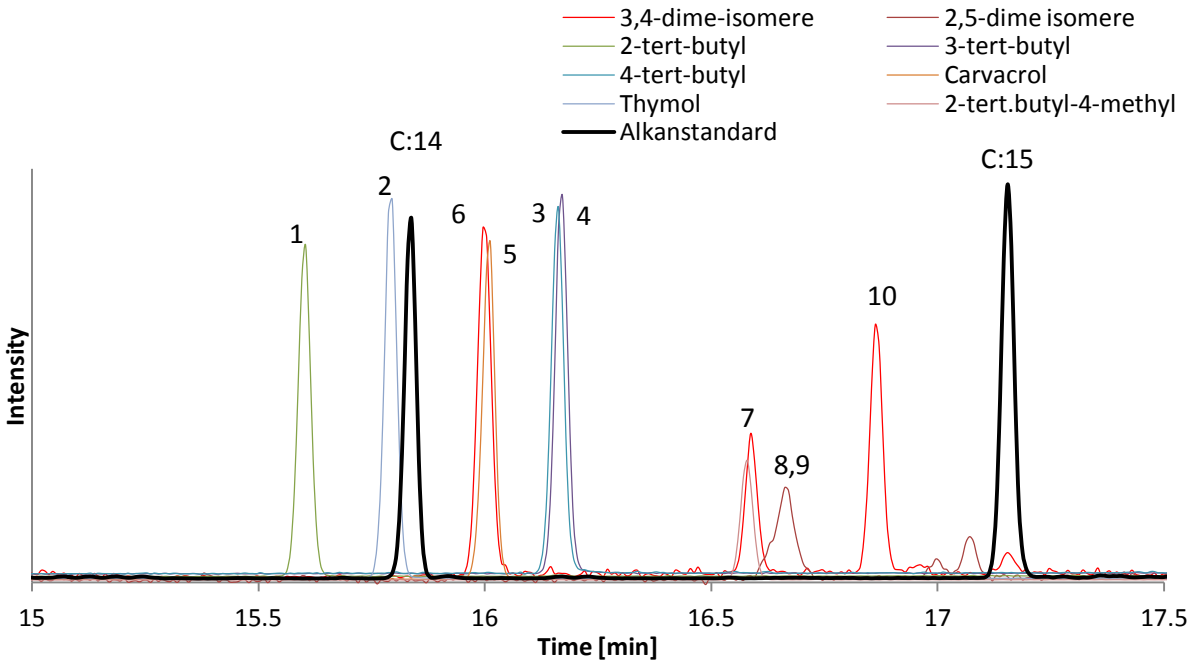

Figure 31 Overlay of the investigated components and the alkane standard, measured with the ZB 624 column; 1: 2 – tert-butylphenol; 2: Thymol; 3: 4 – tert-butylphenol; 4: 3 – tert-butylphenol; 5: Carvacrol; 6: 2,3-dimethyl-6-ethylphenol; 7: 3,4-dimethyl-6-ethylphenol; 8: 2,5-dime-1; 9: 2,5-dime-2; 10: 2,3-dimethyl-5-ethylphenol

When using the ZB-624 column, the situation did not change significantly. Especially the components of interest (6-10) showed no change in separation. The tert.butylphenol isomers 3 and 4 again could not be separated on this column. Component number 6 co-eluted with number 5 under this setup, whereas these compounds were separated on the ZB-5 column. A list of the retention times and the calculated RI´s is shown in Table 8.

