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Determination of Abietic Acid and its Degradation Products in Cellulose Based Materials

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Vorwort

Während der Produktion von auf Cellulose basierenden Materialen werden Harze oft als technische Hilfsstoffe zu unterschiedlichsten Zwecken eingesetzt. Diese Harze sind zum Teil komplexe Gemische, zu deren Hauptbestandteilen Abietinsäure und ihr häufigstes Abbauprodukt Dehydroabietinsäure zählen. Im Laufe der Herstellung, Weiterverarbeitung und Lagerung kann es durch Oxidation und weitere chemische Reaktionen zum Abbau dieser zwei Hauptbestandteile kommen. Das Problem dabei ist, dass im Gegensatz zu den zwei Hauptprodukten, die kein allergenes Potential aufweisen, den Abbauprodukten ein kontaktallergenes Potential zuzuschreiben ist. Ziel dieser Arbeit war es den aktuellen Stand des Wissens und der Technik im Hinblick auf allergenes Potential von Abietinsäure und deren Abbauprodukte zu ermitteln und die gängigen Analysenmethoden zu vergleichen und zusammenzufassen. Mit Hilfe dieses Wissen wurden Methoden für die qualitative und quantitative Analyse von Abietinsäure und Abbauprodukten in auf Cellulose basierenden Materialien entwickelt. Dafür wurden die beiden analytischen Methoden Gaschromatographie mit Massenspektrometrie und Flüssigchromatographie mit Massenspektrometrie verwendet und miteinander verglichen. Am Ende wurde mit den entwickelten Methoden eine Warenkorbstudie von Hygieneartikeln und ihren Verpackungen durchgeführt und die Belastung dieser mit den möglichen Allergenen untersucht.

Abstract

During the production of cellulose based materials resins are added as technical auxiliaries often. This resins could be complex mixtures with abietic acid and its main degradation product dehydroabietic acid as major constituents. During production, further processing and storage oxidation and other chemical reactions could take place resulting in the degradation of abietic acid and dehydroabietic acid. This could be a problem since the degradation products contrary to the major substances are considered to have a contact allergenic potential. Aim of this work was to find the state-of-the-art of science and technology of the allergenic potential of abietic acid and its degradation products and to summarise and compare the analytical methods used for analysis. Using this knowledge, methods for the qualitative and quantitative analysis of abietic acid and its degradation products in cellulose based materials were developed using gas-chromatography mass spectrometry and liquid-chromatography mass spectrometry. At the end a market analysis of hygienic products was made using the developed methods to point out the concentrations of the potential allergens in the products.

Danksagung

An dieser Stelle möchte ich an alle, die mir in den letzten fünf Jahren zur Seite gestanden sind und mich unterstützt haben ein großes Dankeschön aussprechen.

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I. Table of Content

II. List of Abbreviations

%	percent
(v/v)	Volume per volume percent
(w/w)	Weight per weight percent
°C	Degree celcius
µg	Microgram
µl	Microliters
15-HPA	15-Hydroperoxyabietic acid
15-HPDA	15-Hydroperoxydehydroabietic acid
AA	Abietic Acid
ACN	Acetonitrile
BSTFA	N,O-Bis(trimtethylsilyl)trifluoracetamide
$CHCl_3$	Chloroform
d	Days
DAD	Diode array detector
DHA	Dehydroabietic Acid
dm^2	Cubic decimeter
EI	Electron impact ionisation
ESI	Electron spray ionisation
EFSA	European food Safety Authority
EU	European Union
et al.	and others (referring to authors)
Et_2O	Diethyl Ether
EtOH	Ethanol
FCM	Food contact material
FID	Flame ionisation detector
g	Gram
GC	Gas chromatography
h	Hours
He	Helium
H_2O	Water
HPLC	High pressure liquid chromatography

IAS	Intentionally added sustances
kg	Kilogram
L	Liter
LC	Liquid chromatography
m	Meter
MDHS	Methods for the Determination of Hazardous Substances
MeOH	Methanol
mg	Milligram
min	Minutes
ml	Milliliters
mm	Millimeters
mmol	Millimol
mol	Mol
MS	Mass spectrometry
MTBE	Methyl tert.- Butylether
NIAS	Non-intentionally added substances
No.	Number
N_2	Nitrogen (gas)
PFB	Pentafluorobenzyl bromide
RI	Retention Index
sec	Seconds
SPE	Solid phase extraction
t_R	Retention time

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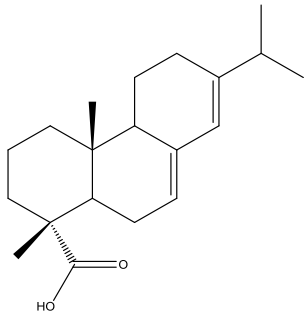
1. Introduction

1.1. Abietic Acid

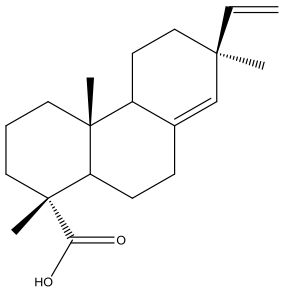
1.1.1. General

The word colophony is derived from Colophon, an ancient town on the west coast of Asia Minor that was famous for its colophony production. The name has been used to name either the crude pine exudate or the residue left after distillation of the more volatile part (1). Today the name colophony is synonymous to colophonium, rosin and pine resin.

Colophony is a yellowish residue appearing after distillation of oleoresin obtained from plants belonging to Pinaceae. It is an inhomogeneous mixture of approximately 90% resin acids and 10% neutral substances. Its precise constituents are not known because of varying constituents depending on species, climate, extraction method and storing. The resin acids are monocarboxylic acids with an alkylated hydrophenanthrene nucleus. They can be classified into two types: the abietic acid type with conjugated double bonds and the pimaric acid type without conjugated double bonds (Figure 1). Examples for resin acids involve abietic acid, dehydroabietic acid, neoabietic acid and other isomers. Neutral substances are for example stilbene compounds, fatty acid esters and other hydrocarbons (1) (2) (3). It has been shown that isomers of abietic acid such as palustric, levopimaric or neoabietic acid isomerize mainly to abietic acid when they are treated thermally (4).



Abietic Acid
$C_{20}H_{30}O_2$/ 302.46 g*mol^{-1}
CAS 514-10-3



Pimaric Acid
$C_{20}H_{30}O_2$/ 302.46 g*mol^{-1}
CAS 127-27-5

Figure 1: Abietic Acid and Pimaric Acid

We differ three ways of production of rosin yielding in gum rosin, wood rosin and tall oil rosin. Gum rosin is obtained from crude rosin (oleoresin) taken directly from living pine trees by tapping the bark of pine species and steam distillation to separate the rosin and the

turpentine afterwards. Wood rosin is obtained from old stumps and tall oil rosin is obtained by fractional distillation of crude tall oil which is a by-product of paper pulp production (1) (2) (3).

Colophony could be used in solder flux, soaps and cutting fluids, as sizing agent in paper and it has many other applications. It could also be manufactured to modified rosins (3). Typical modifications are for example hydrogenation (for production of plasticizers and tackifiers), disproportionation (simultaneous hydrogenation and dehydrogenation for use in printing inks and adhesives) or esterification in presence of glycerol or ethylene glycol. For paper sizing rosin is often reacted with maleic anhydride or fumaric acid. Modified rosins are rarely pure substances and will contain also unmodified rosin. Autoxidation of resin acids can take place and change unmodified as well as modified products (1).

1.1.2. Contact Dermatitis due to Colophony

Since colophony is used in such a broad range of applications, many clinical cases related to colophony has been reported. They could be classified into bronchial asthma and contact dermatitis (2). This work focus on contact dermatitis due to colophony.

Development of colophony sensitivity depends on length of exposure, the concentration of the allergen, exposure sites, skin integrity and the chemical constituents of the colophony (5). The mechanism of developing allergic contact dermatitis can be divided into afferent and efferent events. The afferent events start, when the allergen penetrates the epidermis and binds to molecules (e.g. proteins) present on the surface of dendritic cells, which are called Langerhans cells. The Langerhans cells are activated by the allergen-molecule complex and carry it via the afferent lymphatics to the regional lymph nodes, where they present the allergen to T-cells. The T-cells are activated, became allergen-specific and lead to the production of antigen-specific effectors, the memory T-cells. They are released into the circulation and enter the peripheral tissues including the skin. At this point the efferent event starts: The individual is sensitized to the allergen and the specific T-cells have increased. When the allergen penetrates the skin a local inflammatory reaction follows, because the allergen-presenting cells and the specific T-cells meet, more memory T-cells arrive at the site of contact and the eczematous reaction follows (6) (7). The binding of the allergen to the molecules in the epidermis is of huge interest, because there are several mechanisms possible. The problem with colophony is that it has a complex chemical composition and is not a chemically defined allergen. Contact allergy due to rosin was reported for unmodified

and modified rosin and for derivatives of rosin. Abietic acid is the main component, but if it is also the main allergen is controversial: Sadhra et al. found out that abietic acid (99% purity according to GLC) has a low allergenic activity and has to be considered because of the high concentration in colophony (8). Hausen and coworkers are also of this opinion (9). Karlberg et al. showed that pure abietic acid is not allergenic: Patients sensitive to gum rosin did not react to purified abietic acid (purity of 99.8%, according to GC-MS), but they did when testing the non-purified sample. One patient also reacts when using purified samples that has been stored in refrigerator before use for four weeks. It was concluded that abietic acid could be considered to be a prohapten or prehapten (10) (11).

Haptens are substances with electrophilic groups, which can react with nucleophilic groups in proteins resulting in a nucleophilic substitution or a nucleophilic addition forming a covalent bond between hapten and protein, creating an antigen. This is considered as the classical model for hapten-protein interaction (6). A prehapten is a substance that has a low or no sensitising potential at its self. It needs to be activated, so that a sensation is possible and this activation has to take place outside the skin by e.g. autoxidation or photoactivation. A prohapten is a substance that also has low or no sensitising potential at its self, but can be activated in the skin by metabolic reactions or enzymatic reactions (10) (11).