Table 8 Overview of the Retention times (RT) and retention indices (I) with the ZB-624 column

#	Compound	CAS #	RT	I
1	2 – tert.-butylphenol	88-18-6	15.6	1377
2	Thymol	89-83-8	15.79	1390
3	4 – tert.-butylphenol	98-54-4	16.16	1415
4	3 – tert.-butylphenol	585-34-2	16.17	1415
5	Carvacrol	499-75-2	16.01	1405
6	2,3-dimethyl-6-ethylphenol	18441-55-9	16	1404
7	3,4-dimethyl-6-ethylphenol	2219-78-5	16.6	1444
8	2,5-dime-1	---	16.66	1448
9	2,5-dime-2	---	16.66	1448
10	2,3-dimethyl-5-ethylphenol	---	16.86	1461

Since there was not one comparable RI found in literature, the values for the medium polar column are omitted from this table. Generally it can be seen that the compounds that showed similar RI values with the ZB-5 column, also did this on the ZB-624 column.

3 Conclusion

For the identification of unknown compounds in complex mixtures GC-MS is a powerful combination. The major drawback of this technique is that authentic reference standards are needed to verify the structure of the components. If these standards are not commercially available, they have to be properly synthesized. As shown in this work, even if the compounds are not very complex, the synthesis may require a well suited laboratory with experienced staff. If the number of possible compounds is too high, it might be almost impossible (mostly because of economic reasons) to identify some compounds. In preliminary investigations to this work with GC-MS analysis and literature research it was possible to restrict the number of possible compounds to 16 structures. The synthesis of the five preferred compounds led to no positive match. This clearly shows that the availability of sophisticated

databases with related GC and MS data would be a great benefit and would also lead to a faster progress in the understanding of natural processes and developments. It is of crucial importance to promote interdisciplinary cooperation for the development of useable data sets for all research fields.

4 Experimental section

4.1 Materials and Methods

Synthesis

Three different microwave reactors were used for the syntheses. One was a Biotage Initiator 2.5 equipped with a robot Eight autosampler (Biotage, Uppsala, Sweden). As second, a Biotage Optimizer (Biotage, Uppsala, Sweden). The third reactor was a Monowave 300, equipped with an MAS 24 Autosampler (Anton Paar GmbH, Graz, Austria). Method: Heat as fast as possible to the desired reaction temperature, stirring at 600 RPM and cooling to 50° C with pressurized air. The temperature was monitored from outside of the vial with IR.

The chromatographic system used for the sample cleanup was a Biotage SP1 flash chromatography purification system (Biotage, Uppsala, Sweden). The columns used were 12+, 25+ and 40+ normal phase silica cartridges. As solvent, a mixture of ethyl acetate and petroleum ether was used.

As GC-MS system to monitor the synthesis progress, a Focus GC coupled with a DSQ II MS (EI, 70 eV) (Thermo Scientific, Massachusetts, USA) was used. As column, a HP5-MS (30 m × 0.25 mm i.d. × 2.5 μm film thickness) with Helium 5.0 at 1 mL/min (constant flow) as carrier gas was used.

For the chromatographic separation an Agilent 1200 series HPLC system was used. The system consisted of a 1260 HiP degasser, a 1260 binary pump, a 1260 autosampler system connected to a 1290 thermostat, a 1260 column oven (Agilent Technologies GmbH, Waldbronn, Germany) and a 1260 VWD VL detector (Agilent Technologies GmbH, Waldbronn, Germany) with a standard detection cell. As analytical-column, the C18 modified reversed phase column ZORBAX SB – C 18 150 x 4.6 mm with 5 µm particle size was used (Agilent Technologies GmbH, Waldbronn, Germany).

The organic solvents used as eluents were: Acetonitrile Rotisolv® HPLC grade (CARL ROTH GMBH + CO. KG, Karlsruhe, Germany), Methanol Rotisolv® HPLC grade (CARL ROTH GMBH + CO. KG, Karlsruhe, Germany), Isopropanol Rotisolv® ≥99,9% UV / IR grade (CARL ROTH GMBH + CO. KG, Karlsruhe, Germany) and Tetrahydrofuran Rotisolv® HPLC grade, not stabilized (CARL ROTH GMBH + CO. KG, Karlsruhe, Germany). The deionized water (Milli-Q, 18.2 MΩ*cm), used for the chromatography was prepared in house by a Milli-Q system (Millipore, Bedford, Massachusetts, USA).