Since the abietic acid contains unsaturated double bonds, which can react with oxygen in air to form hydroperoxides it is considered to be a prehapten. For example, abietic acid is autoxidized on air, forming the allergenic oxidation products 15-Hydroperoxyabietic acid (15-HPA) that has been identified as a major allergen in colophonium (12). The hydroperoxid can undergo homolytic cleavage of the peroxy group to form a free radical which can form a covalent bond with the proteins in the skin leading to specific antigens and cause the allergic reaction. This was found by Christensson et al. who studied the cross-reactions of 15-Hydroperoxyabietic acid with limonene hydroperoxide and linalool hydroperoxide. Cross reactivity takes place when haptens form antigens that have very similar or identical epitopes on the antigen, which let them react to the same T-cells. They assumed, if there is cross-reactivity between these three substances a non-specific mechanism of antigen building has to take place, which reacts with structurally different hydroperoxides. However, they did not find cross-reactivity and concluded that hydroperoxides form specific antigens, because only structurally closely related hydroperoxides cross-reacted. A second possible way of forming antigens may be the intramolecular rearrangement of the alkoxy-radicals resulting from the hydroperoxides to form epoxides, which act as electrophilic haptens (13). This was studied by Gäfvert et al.:

15-Hydroperoxydehydroabietic acid (15-HPDA) and 13,14-Epoxides of abietic acid showed sensitization at concentrations of 0.03-0.15 mol kg^{-1}. Cross-reactivity between 15-Hydroperoxydehydroabietic acid and 15-Hydroperoxyabietic acid and between the epoxides and 15-Hydroperoxyabietic acid was detected. This implies that 15-Hydroperoxyabietic acid may form an epoxide by oxidation of the double bond in position 13 and 14, which would act as an electrophile and could be easily attacked by the nucleophilic groups present in the skin proteins. 15-Hydroperoxydehydroabietic acid did not show cross-reactivity with the epoxides, but with 15-Hydroperoxyabietic acid. This is probably due to breakage of aromaticity if an epoxide would be formed from 15-Hydroperoxydehydroabietic acid (13). Therefore, 15-Hydroperoxyabietic acid may react as skin sensitizer through radical or epoxide mechanism and 15-Hydroperoxydehydroabietic acid is considered to react only as alkoxy-radical (Figure 2) (14).

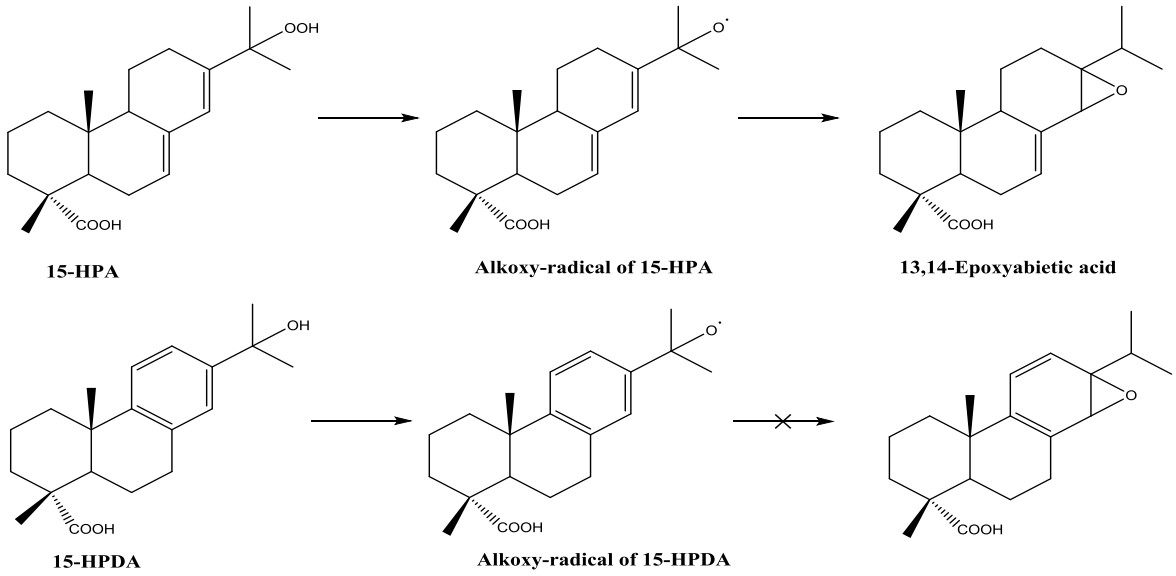


Figure 2: Reaction pathways for protein-hapten complexes, explaining cross-reactivity between 15-HPA, 15-HPDA and epoxides

Oxidation products of dehydroabietic acid were also found to be allergenic: 7-Oxodehydroabietic acid is a moderate allergen, 15-Hydroxydehydroabietic acid gave a response in animal test as well as in human patients and 15-Hydroxy-7-Oxodehydroabietic acid gave a response in human patients but not in animal tests (15) (8).

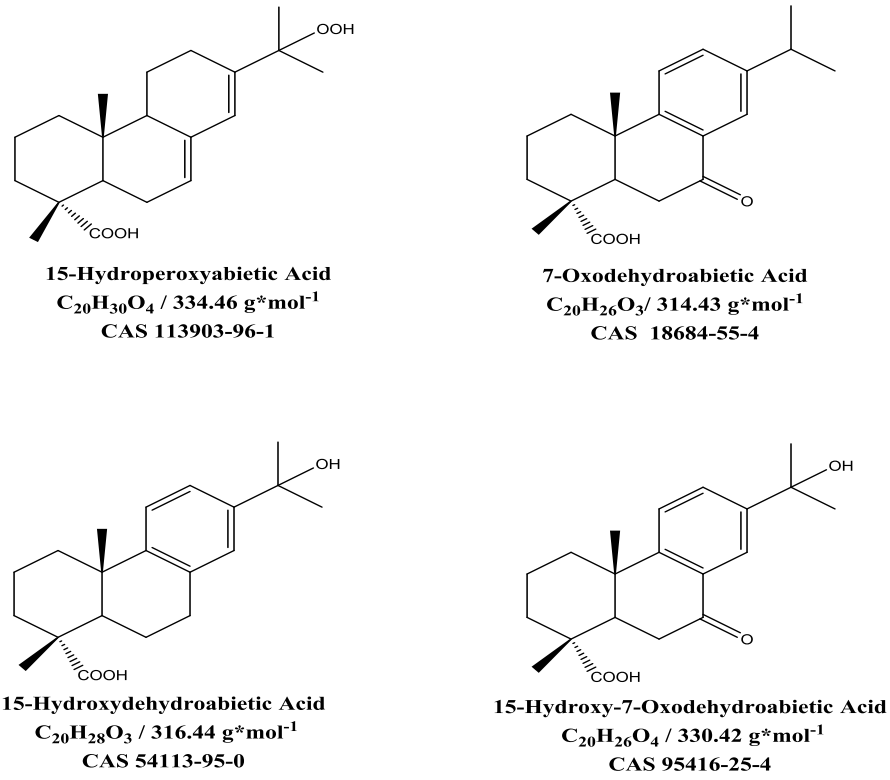


Figure 3: Oxidation products of abietic acid and dehydroabietic acid that were identified as contact allergens

Other resin acids like levopimaric acid or tetrahydroabietic acid are also considered to be weak contact allergens, but their concentration in rosin is low. Neoabietic aicd, dehydroabietic acid and isopimaric acids are considered to be no contact allergen. The neutral fraction shows low allergenic potential too but is considered to be irrelevant for development of contact dermatitis, because of the low concentrations (9).

Karlberg et al. stated in 1996 that there were no studies demonstrating the lowest concentration of rosin to sensitize in humans and therefore it is difficult to say which amount of rosin cause sensitization (16). Patch testing is usually done with gum rosin in the concentration of 20% (w/w) in petrolatum, also 60% has been done, but no further cases picked up at the higher concentration. A dilution test with concentration from 20-0.001% (w/w) in petrolatum gave a dose-response relationship; the highest response was seen between 10-20% (17). In guinea pig studies, 15-Hydroperoxyabietic acid shows a high allergenic potential. For 5% (w/w) solution in petrolatum elven of elven animals show sensation, for 1% (w/w) eight out of eleven (12). Similar are the test results for 15-Hydroxydehydroabietic acid, 7-Oxodehydroabietic acid and 15-Hydroxy-7-Oxodehydroabietic acid (18).

Whether abietic acid is also a prohapten or not has to be further investigated. However, it could be said that it has no or low sensitizing potential at its self and therefore activation through oxidation in the skin is unlikely or not efficient enough (19).
Hydrogenation eliminates the hydroperoxy group, decrease the level of air oxidation of the conjugated double bonds and therefore decrease the allergenicity of gum rosin (20).

1.1.3. Degradation Pathways

Abietic acid is main compound in the acid resins and used for example for the manufacture of hot melt adhesives. It is added as tackifier to reduce viscosity, improve wetting and adhesion (21). The acid underwent thermal degradation when the adhesive is heated for curing (22). Simoneit et al. and Rogge et al. found that resin acids are released unaltered, partially altered or completely compusted after wood burning in fireplaces (23) (24). They found the altered resin acids dehydroabietic acid, 7-Oxodehydroabietic acid and retene in smoke after combustion of coniferous wood as well as the unaltered abietic, sandaracompimaric, pimaric and isopimaric acids. Marchand-Geneste and Carpy made thermodynamic calculations and came to the results that degradation starts with dehydrogenation of abietic acid to dehydroabietic acid (dehydroabietic acid) and a second degradation to from 13-isopropyl-5a-podocarpa-6,8,11,13-tetraen-16-oic acid, demethylation yielding 7-isopropyl-1-methyl-1,2,3,4-tetrahydro-phenanthrene-1-caboxylic acid, followed by decarboxylation giving tetrahydroretene and two more dehydrogenations for full aromatization to retene (25). Marchand-Geneste and Carpy gave the thermodynamic justification for the thermal degradation scheme proposed by Standley and Simoneit based on GC-MS results (26).
Besides the combustion of resin acids by burning, several authors have studied the autoxidation: Gigante et al. irradiated a solution of dehydroabietic acid methyl ester ***(2)*** in t-BuOH for 38h with a mercury vapour lamp with exposure to air (27). They isolated as methyl esters 7-Oxodehydroabietic acid ***(4)*** and 13-Acteyl-7-Oxodehydroabietic acid ***(9)***. Irradiation of Methyl 7-Oxodehydroabietate ***(4)*** gave 15-Hydroxy-7-Oxodehydroabietic acid ***(6)***, 13-Acetyl-2,7-Dioxo-podocarpa-8,11,13-trien-15-oic acid ***(10)*** and also 13-Acteyl-7-Oxodehydroabietic acid ***(9)*** (isolated as methyl esters). Increasing the period of irradiation to three weeks gave 2,15-Dihydroxy-7-Oxodehydroabietic acid ***(12)***, 13-Acetyl-2-Hydroxy-7-Oxodehydroabietic acid ***(13)***, 13-Acetyl-12-Hydroxy-7-Oxodehydroabietic acid ***(16)***, 7-

Oxo-podocarpa-8,11,13-trien-13,15-dioic acid ***(15)***, 13-Isopropenyl-7-Oxodehydroabietic acid ***(8)*** and two lactones ***(11, 14)***.

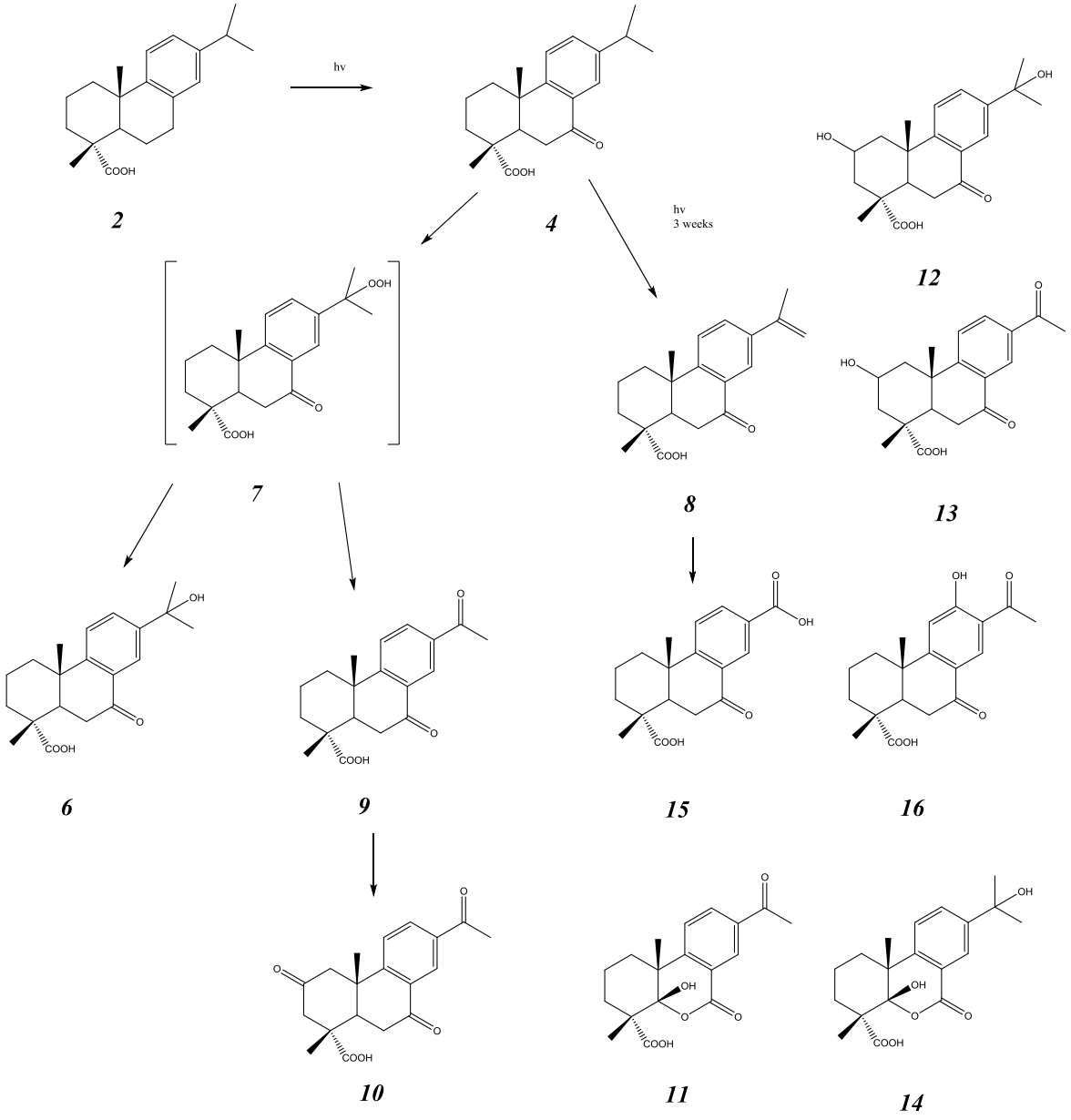


Figure 4: Degradation Pathway and Products of Gigante et. al.

Krohn et al. tested the autoxidation of abietic acid ***(1)*** in dichloromethane and under day light for three months (28). The nonpolar fraction gave dehydroabietic acid ***(2)***, the polar fraction was treated with diazomethane for esterification and separated with chromatography. They isolated as their methyl esters 7-Oxodehydroabietic acid ***(4)***, 12α-Hydroxyabietic acid ***(24)***, 15-Hydroperoxyabietic acid ***(25)***, 13,14-Dihydro-13α-Hydroxy-14-Oxoabietic acid ***(22)***, 13-Oxo-8(14)-Podocarpen-18-oic acid ***(23)*** and 7α-Hydroxydehydroabietic acid ***(3)***. Autoxidation of dehydroabietic acid ***(2)*** for six months yielded 7-Oxodehydroabietic acid ***(4)*** and 7α-Hydroxydehydroabietic acid ***(3)***. Therefore, they concluded that hydroxylation could

take place before and after aromatisation. The last step was the autoxidation of tall oil where they found 15-Hydroxy-7-Oxodehydroabietic acid ***(6)*** besides the already known acids. For analysis, they used NMR-spectroscopy because the acids are easily isomerised under thermic conditions.

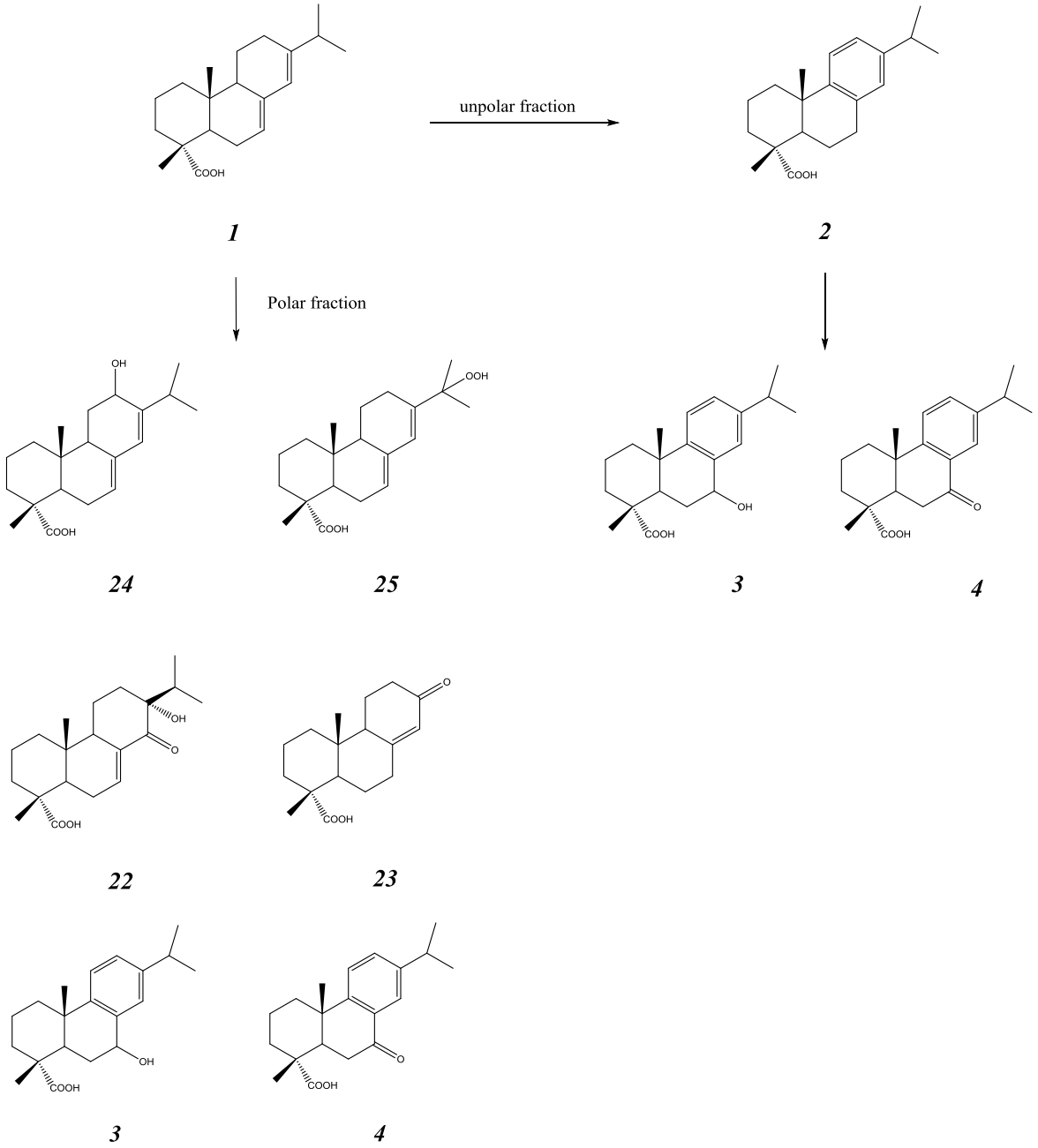


Figure 5: Degradation products of Krohn et. al.

Corin et al. tested the effects of UV_{254}-radiation and artificial solar radiation on the degradation of dehydroabietic acid ***(2)*** in humic water (29). They identified several compounds (including three pairs of isomers) after silylation and detection with GC-MS, which can be divided into two groups: the first group consists of dehydroabietin (18-

norabieta-8,11,13-triene; decarboxylation product of dehydroabietic acid) ***(17)*** and its oxidation products, were the most abundant one is 7-Hydroxydehydroabietin ***(18)*** followed by 7-Oxodehydroabietin ***(19)***, 15-Hydroxydehydroabietin ***(20)*** and 15-Hydroxy-7-Oxodehydroabietin ***(21)***. The second group consists of oxidation products of dehydroabietic acid. The most abundant one is 7-Hydroxydehydroabietic acid ***(3)*** followed by 7-Oxodehydroabietic acid ***(4)***, 15-Hydroxydehydroabietic acid ***(5)*** and 15-Hydroxy-7-Oxodehydroabietic acid ***(6)***.