All samples were prepared in 1,5 ml HPLC glass vials with screw caps (Agilent Technologies GmbH, Waldbronn, Germany) with a 1:1 mixture of the solvent used for the specific chromatography and deionized water.

Analysis

The samples were examined with 2 GC-MS systems. System number one was a Voyager GC-MS (EI, 70 eV) with a Trace 2000 GC (Thermo Scientific, Massachusetts, USA), equipped with a ZB 624 fused silica capillary column (30 m x 0.25mm i.d., 1.4 μm film thickness) from Phenomenex (Phenomenex, Torrance CA, USA). As carrier gas, Helium 6.0 was used (Messer, Graz, Austria). The injector temperature was set to 240° C. The starting temperature was 50° C, which was held for 1 minute. Then heating with 10° C / min to 240° C and held again for 5 minutes at 240° C. The interface temperature was 245° C. System number two was a Focus GC coupled with a DSQ II MS (EI, 70 eV) (Thermo Scientific, Massachusetts, USA). For the separation, a ZB5-MS column (30 m × 0.25 mm i.d. × 0.025 mm film thickness) (Phenomenex, Torrance CA, USA) with Helium 6.0 (Messer, Graz, Austria) at 1 mL/min (constant flow) as carrier gas. The injector temperature was set to 240° C. The starting temperature was 50° C, which was held for 1 minute. Then heating with 10° C / min to 300° C and held again for 5 minutes at 300° C. The interface temperature was 300° C.

The Samples were prepared in 1,5 ml screw cap glass vials with Hexane (CHROMASOLV®, ≥97.0% (GC) Sigma-Aldrich) as solvent.

4.2 Synthesis overview

In this work 5 different phenol isomers were synthesized in order to obtain standards for the GCMS analysis. The syntheses were done with two different starting materials. One was a 3,4-dimethylacetophenone, the other was a 2,5-dimethylacetophenone. Each of these substrates gives three possible phenol isomers. The resulting isomers are listed in Table 9.

Table 9 Overview of the synthesis products

Starting material	Product
3,4-dimethylacetophenone	2,3-dimethyl-6-ethylphenol
	2,3-dimethyl-5-ethylphenol
	4,5-dimethyl-2-ethylphenol
2,5-dimethylacetophenone	2,5-dimethyl-3-ethylphenol
	2,5-dimethyl-4-ethylphenol
	2,5-dimethyl-6-ethylphenol

In principle the synthesis was done in four steps according to a paper of Morgan and Pettet (1934). The first step was the nitration of the starting material, followed by a reduction of the nitro group to the amine. The amine group was then changed to a hydroxyl group by diazotization and cooking. Finally, the carboxyl group was reduced to an ethyl group to reach the final product. The detailed procedure is given in the following paragraphs.

4.3 Synthesis procedures

Nitration

with H_2SO_4/HNO_3

A solution of 6.5 ml (48.4 mmol) dimethylacetophenone and 15 ml concentrated sulfuric acid was prepared in a two necked flask. The solution was stirred well and cooled on an ice / salt bath. A mixture of 4 ml concentrated HNO_3 and 6 ml concentrated H_2SO_4 was added dropwise, that the temperature never exceeded -5°C. After finishing the addition the reaction mixture was stirred for about 15 minutes at -8°C. Pouring the solution on ice and water led to a precipitate, which was

extracted with ethylacetate. The organic phase was washed free of acid with a Na_2CO_3 solution and dried with Na_2CO_3. The dry solution was then concentrated under vacuum to obtain the final nitro compounds as a brown oil (Morgan and Pettet 1934).

with P_2O_5 on SiO_2

Preparation of the P_2O_5 on silica: 4.5 g (31.7 mmol) P_2O_5 were mixed with 2.5 g SiO_2 (60-100 mesh) in a mortar and ground to homogeneity.
Nitration: 2 g P_2O_5 on SiO_2, were mixed with 0,15 g dimethylacetophenone in a mortar and ground to homogeneity. This resulted in a yellow paste. 0.5 ml HNO_3 were added dropwise while constantly grinding the mixture with a pestle. The grinding was continued for two minutes after the addition of HNO_3 was finished. The product was extracted with 15 ml diethylether, washed with Na_2CO_3 and dried with $MgSO_4$. The dry solution was then concentrated under vacuum to obtain the final nitro compounds as a brown oil (Hajipour and Ruoho 2005).