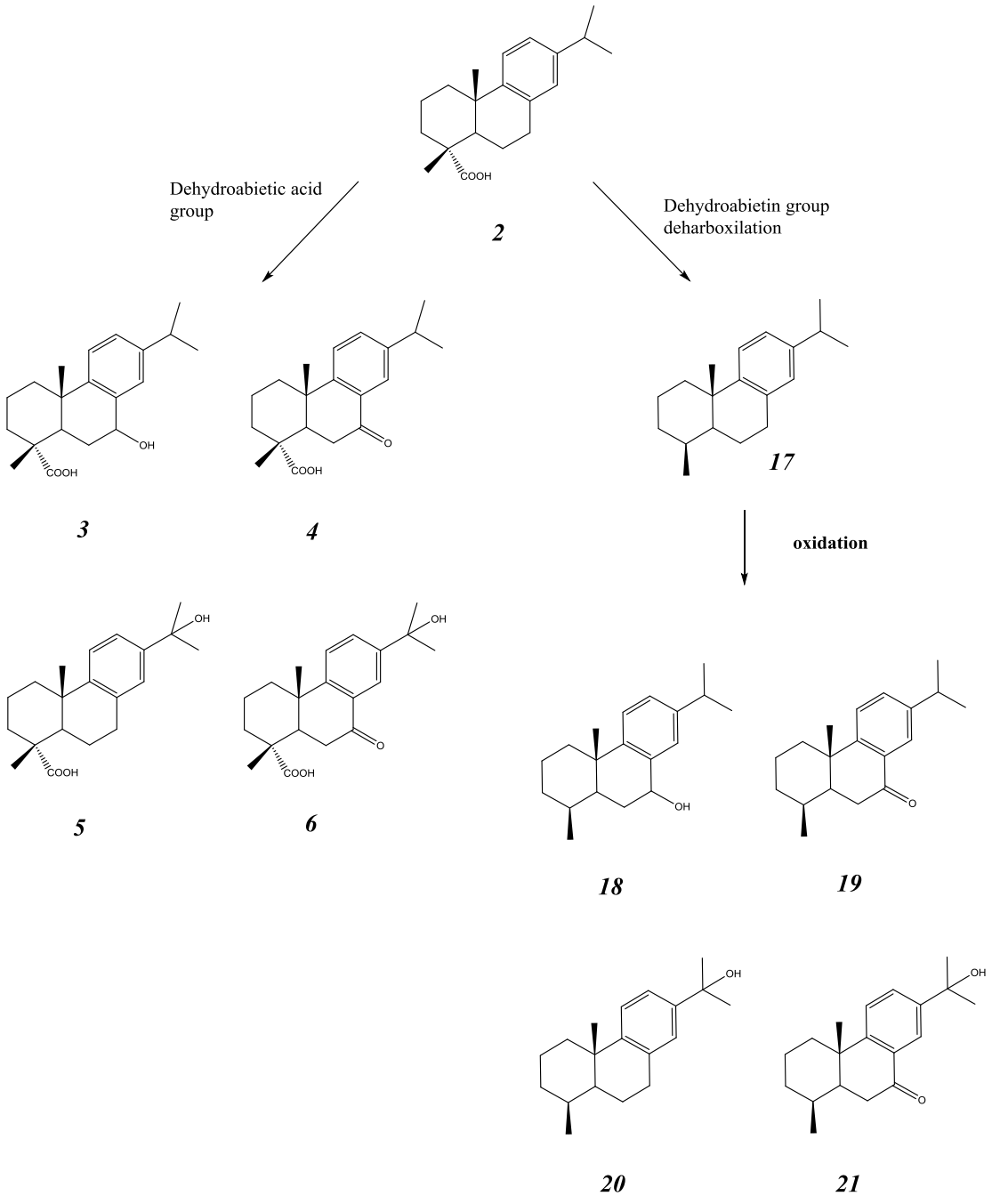


Figure 6: Degradation products of Corin et. al.

Prinz et al. stored abietic acid and its methyl ester under various conditions to provide an indication of their possible oxidation mechanisms and the favoured oxidation positions in the abietane skeleton (30). They isolated the following compounds as their methyl esters: 7α,13β-Dihydroxyabiet-8(14)-enoic acid ***(27)***, 7α,13α-Dihydroxyabiet-8(14)-enoic acid ***(27)***, 12-Oxoabietic acid ***(26)***, 7-Oxodehydroabietic acid ***(4)***, 7α-Hydroxydehydroabietic acid ***(3)*** and 13,14-Dioxoabiet-7(8)-enoic acid (11). Following new oxidation products were isolated as their methyl esters: 13β-Ethoxy-7α-Hydroxyabiet-8(14)-enoic acid (3a), 13α-Ethoxy-7α-Hydroxyabiet-8(14)-enoic acid (3b), 7α-Hydroperoxy-13α-Hydroxyabiet-8(14)-enoic acid or (5) 13α-Hydroperoxy-7α-Hydroxyabiet-8(14)-enoic acid, and 7α,15-Dihydroxydehydroabietic acid ***(28)***. In conclusion, they found out that C-7 and C-13 were the most favoured positions for oxidation.

Ren et al. investigated the oxidation mechanism of abietic acid (31). They concluded that the C-7 is first transformed to a hydroxyl by the attack of oxygen, including the isomerization of the conjugated band. The methylene at C-12 is converted by an oxygen atom to a hydroxyl intermediate. Hydrogen continues to react with oxygen to from C=O and water. Finally, the conjugated band is converted into a peroxide before transforming into an oxidant. In a second study they concluded that the peroxide is formed in the first step, followed by further oxidation, which forms hydroxyl-containing abietic acid oxide (32).

Several other authors presented their results on the possible degradation pathway of abietic acid and isomers. However, the most important degradation products and pathways has been mentioned and are summarised in Figure 7.

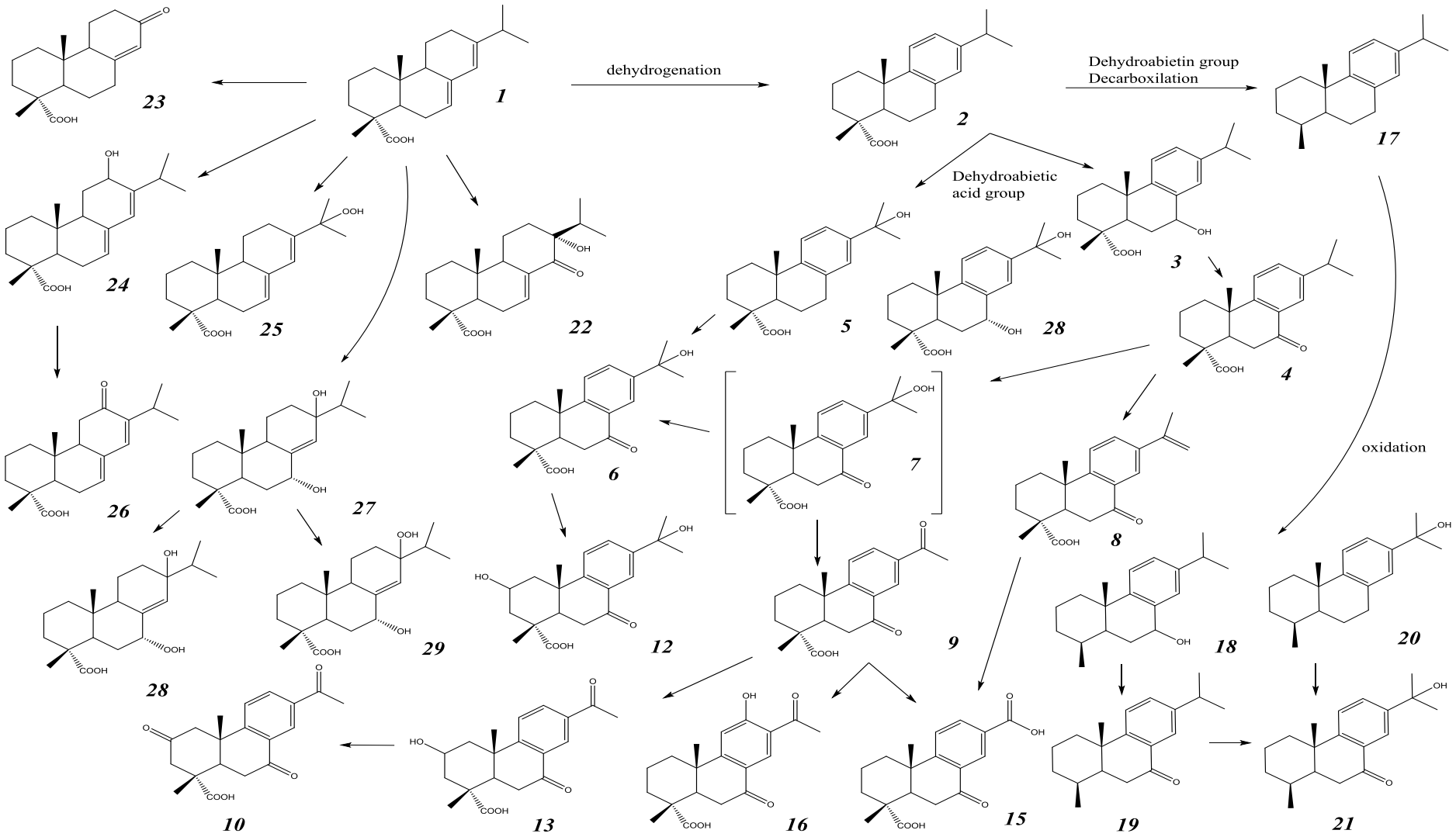


Figure 7: Summary of degradation pathways and products

1.1.4. Analysis of Abietic Acid and Degradation Products

1.1.4.1. General

For analysis of abietic acid and degradation products chromatography with mass spectrometry detection is the method of choice because it is able to separate the different analytes efficiently and to give structural information about them. Liquid chromatography (LC) and gas chromatography (GC) are used and described in literature. Both methods have advantages and disadvantages, which are described in literature and are summarised below.

a) Advantages and Disadvantages of Liquid Chromatography

According to literature, high pressure liquid chromatography (HPLC) techniques are in preference because of separation at room temperature and therefore avoiding isomerisation and degradation of resin acids. Furthermore, no derivatisation is needed and sample manipulation and analyte loss is reduced, also leading to quicker and easier methods than GC. Another advantage in comparison to gas chromatography is that separation and fraction collection is possible with HPLC. Separated fractions/acids could be further examined e.g. in toxicology tests (8) (33).

Difficulties for HPLC are the separation of the various resin acids and isomers, because of similar retention and coelution of the non-aromatic resin acids. Furthermore, due to their similar structure differentiation by mass spectra is difficult and leads to the impossibility of LC-MS to quantify them individually (34).

Latorre et al. compared LC-MS with GC-MS for the determination of resin and fatty acids in paper mill process waters. He conclude that liquid chromatography is highly sensitive and quantification is possible at the low $\mu g\ L^{-1}$ level, repeatability and reproducibility was slightly better for LC-MS and it was more robust (34).

Kersten et al. reviewed that for the determination of resin acids in paper mill process waters reversed-phase HPLC of underivatised samples has been used but with the disadvantage, that structural information has not been resolved. Furthermore, HPLC has most often been used for the identification of abietic acid and dehydroabietic acid only and not for complex mixtures (35).

b) Advantages and Disadvantages of Gas Chromatography

Analysis of resin acids is usually carried out using gas chromatography coupled with flame-ionization, electron-capture or mass spectrometry with or without derivatisation (33). Derivatisation methods are described later in 1.1.4.2. Sample Preparation.

Latorre et al. claimed that in his study GC-MS showed better selectivity and lower detection limits, linearity ranges are longer, recoveries were slightly higher and GC-MS provides more information but also more complicate spectra.

Disadvantages are that extensive sample preparation including derivatisation is often necessary, which increases the risk of sample manipulation (isomerisation or oxidation of resin acids), analyte loss and the health risk from certain methylation agents has to be considered. Furthermore, derivatised samples have a short lifetime of 12-24h (34) (35).

1.1.4.2. Sample Preparation

As sample preparation method for cellulose based materials, solid-liquid extraction is often done to extract the analytes from the matrix. For the polar abietic acid polar solvents like methanol, acetone or ethyl acetate could be used. After extraction the solvent is evaporated partly or completely if derivatisation has to be done. For gas chromatography, derivatisation is needed to increase volatility of the analytes to make evaporation possible, because separation is done between the gaseous phase and the column. For liquid chromatography, this step is not needed because chromatography is done between a liquid phase and the column and the analytes has to be in solution.

Different sample preparation methods for analysis of abietic acid and degradation products with liquid or gas chromatography that are described in literature are summarised below.

a) Liquid Chromatography

Different authors described extraction of their samples with methanol as sample preparation method for liquid chromatography: Axelsson et al. analysed different resin acids in air filter samples, Hrobonova et al. analysed abietic acid and dehydroabietic acid in propolis tincture and Kersten et al. analysed the composition of conifer oleoresins. All of them used extraction with methanol prior analysis with HPLC (33) (35). Axelsson et al. also used diethyl ether or toluene for the extraction of wood pellet dust (36). Latoree et al. introduced water samples directly into HPLC after dilution with methanol for the analysis of pulpmill effluents (34).

Lee et al. analysed Chinese medical products. Samples were extracted with pure acetonitrile or acetonitrile with diethyl ether. The extract was partitioned with water and further

purification was done with solid phase extraction (SPE; 1 ml tube; 100 mg C_{18}) (37). A similar method was used by Nilsson et al. who dissolved rosin samples in 100% acetonitrile (ACN), to a concentration of 1.5 mg ml^{-1} and analysed them directly after syringe filtration by HPLC. For cosmetic products, the samples were sonicated in ACN and diluted with water. Purification has been done with SPE (Oasis MAX columns; 6 ml, 500 mg) (38).

Castle et al. used paper or board samples, cut into small pieces and extracted them with water, chloroform or ethanol for 24 h at 95, 40 and 60°C (39). Ozaki et al. and Bengtström et al. also used extraction of paper and board samples with ethanol (40) (41) (42). Ozaki et al. did migration tests under various conditions and with different food simulants: for distilled water, 4% acetic acid and 20% ethanol the samples were incubated 30 min at 60 and 95°C and 1 day at 40°C. For 95% ethanol incubation was 30 min at 60°C, for heptane 1 h at 25°C, for Tenax TA 30 min at 60, 100 and 150°C and 1 and 2 days at 40°C. The ratios of liquid contact area were 2 ml cm^{-2} or 0.04 g cm^{-2} for Tenax TA respectively (41).

A summary of possible sample preparations and used conditions can be seen in Table 1.

Table 1: Sample preparation for liquid chromatography

Source	Sample	Analyte	Amount of Sample	Extraction	Description	Internal Standard
(33)	propolis tincture	AA, DHA	2 ml	MeOH	Sample dried, dissolved in 0.5 ml MeOH, centrifuged; injected directly or after dilution	-
(35)	conifer oleoresins	resin acids	500 mg	MeOH	Samples cut into pieces; 4 ml MeOH, filtered through glass wool and syringe	-
(36)	Air filter samples	AA, DHA, 7-Oxo-DHA	1/3 of filter	MeOH	MeOH + d_2-dehydroabietic acid (2 ml of 1 mg L^{-1}); shaken 25 min; syringe filtration; dilution with water 1:1 (v/v)	dehydroabietic acid-6,6-d_2 1 mg L^{-1}
	Wood pellet dust collected on filters	AA, DHA, 7-Oxo-DHA	1/3 of filter	Et_2O	Ether + d_2-dehydroabietic acid (1.2 ml of 1 µg ml^{-1}); ultrasonication 30s; 1 ml transferred, evaporated, 1 ml MeOH, ultrasonication 1 min, syringe filtration	dehydroabietic acid-6,6-d_2 1 mg L^{-1}
	Wood pellet dust collected on filters	AA, DHA, 7-Oxo-DHA	1/3 of filter	toluene	Toluene + d_2-dehydroabietic acid (1.2 ml of 1 mg L^{-1}); 75°C; 30 min; 1 ml transferred, evaporated, 1 ml MeOH, ultrasonication 1 min, syringe filtration	
(37)	Chinese medical products (oil)	AA, DHA	80-100 mg	ACN	100 µL sample + 900 µl ACN ultrasonication 20 min; centrifugation, dilution with water 1:1 (v/v), centrifugation; 1 ml for SPE (1 ml; 100 mg C_{18}), eluted with1 ml mobile phase	-
	Chinese medical products (ointment)	AA, DHA	50 mg	Et_2O/ACN	50 mg sample + 0.2 ml ether + 0.8 ml ACN; ultrasonication 15 min, centrifugation, dilution with water 1:1 (v/v), centrifugation; 1 ml for SPE (1 ml; 100 mg C18), eluted with 1 ml mobile phase	
(38)	rosin	Resin acids	2 ml of 1.5g L^{-1}	ACN	syringe filtration	EPA* 186 mg/2 L

	cosmetic products	Resin acids	1g	ACN	20 ml ACN, ultrasonication 30 min; centrifugation; twice; solvent evaporated to 7 ml + 3 ml water; SPE (6 ml, 500 mg; Oasis MAX)	EPA* 464 mg/20 L
(39)	Paper & board	AA	10g	H_2O	Cut into pieces; 200 ml solvent; 24h at 95, 40, 60°C; 2 ml diluted with 1 ml EtOH	-
				$CHCl_3$	Cut into pieces; 200 ml solvent ; 24 h at 95, 40, 60°C; 2 ml evaporated and re-dissolved in ACN/water (1:1)	
				EtOH	Cut into pieces; 200 ml solvent ; 24 h at 95, 40, 60°C; mixed with water 1:1 (v/v)	
(40)	paper & board	AA	900 dm^2	EtOH	Cut in pieces; soxhlet extraction (500 ml EtOH; 2 h); evaporated to 0.5 ml, diluted 1:10 v/v with EtOH	-
(42)	paper & board	AA, DHA	10g	EtOH	Cut into pieces; refluxed (200 ml EtOH, 2 h), evaporated, 2 ml ethyl acetate; further partitioning for toxicity test ending with methanol; SPE (6 ml; 500 mg, graphitized non-porous carbon); eluted with 1 ml THF	-
(41)	paper & board (extraction)	AA, DHA	5g	EtOH	Cut into pieces; refluxed (100 ml EtOH, 2 h), evaporated, 10 ml MeOH; diluted 1:100 (v/v) with mobile phase	-
	paper & board (migration)	AA, DHA		H_2O	Various conditions	-
				4% acetic acid		
				EtOH		
				heptane	1h; 25°C	-
				Tenax TA	2 g Tenax; extracted twice (20 ml acetone), evaporated, 1 ml mobile phase	-
(34)	pulpmill effluents	resin & fatty acids	0.8 ml	MeOH	Sample diluted with MeOH: 0.8 sample + 0.2 MeOH	-

*EPA= cis-5,8,11,14,17-Eicosapentaenoic acid

b) Gas Chromatography

Several authors described the extraction of samples using methyl tert.- butyl ether (MTBE): Voss et al. established a method for the extraction of resin and fatty acids in pulp mill effluents using methyl tert.-butyl ether (43). This method was also used by Lee et al. and Latorre et al. for the determination of resin and fatty acids in pulpmill effluents using gas chromatography mass spectrometry after derivatisation with pentafluorobenzyl bromide and N,O-bis(trimethylsilyl)trifluoracetamide (BSTFA) and trimethylchlorosilane, respectively (44) (34). Extraction with MTBE and derivatisation with BSTFA was also used by Corin et al. for the investigation of the effect of UV-radiation and solar radiation on the degradation of dehydroabietic acid in humic water (29).

Karlberg et al. prepared acetone or methanol extracts from newspaper and magazine samples by cutting them into small pieces and stirring in ultrasound (45) (46). A similar method was used for the extraction of abietic acid, dehydroabietic acid and 7-Oxodehydroabietic acid from diapers, where the different layers of diapers were cut in small pieces' prior extraction. For GC analysis, the extracts were methylated with diazomethane in dichloromethane (16).

Sadhra et al. examined the autoxidation of rosin preparations for patch testing (20% w/w in petrolatum) using gas chromatography after derivatisation. Derivatisation was done following a method by Pastorova et al. using unmodified rosin and gum rosin which was dissolved in methanol and tetramethylammonium hydroxide in methanol was added in excess to produce the tetramethylammonium derivatives (47) (48).