Reduction to the Amine

Fe/HCl (oil bath)

400 mg dimethyl-nitro-acetophenone was suspended in 100 ml 1% HCl at 95-100°C in a three necked flask. During 1 hour, 550 mg of iron fillings were added to the stirred suspension. After finishing the addition, the mixture was made alkaline by the addition of ammonia which led to a precipitation. The precipitate was extracted with ethyl acetate, dried with $MgSO_4$ and concentrated under vacuum (Morgan and Pettet 1934).

Pd/C catalyzed (MW-heating)

965 mg (5 mmol) dimethyl-nitro-acetophenone, 82 mg (0.08mmol) Pd/C, 2 ml cyclohexene and 15 ml of ethanol were mixed in a 20 ml microwave vessel, assembled with a magnetic stir bar. The reaction was carried out in the microwave reactor at 160°C for 15 minutes. After cooling down to 50° C another 40 mg (0.04mmol) Pd/C and 1 ml cyclohexene were added and the heating process was repeated for another 5 minutes. After filtration through a plug of celite, the product was concentrated under vacuum (Glasnov et al. 2009)

Diazotisation

166 mg (1.02 mmol) 3-amino-4-methylacetophenone was dissolved in 32 ml 3% sulfuric acid and cooled on an ice bath. After the addition of 76 mg (1.1 mmol) $NaNO_2$ in 6 ml water, the reaction mixture was stirred for 1 hour at -6° C. Then the acid concentration was increased to about 30% by adding some concentrated sulfuric acid. The acidic solution was then heated to 70°C for 15 minutes. After cooling to room temperature, the product was extracted with ethyl acetate washed free of acid with a Na_2CO_3 solution and dried with Na_2CO_3. The solvent was evaporated under vacuum and the product was purified with flash chromatography to obtain a dimethyl-hydroxy-acetophenone isomer mixture (Wiedermannová et al. 2003).

Reduction of the keto function

To a solution of 0.15 mmol hydroxy-dimethylacetophenone in 4 ml DCE, 0,015 mmol $FeCl_3$ and 0.4 mmol PMHS (viscosity 15-40 mPa*s) were added in a 5 ml microwave vessel, equipped with a magnetic stir bar. The reaction was done in a microwave reactor for 1 h at 120°C. After cooling to 50°C another 0.015 mmol $FeCl_3$ and 0.4 mmol PMHS were added and the heating step was repeated. After checking the success of the reaction with GC-MS, the mixture was filtered through a plug of celite and concentrated under vacuum. Finally the crude material was cleaned with flash chromatography to obtain a mixture of dimethyl-ethylphenols (Dal Zotto et al. 2009).

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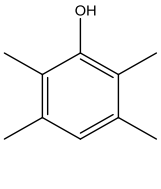
6 Appendix

Structures, IUPAC names and CAS numbers of all possible alkyl substituted phenol isomers with a mass of 150 amu

Tetramethylphenol



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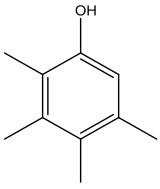

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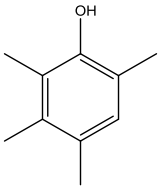
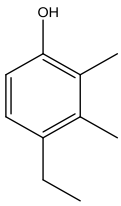


(3)

Dimethyl – ethylphenol



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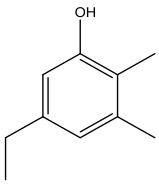

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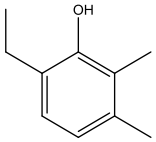

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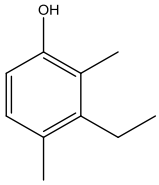
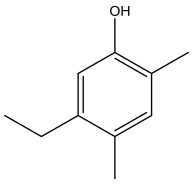


(7)



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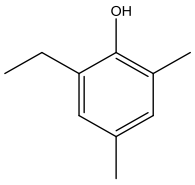

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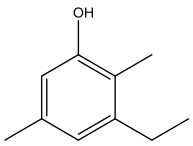

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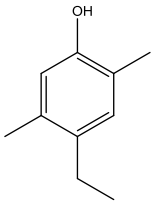

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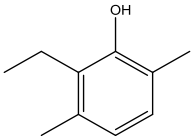

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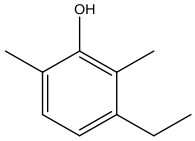

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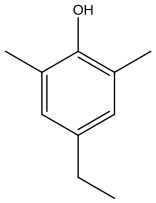

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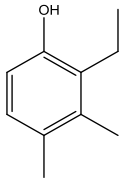

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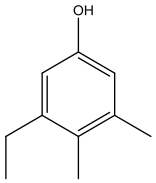

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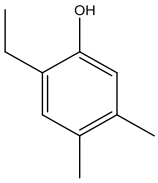

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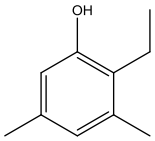

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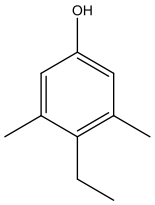
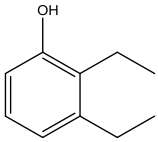


(19)

Diethylphenol



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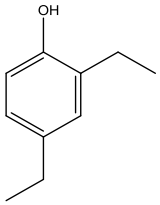

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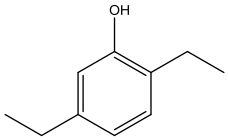

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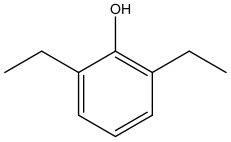

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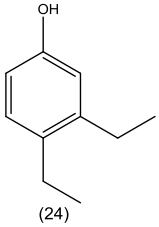

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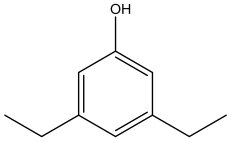
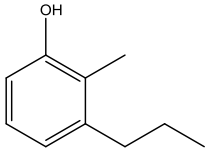


(25)

Methyl-Propylphenol



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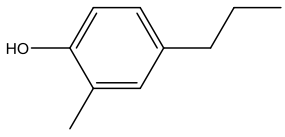

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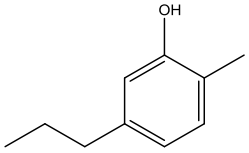

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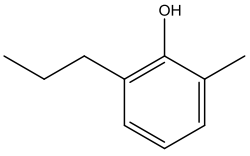

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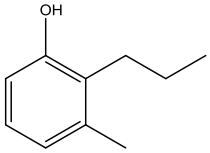

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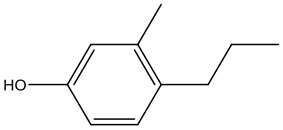

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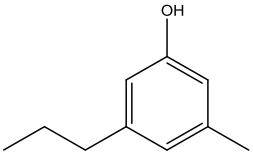

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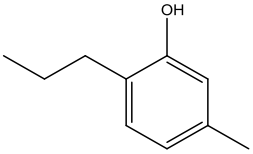

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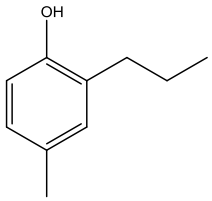

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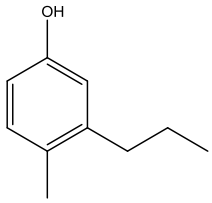
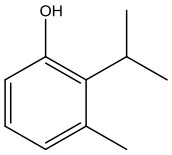