Castel et al. tested paper or board samples, cut them into small pieces and extracted them with water, chloroform or ethanol. In addition, headspace analysis was done as diffusion trapping and direct headspace sampling. For diffusion, trapping the sample was placed in a vial alongside an open tube containing Tenax, which was transferred to a fresh vial for analysis using direct headspace after incubation for 40 days at 45°C (39). Bradley et al. tested nineteen food contact papers and board onto their in vitro toxicity. Samples were extracted in hot or cold water, aqueous ethanol and Tenax. Tenax extracts has been done from twelve 1.5 dm^2 circles of paper. The first one was placed in a Petri dish and covered with 3 g of Tenax; two more were added with the food contact surface in contact to each other and further 3 g of Tenax were added. This results in a stack with single-sided contact to Tenax. Tenax was extracted with ethanol. Water extracts has been done as described in EN645 1994 and EN647 1994. Derivatisation was made with BSTFA (49) (50).

Binderup et al. made toxicity testing and chemical analysis of recycled fibre-based paper for food contact, Ozaki et al. analysed virgin and recycled paper samples onto their

genotoxicants. They also cut the sample into small pieces and extracted them with ethanol. Ozaki et al. diluted the extracts with ethyl acetate afterwards and several partitioning steps with e.g. ethyl acetate, hydrochloric acid and alkali buffer solution followed for the analysis of genotoxicity followed. At the end, SPE was used for purification (6 ml tube, 500 mg graphitized non-porous carbon) (51) (42).

Van den Berg et al. tested a resin/oil painting sample and derivatised it with trimethylsilyl (TMS) diazomethane in hexane in a methanol-benzene solution, with BSTFA in benzene-pyridine or with BSTFA after methylation with TMS-diazomethane (52).

The Health and Safety Executive UK established a GC-FID method for the analysis of resin acids in air after collection of air samples on membrane filters. Belonging to this method, called MDHS 83/2, samples are extracted with cyclohexane in ultrasonic bath, solvent is evaporated to dryness and methylation agent is added for 30 min at 75°C. As methylation agent a solution of toluene and dimethylformamide dimethylacetal is prepared (53). For analysis of air filter samples and wood pellets with GC-FID a modification of method MDHS 83/2 was used by Axelsson et al. They used diethyl ether for extraction instead of cyclohexane because 7-Oxodehydroabietic acid is not well dissolved in cyclohexane (36).

Sample preparation methods and applied conditions that has been used in the last decades are summarised in Table 2.

Table 2: Sample preparation methods for gas chromatography

Source	Sample	Analyte	Amount of Sample	Extraction	Description	Derivatisation	Internal Standard
(44)	pulpmill effluents	resin & fatty acids	25 ml	MTBE	2x50 ml MTBE 30 min; dried, evaporated; 3 ml acetone; evaporated to 0.5 ml	100 µl PFBBr + 30 µl 30% K_2CO_3; 60°C 30 min; evaporation and SPE	-
(45)	newspaper	AA	10 mg	Acetone or MeOH	cut into pieces, solvent; ultrasonication 10 min twice; evaporated partly	-	-
(46)	newspaper	AA, DHA, 7-Oxo-DHA	10 mg	Acetone or MeOH	cut into pieces, solvent; ultrasonication 10 min twice; evaporated	diazomethane	Methyl stearate
(16)	diapers	AA, DHA, 7-Oxo-DHA	2.5-12 g	acetone	cut into pieces; 100 ml acetone, stirred and ultrasonication 10 min twice; evaporated	diazomethane; CH_2Cl_2	Methyl stearate
(39)	paper	AA	10g	H_2O	cut into pieces; 24h at 95, 40, 60°C; extracted with ethyl acetate	-	Methyl stearate; 250-350 mg L^{-1} extract
			10g	$CHCl_3$ or EtOH	cut into pieces; 24h at 95, 40, 60°C	-	
			2.5g	Tenax	0.1g Tenax; 40 days; 45°C	-	-
			2.5g	direct headspace	Equilibrated in headspace sampler; 70°C 30-60 min	-	
(47)	rosin 20% (w/w) pet.	Resin acids	50 mg	MeOH	1 ml MeOH; syringe filtration; evaporated to 0.5 ml	MeOH+TMAH	-
	rosin	Resin acids	10mg	MeOH	1 ml MeOH;	MeOH+TMAH	
(29)	humic water	AA, DHA, 7-Oxo-DHA	40-50 ml	MTBE	4x10 ml MTBE; water frozen out; evaporated to 0.5 ml	BSTFA + pyridine; 60°C; 30 min	heptadecanoic acid; 50 µg L^{-1}

(52)	resin/oil paint	Oxidation products	50µg		TMS-diazomethane; in MeOH-benzene, 15 min; evaporated, CH_2Cl_2		-
					BSTFA in benzene/pyridine; 70°C 60 min		
					TMS-diazomethane after BSTFA		
(51)	paper	DHA	50g	EtOH	Reflux (EtOH 1500 ml, 3h), centrifugation, evaporation (10 ml); final conc. 5g paper/ml	-	C_{12}, C_{20}, C_{24}; 30 µg ml^{-1}
(34)	pulpmill effluents	resin & fatty acids	4 ml	MTBE	2 ml MTBE shaking 2 min twice; centrifugation; evaporation	80 µl BSTFA + 40 µl trimethylchlorosilane; 70°C 20min	Margaric acid 7 mg L^{-1}
(42)	paper	AA, DHA	10g	EtOH	cut into pieces; refluxed (200 ml EtOH, 2 h), evaporated, 2 ml ethyl acetate; partitioning ending with MeOH; SPE, eluted with THF	-	-
(49)	paper	Resin acids		H_2O	hot or cold after EN645 and EN647; extract dissolved in 1 ml CH_2Cl_2	BSTFA; 70°C; 30min; acetone	14-methylpentadecanoic acid; cholesterol; 2 mg L^{-1}
			200g	EtOH/H_2O (95:5, v/v)	cut into pieces, 2 L solvent, RT 24 h; evaporated; re-dissolved in 80 ml EtOH; with and without deriv.	BSTFA; 70°C; 30min; acetone	Hexadecanoic acid; 40 mg L^{-1}
				Tenax	2x extracted with 100 ml EtOH; concentrated to 80 ml; with and without deriv.	-	1,9-dichlorononane; 1-fluorononane; 2 mg L^{-1}

1.1.4.3. Analysis Parameter for LC and GC

In the following chapter analysis parameter like column, oven program, mobile phases used for liquid or gas chromatography that has been described in literature are summarised:

a) Liquid Chromatography

Hrobonova et al. analysed abietic acid and dehydroabietic acid in propolis samples using HPLC equipped either with a Separon SGX C_{18}-Column or with a Lichrosorb RP-8 coloumn. dehydroabietic acid and abietic acid are well dissolved in MeOH or ACN and very poor in water, so reversed phase was used. The Separon SGX C_{18} column gave better result and the mobile phase had to contain at least 70% (v/v) acetonitrile or 80% (v/v) methanol. Addition of 0.05% formic acid improved chromatographic efficiency (33).

Ozaki et al. investigated the presence and migration of dehydroabietic acid and abietic acid in virgin and recycled paper and paperboard products used in contact with food. As food-simulating agents, they used distilled water, 4% acetic acid and 20% ethanol as aqueous simulants, 95% ethanol and heptane as fatty simulants, 20% ethanol as alcoholic simulant and Tenax TA as dry food simulant. After extraction and migration, analysis was done with LC-ESI-MS. They found dehydroabietic acid and abietic acid in five of ten virgin paper samples at levels of 14-500 and 110-1200 mg kg^{-1}, while the acids were present in all of the 11 recycled samples in levels of 55-230 and 260-880 mg kg^{-1}. Under the same test conditions (30 min at 60°C), dehydroabietic acid and abietic acid migrated most to 95% ethanol, followed by 20% ethanol and water. In 4% acetic acid, there was no detectable migration under any conditions, heptane (1 h at 25°C) and water (30 min at 60 or 90°C) behaved similar than 20% ethanol. Using the same simulant migration level increased when the temperature was reduced but the time was increased. However, after incubation for 1 day at 40°C, the samples had absorbed too much simulant and findings were concluded to be inconsistent with reality. They concluded that 30 min at 60°C are best suitable for water, acetic acid and ethanol and 1 h at 25°C is best for heptane. For Tenax TA incubation for 1 and 2 days at 40°C and 30 min at 60°C were chosen (41). In a second study for the identification of genotoxicants, determination of quantity was done with LC-MS as described above in (41). As Virgin paper products coffee filters, tissues and dish were used, which show a concentration of dehydroabietic acid or abietic acid as not detected (below 1.0 mg kg^{-1}), 38-77 mg kg^{-1} dehydroabietic acid, 200-910 mg kg^{-1} abietic acid and again not detected. Recycled products has been cardboard and food boxes, newspaper and paperboard.

The concentrations were for dehydroabietic acid 59-170, 53-370, 210 and 140 mg kg^{-1}, for abietic acid 380-580, 150-840, 590 and 370 mg kg^{-1} (42).

Kersten et al. reported a simple method with the advantages mild extraction and chromatographic condition, which did not change components and their biological activities and they used only volatile component for the mobile phase, therefore recovery of compounds from fractionated sample is easy (35).

Nilsson et al. established a method for the quantification of different resin acids and oxidation products in cosmetic products and rosin: a urea-embedded C_{12}-HPLC column was used for separation of resin acids. Purification was made by SPE utilised with a mixed mode hydrophobic and anion exchange retention mechanism, which made it possible to separate the lipophilic resin acids from the complex matrix by elution with acidified methanol. The urea-embedded column has a higher selectivity due to the polar urea function, which interact with the resin acids by ionic interactions and hydrogen bonding, between the spacer and the C12 chains. The concentration of abietic acid was 26% (by weight) in unmodified gum rosin, 69% in unmodified gum rosin and 7% in rosin modified with maleic anhydride. For dehydroabietic acid the concentrations were 4, 2 and 5% respectively. Advantages were the quick separation within 20 min and no need of sample preparation for the pure rosin. The method is considered to be used universally, limited by sample preparation. LODs were between 7-13 mg kg^{-1}, LOQs 22-39 mg kg^{-1}. Recovery was between 92 and 94%. Linearity range was 1-400 mg kg^{-1}. As disadvantage, they named the large number of resin acids, which could not be separated completely. Reference solutions were stable for at least 14 days if stored in refrigerator (38).