(35)

Isopropyl-methylphenol



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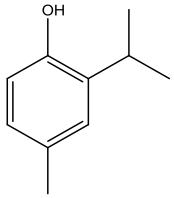

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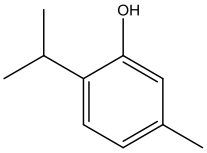

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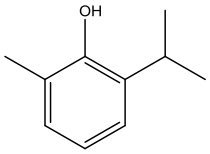

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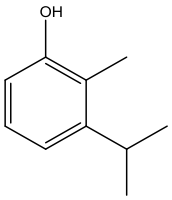

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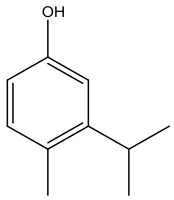

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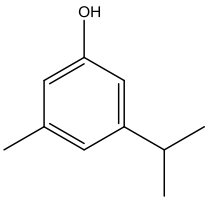

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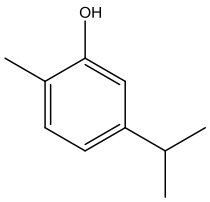

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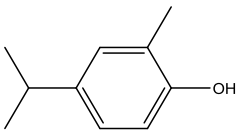

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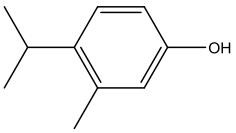
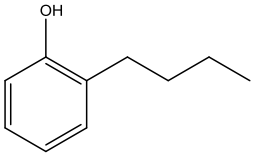


(45)

Butylphenol



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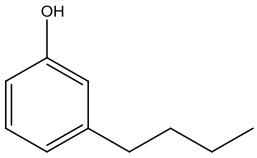

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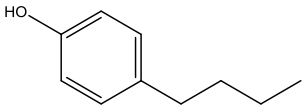
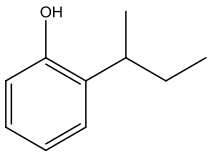


(48)

Sec.-butylphenol



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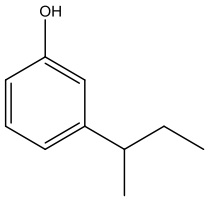

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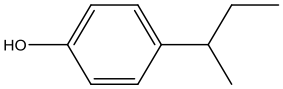
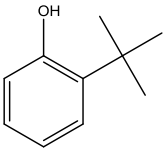


(51)

Tert.-butylphenol



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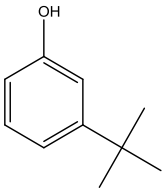

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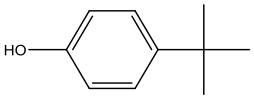
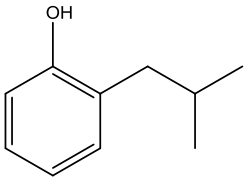


(54)

Isobutylphenol



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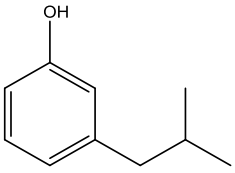

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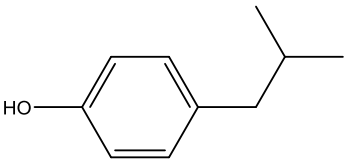

(57)