Axelsson et al. compared HPLC-ESI-MS with GC-FID for the determination of resin acids in wood dust-containing air samples collected on filters during the production of wood pellets and from extraction of wood pellet dust. For HPLC they used a PRISM RP^{TM} column with a corresponding guard column. Recoveries from spiked filter samples were about 99% (36).

In order to ensure the safety of paper and board FCMs Bengtström et al. analysed a virgin fibre paper and a recycled corrugate fibreboard for in vitro toxicity. They extracted the paper and board samples and fractionated them for in vitro analysis with HPLC. Mobile phase varied according to the fractions that should be collected. They found abietic acid in their samples, but the concentration was stated to be too high for quantification in recycled board and to low in virgin fibre paper (40).

A summary of LC methods that has been used in the last years can be seen in Table 3.

Table 3: analysis parameter liquid chromatography

Source	Sample	Analyte	Injection [µL]	Mobile Phase and Flow Rate	Column	Detection
(39)	paper	AA	250	ACN/ H_2O; 60% (5min) rising at 5%/min to 100% ACN; (0.4 ml/min)	ODS-2 (two cartridges of 100×3 mm; 5 µm);	DAD
(37)	Chinese medical products	DHA, AA	20	MeOH/ H_2O (87:13 v/v) + 0.02% phosphoric acid; (1 ml/min)	PartiSphere 5 C_{18} (110×4.6 mm; 5 µm)	variable wavelength
(34)	pulpmill effluents	resin & fatty acids	100	MeOH/H_2O (70% to 100% MeOH in 0.3min) +25 mM CH_3COONH_4; (0.8 ml/min)	Lichrospher 100 RP-18 (250×4 mm; 5 µm) + guard column; 40°C	APCI-MS
(33)	propolis tincture	AA, DHA	20	MeOH/ H_2O (75:25-90:10 v/v) or ACN/H_2O (60:40-90:10 v/v) +0.05% formic acid; (0.5 ml/min)	Separon SGX C_{18} (125×3.9 mm: 5 µm) or Lichrosorb RP-8 (250×4 mm; 7 µm); 30°C	variable wavelength
(41)	paper & board	AA, DHA	10	50mM CH_3COONH_4 in H_2O /ACN (2:8); (0.2 ml/min)	ZORBAX Eclipse XDB-C_{18} (150×2.1 mm; 5 µm); 30°C	ESI-MS
(42)	paper & board	AA, DHA	10	50mM CH_3COONH_4 in H_2O /ACN (2:8); (0.2 ml/min)	ZORBAX Eclipse XDB-C_{18} (150×2.1 mm; 5 µm); 30°C	ESI-MS
(35)	oleoresin	resin acids	-	MeOH 85, 5 or 10%/acetic acid 5%/ H_2O respectively; (1 ml/min)	Alltima C_{18} (250×4.6 mm; 5 µm),	diode array
(38)	cosmetics & rosin	resin acids	20	MeOH/H_2O (8:2 v/v) +0.05% formic acid; (1 ml/min)	Prism RP-12 (150×4.6 mm; 3 µm) with urea-embedded C_{12}; 25°C	UV/DAD
(36)	air filter samples	AA, DHA, 7-oxo-DHA	20	ACN/H_2O (59:41, v/v) + 0.05% formic acid; (0.3 ml/min)	PRISM RP™ (100×2.1 mm, 3µm) + guard column	ESI-MS
	wood pellet dust	AA, DHA, 7-oxo-DHA	20	ACN/H_2O (59:41, v/v) + 0.05% formic acid; (0.3 ml/min)	PRISM RP™ (100×2.1 mm, 3µm) + guard column	ESI-MS
(40)	paper	AA	100	varied due to fractionation; (0.8 ml/min)	XTerra C_{18} (250×4.6 mm; 5 µm)	-

b) Gas Chromatography

A method for the analysis of underivatised resin acids in gum rosin (dissolved in hexane/ ethyl acetate 80:20, v/v) was developed by Gref. Monocarboxylic resin acids were nearly equally well resolved than the derivatised one, although the non-polar DB-1 column did not separate some resin acids completely. Dicarboxylic acids were not eluted unmethylated. Overall analysis time was completed within 5 min (54)

Lee et al. established a method for the determination of resin and fatty acids in pulpmill effluents using GC-MS after derivatisation with pentafluorobenzyl bromide (PFB). The EI-MS spectra all show the very strong ion for the PFB-ester (m/z 181) (44).

Brites et al. analysed dehydroabietic acid in disproportionated resin using GC/FID and derivatisation with diazomethane. As advantages they mention the easy and fast derivatisation, without need of purification and minimal losses, the DB-1 column proved to have good resolution and short analysis time and the overall method is simple, specific, exact and reproducible (55).

Karlberg et al. tested the sensitization potential of extracts derived from newspaper. Extracts were analysed with GC-FID. abietic acid, dehydroabietic acid and 7-Oxodehydroabietic acid were identified as their methyl esters in the extracts in concentrations of 1-87, 2-191 and 9-63 mg kg^{-1} in the papers. The extracts were used for patch testing with a dilution series of 6, 3, 1 and 0.3 mg of the extracted material from the paper samples. Irritation was seen by 6 mg (46). Karlberg et al. also tested disposal diapers and its materials, which are used for infant diapers, incontinence products and feminine hygiene products with GC-FID. Concentrations found reached from not detected to 104 mg kg^{-1} of abietic acid and 225 mg kg^{-1} of dehydroabietic acid in the top layers of diapers. In the fluff, the concentration range was between 3 and 20 mg kg^{-1} abietic acid and not detected to 225 mg kg^{-1} for dehydroabietic acid. 7-Oxodehydroabietic acid was only detected in one fluff sample of one diaper in the concentration of 2 mg kg^{-1}. They considered the glue used in some diapers to be the biggest source of rosin allergens, but also minimal amounts are derived from the pulp produced from coniferous woods (16).

Thirty-two paper and board raw material samples intended for food contact has been an analysed by Castle et al. to identify potential migrants, including determination of volatiles by headspace GC/MS, extraction using water, ethanol and chloroform with analysis by GC-MS and HPLC. Abietic acid could be identified in the ethanol extracts of unlaminated liquid carton board samples at levels from 2 to 22 mg kg^{-1} with GC-MS. Findings were supported by HPLC retention time and corresponding UV spectra (39).

Van den Berg et al. analysed resin compounds in a 200-year old resin/oil painting by GC-MS using different off-line derivatisation methods. They concluded that the use of TMS-diazomethane yield the most highly oxidized compounds. However, hydroxyl-groups are not derivatised by this method, resulting to high polarity and remaining of these substances on the coloumn. Additional derivatisation with BSTFA can avoid this problem, but with the risk of contaminations and loss of analytes and the formation of TMS-CH_2-esters. Derivatisation with TMS facilitates the distinction between free acids and methyl esters, but mass spectra contain less information since the predominant fragmentation if loss of a methyl group. They presented EI mass spectra for several resin acids and oxidation products with the use of different derivatisation methods and concluded that derivatisation of highly oxidized compounds with BSTFA for analysis with GC-MS is the most suitable (52).

Analysis of ethanol extracts of newspaper samples was done with GC-MS without derivatisation by Binderup et al.. Dehydroabietic acid and dehydroabietic acid-methyl ester were found in the newspaper samples in a concentration range of 5-21 mg kg^{-1} and 7-11 mg kg^{-1} (51).

Ozaki et al. identified genotoxicants in virgin and recycled paperboard and products. The samples were fractionated using liquid/liquid extraction and gel permeation chromatography with analysis by gas chromatography and liquid chromatography. Identification of genotoxicants was done with GC-MS. The fractions that showed DNA-damaging activity in GPC were analysed with GC-MS. The biggest peak identified was dehydroabietic acid with smaller peaks nearby that show similar mass spectra. A second large peak was identified as abietic acid. LC-MS was used for quantification (42).

Bradley et al. investigated the in vitro toxicity of paper sample. Analysis was done with GC-MS after extraction with water, ethanol and Tenax. The main substances extracted into water were pulp-derived natural products like fatty acids, resin acids and natural wood sterols and alkanols. Substances extracted into ethanol were alkanes, phthalic acid ester and diisopropylnaphthalenes. Analysis of water extracts was done with GC-MS, ethanol extracts were analysed with and without derivatisation. Derivatisation and analysis of derivatised ethanol extract was done as described above. Tenax was extracted with ethanol and analysed with or without derivatisation as described above. Only substances in a concentration higher than 10 µg dm^{-2} were identified. In the water extracts of the samples abietic acid was detected in a concentration range of 12-168 µg dm^{-2}, dehydroabietic acid in a range of 10-159 µg dm^{-2}, isopimaric acid at 17 µg dm^{-2}, neoabietic acid at 42 µg dm^{-2}, and other unspecified resin acids at concentration between 12 and 82 µg dm^{-2}. In one sample (board;

water resistant treated) approximately 80% of the whole GC/MS identifiable fraction consists of resin acids. In the Tenax extracts only dehydroabietic acid-methyl ester could be detected in two samples in the concentrations 12 and 18 µg/dm^2. In the ethanol extracts dehydroabietic acid (2.8-38 µg dm^{-2}) dehydroabietic acid-methyl ester (1.1-16 µg dm^{-2}) and 7-Oxodehydroabietic acid methyl ester (2.9-3.5 µg dm^{-2}; in two samples) 15-Hydroxydehydroabietic acid methyl ester (3 µg dm^{-2}, in one sample) has been found (49).

A method for the detection of resin acids with GC/FID, called MDHS 83/2, is described by the Health and Safety Executive UK (53). A modification of this methods was done by Axelsson et al.. Comparison of LC and GC show good correlation despite for 7-Oxo-dehydroabietic acid, where the concentrations were consistently lower with LC (about half of GC). Different solvents were tested to exclude extraction discrimination, with the results that methanol gave higher extraction efficiency for abietic acid than with ether or toluene, and the extraction efficiency is more than twice better for 7-Oxodehydroabietic acid using ether. They concluded that the higher concentration is due to oxidation of abietic acid during sample preparation, confirmed by equivalent decrease of abietic acid. However, they found out that the oxidation is possibly facilitated by other soluble substances extracted from the wood and not because of sample preparation (36).

A summary of gas chromatography methods that has been applied for the analysis of abietic acid and degradation products can be seen in Table 4.