Number	Name	CAS #	RI/MS (NIST)
	Tetramethylphenol		
1	2,3,4,5 - Tetramethylphenol	788-70-0	N
2	2,3,4,6 - Tetramethylphenol	28449-98-1	N
3	2,3,5,6 - Tetramethylphenol	527-35-5	Y
	Dimethyl - Ethylphenol		
4	2,3- Dimethyl - 4 - Ethylphenol	66142-72-1	N
5	2,3- Dimethyl - 5 - Ethylphenol	---	N
6	2,3- Dimethyl - 6 - Ethylphenol	18441-55-9	N
7	2,4- Dimethyl - 3 - Ethylphenol	62126-74-3	N
8	2,4- Dimethyl - 5 - Ethylphenol	18441-55-9	N
9	2,4- Dimethyl - 6 - Ethylphenol	2219-79-6	N
10	2,5- Dimethyl - 3 - Ethylphenol	---	N
11	2,5- Dimethyl - 4 - Ethylphenol	32018-76-1	N
12	2,5- Dimethyl - 6 - Ethylphenol	---	N
13	2,6- Dimethyl - 3 - Ethylphenol	---	N
14	2,6- Dimethyl - 4 - Ethylphenol	10570-69-1	N
15	3,4- Dimethyl - 2 - Ethylphenol	---	N
16	3,4- Dimethyl - 5 - Ethylphenol	62126-75-4	N
17	3,4- Dimethyl - 6 - Ethylphenol	2219-78-5	Y
18	3,5- Dimethyl - 2 - Ethylphenol	62126-76-5	N
19	3,5- Dimethyl - 4 - Ethylphenol	62126-77-6	N
	Diethylphenol		
20	2,3 - Diethylphenol	66142-71-0	N
21	2,4 - Diethylphenol	936-89-0	N
22	2,5 - Diethylphenol	26967-65-7	N
23	2,6 - Diethylphenol	1006-59-3	Y
24	3,4 - Diethylphenol	875-85-4	Y
25	3,5 - Diethylphenol	1197-34-8	N
	Methyl - Propylphenol		
26	2 - Methyl - 3 - Propylphenol	66142-78-7	N
27	2 - Methyl - 4 - Propylphenol	18441-56-0	Y
28	2 - Methyl - 5 - Propylphenol	7786-21-2	N
29	2 - Methyl - 6 - Propylphenol	3520-52-3	N

Number	Name	CAS #	RI/MS (NIST)
30	3 - Methyl - 2 - Propylphenol	62744-64-3	N
31	3 - Methyl - 4 - Propylphenol	---	N
32	3 - Methyl - 5 - Propylphenol	36186-96-6	N
33	3 - Methyl - 6 - Propylphenol	31143-55-2	N
34	4 - Methyl - 2 - Propylphenol	4074-46-8	N
35	4 - Methyl - 3 - Propylphenol	61783-87-7	N
Isopropyl- Methylphenol			
36	2 - Isopropyl - 3 - Methylphenol	3228-01-1	N
37	2 - Isopropyl - 4 - Methylphenol	4427-56-9	Y
38	2 - Isopropyl - 5 - Methylphenol	89-83-8	N
39	2 - Isopropyl - 6 - Methylphenol	3228-04-4	N
40	3 - Isopropyl - 2 - Methylphenol	4371-48-6	N
41	3 - Isopropyl - 4 - Methylphenol	4371-46-4	N
42	3 - Isopropyl - 5 - Methylphenol	3228-03-3	N
43	3 - Isopropyl - 6 - Methylphenol	499-75-2	N
44	4 - Isopropyl - 2 - Methylphenol	1740-97-2	N
45	4 - Isopropyl - 3 - Methylphenol	3228-02-2	Y
Butylphenol			
46	2 - Butylphenol	3180-09-4	N
47	3 - Butylphenol	4074-43-5	Y
48	4 - Butylphenol	1638-22-8	Y
Sec- Butylphenol			
49	2 - Sec - Butylphenol	89-72-5	Y
50	3 - Sec - Butylphenol	3522-86-9	N
51	4 - Sec - Butylphenol	99-71-8	Y
Tert - Butylphenol			
52	2 - tert - Butylphenol	88-18-6	Y
53	3 - tert - Butylphenol	585-34-2	Y
54	4 - tert - Butylphenol	98-54-4	Y
Isobutylphenol			
55	2 - Isobutylphenol	31195-95-6	N
56	3 - Isobutylphenol	30749-25-8	N
57	4 - Isobutylphenol	4167-74-2	Y