Table 4: analysis parameter gas chromatography

Source	Sample	Analyte	Injection	Carrier gas	Column	Oven Program	Detection
(36)	air filter samples	AA, DHA, 7-oxo-DHA	2 µl		VF-1ms (30 m × 0.32 mm, 0.5 µm).	180°C →195 C (1°C/min) → 285°C (20°C/min; 5min)	FID
(39)	paper & board	AA	1 µl	He	CP-Sil 5CB (17 m × 0.25 mm; 0.25 µm)	100°C (4min) → 310°C (20°C/min)	EI-MS
	headspace paper & board	AA	-	He	CP-Sil 8CB (50 m × 0.32 mm; 1.2 µm)	70°C (2min) → 110°C (5°C/min)	EI-MS
(42)	paper & board	AA, DHA	1 µl	He	HP1-MS (30 m × 0.25 mm; 0.25 µm)	50°C (2min) → 280°C (10°C/min)	EI-MS
(44)	pulpmill effluents	resin & fatty acids	2 µl	He	SPB-5 (30 m × 0.25 mm; 0.25 µm)	70°C (0.75 min) → 210°C (30°C/min) → 290°C (2°C/min)	various MS
(45)	newspaper	rosin	-	N_2	SE-30 (25 m × 031 mm; 0.30 µm)	250°C	FID
(46)	newspaper	AA, DHA, 7-oxo-DHA	0.5 µg/µl	N_2	HP1 (25 m × 0.31 mm; 0.52 µm)	35°C → 210°C (20°C/min) → 240°C (5°C/min)	FID
(16)	diapers	AA, DHA, 7-oxo-DHA	1µg/µl	N_2	HP1 (25 m × 0.31 mm; 0.52 µm)	35°C → 210°C (20°C/min) → 240°C (5°C/min)	FID
(47)	rosin	resin acids	-	-	glass column with 5.0% sersamid 900 (2.9 m × 2.0 mm; 0.52 µm)	220°C	FID
(29)	humic water	AA, DHA, 7-Oxo-DHA	-	-	HP1 (25 m × 0.2 mm; 0.33 µm)	100°C (2min) → 300°C (8°C/min; 4min)	EI-MS
(52)	oil painting	oxidation products	-	He	SGE BPX5 (25 m × 0.32 mm; 0.25 µm)	50°C → 320°C (6°C/min)	EI-MS
(51)	paper & board	DHA	2 µl	He	DB5-MS (30 m × 0.32 mm; 0.5 µm)	90°C (1min) → 280°C (10°C/min; 5min)	EI-MS
(34)	pulpmill effluents	resin & fatty acids	1	He	HP5-MS (30 m × 0.25 mm; 0.25 µm)	120°C (2min) → 300°C (4°C/min; 5 min)	EI-MS

1.1.4.4. Summary

Different methods for the analysis of colophony and its components and degradation products in water, cosmetics, paper and board has been established. Sample preparation is done via liquid/liquid or solid/liquid extraction of the analytes using different solvent like ethanol, methanol or acetonitrile. Derivatisation of resin acids for GC analysis has been done using diazomethane, trimethylsilyl- diazomethane, pentafluorobenzyl bromide (PFBBr), N,O-bis(trimethylsilyl)trifluoracetamide (BSTFA) , tetramethylammonium hydroxide or combinations of the mention agents. Analysis with GC and LC has both advantages and disadvantages: while GC need derivatisation and is therefore more complicated, time intensive and risk of sample manipulation and analyte risk is increased, but it has the better sensitivity, low detection and quantification limits and spectra gave more information. LC has the advantages that no sample derivatisation is needed but it has not been used for complex mixtures until now, because of coelution.

Summing up, every method has its advantages and disadvantages. Which method is applied always depends on the analytical problem and the aim of the analysis.

1.2. Non-Intentionally Added Substances (NIAS) in Food Contact Materials

Food contact materials and articles (FCMs) are defined as materials and articles intended to come into direct or indirect contact with foodstuff (56). During the contact, FCMs may transfer their constituents to the packed foodstuff, depending on the physical and chemical parameters and composition of the FCM and the food. This mass transfer phenomenon is called migration and can lead to changes in the composition and properties of the food, to exposure to certain chemicals and to adverse health effects (57). Plastic materials and articles, including plastic layers in multi-layer materials, that come into contact with food are regulated by Regulation EU 10/2011 (56). Multi-layer-materials can be composed of different materials, may be held together by adhesives and can also be printed or coated. These materials are allowed to contain other substances than those regulated for plastics and are subject to other EU or national rules (e.g. Regulation (EC) No 1935/2004). The European Food Safety Authority (EFSA) has assumed that only migrants with a molecular weight below 1000 Da have to be considered in risk assessment, because substances with a higher molecular weight cannot be absorbed by the body (58). Risk assessment should cover the potential maximum migration and toxicity. The overall migration limit is set as 10 mg of substances per 1 dm^2 surface area of material. 1 dm^2 is referred to a cubic packaging containing 1 kg of food. The migration should be less than 60 mg per kg food when the assumption is taken that a person of 60 kg bodyweight consumes 1 kg of food daily and that this food is packed in a cubic container of 6 dm^2. Migration should be tested using standardised test conditions und methods. The specific migration limit (SML) gives the maximum permitted amount of a given substance released from a FCM into the food and is expressed in mg of substance per kg food (mg kg^{-1}). The SML is set as 60 mg kg^{-1} if no specific migration limit is provided. The total specific migration limit (SML(T)) is the maximum permitted sum of given substances released (56).

As mentioned above, possible migration depends mainly on the composition of the Food contact materials. This composition can be very complicated especially for multi-layer materials. In general, there has to be a base material, like paper, plastics or metals, to which other materials might be added. These other materials are called intentionally added substances (IAS) and are chemicals that are used during the production process. They have a defined function in the manufacturing process or in the final product and are therefore essentials. IAS can be divided into two classes: Well-defined substances and UVCB

substances. Well-defined substances are substances with defined qualitative and quantitative composition that can be easily identified based on several chemical and physical parameters. Substances of unknown or variable composition, complex reaction products or biological materials (UVCB substances) are not well defined and their identification based on chemical and physical parameters is difficult or not possible (57).

Non-intentionally added substances are defined as "*an impurity in the substances used or a reaction intermediate formed during the production process or a decomposition or reaction product*" in Article 3 of Regulation EU 10/2011 (56). The term was not used for non-plastic-FCMs in European legislation until now, but it can be assumed that the regulations can also be considered for non-plastic materials (57). Considering the mentioned definitions abietic acid or colophony could be considered to be an intentionally added substance, while their oxidation and degradation products are non-intentionally added substances.

The sources of NIAS could be classified as follows (59):

1. Degradation processes:

   Degradation of base materials and additives (IAS) is one of the most important pathways to the formation of NIAS. The main source of degradation are the conditions during packaging manufacturing. High temperatures and irradiation leads to degradation, forming molecules with lower molecular weight, which can migrate easier. In case of abietic acid, autoxidation takes place, leading to oxidised products that have a high contact allergenic potential.

2. Degradation of additives:

   Antioxidants or light stabilizers are added to improve the properties of the material and with the purpose to protect the material e.g. from oxidation. Their degradation products are present in packaging materials and are potential migrants. Commonly used antioxidants has been widely studied (60) (61) (62). In addition, degradation products from adhesives, coatings or inks can suffer degradation. (63)

3. Impurities

   NIAS are often derived from impurities present in base materials or additives. Impurities are never described in information data sheet and therefore their identity is unknown.

4. Neoformed compounds

   Neoformed compounds are substances derived from a chemical reaction between different intentionally added substances in the material. For example, alkoxy-radicals

derived from abietic acid or from one of its oxidation products can react with each other forming dimers.

5. Contaminants

   Contaminants can be derived from recycled materials used in food packaging, by misuse prior discarding or by intrinsic contaminants from the recycling process (64). They may also be process contaminants such as lubricants or contaminations from storage and transport or they may be unpredictable environmental contaminants (57).

Considering the sources of NIAS they can be divided into predicted NIAS and unpredicted NIAS. The assessment of NIAS in food contact materials should start with the characterisation of the sample (base material of FCM, kind of FCM, manufacturing process…) and the IAS used. With this information predicted NIAS like breakdown and reaction products formed from base material and IAS could be identified, including previously detected NIAS. The last step is the assessment of unpredicted NIAS that have not been found before and are not predictable from the chemistry of the system (57).

In general, identification of NIAS is very difficult since there is often a lack of information about the composition and ingredients used in a food contact material. Furthermore, the materials can have a very complex structure with different layers that are coextruded or laminated, using different adhesives and polymers, which can be coated by varnishes and printing inks. Also there is never one producer of a material, there are always several partners involved during the packaging manufacture and the storage and using conditions can lead to contaminations and changes of ingredients. This brings us to the point that it is never possible to know the exact composition of a material (59).

For the analysis of NIAS (57) two types of analytical methods may be considered: Targeted analytical methods for predicted NIAS and non-targeted analytical methods to screen and analyse for a wide range of substances with differing properties.

One kind of method for analysing unpredicted NIAS is solvent extraction to gain high concentrations of substances and therefore high concentration of potential NIAS. Each peak present in GC or LC, which exceeds a level of interest, should be identified if possible. If full identification is not possible, classification into specific chemical groups could give information about potential health risks.

Volatile substances could be analysed by cutting the sample into pieces, transfer them to headspace vials and use solid phase micro extraction with GC-MS for analysis. For semi

volatile substances GC or LC are possible screening methods. Extracts or migrates of non-volatile substances can be analysed using LC or after derivatisation GC-MS may be used. Quantification of known NIAS is possible by calibration standards, containing known amounts of substance of interest. Unknown NIAS could be compared and estimated with the concentration and signal response of an internal standard. Therefore, responses should be similar and relatively comparable. This could be achieved by using a universal detector and differing internals standards. For GC universal detectors can be flame ionisation detector, electron impact mass spectrometry and chemical impact mass spectrometry. EI-MS and CI-MS are advantageous because of easy identification of substances through library search. For LC electrospray ionisation is a technique widely used for analysis of non-volatile substances. Alternatives are atmospheric pressure chemical ionisation and atmospheric pressure photo ionisation. The internal standard should be chosen in a way, that it has a smaller detector response compared to the substances of interest. This makes sure, that the detected levels of substances are always the worst case of migration, because they are always overestimated.

1.3. Methods

1.3.1. Gas-Chromatography Mass-Spectrometry

Gas chromatography mass spectrometry (GC-MS) is an analytical method where a mixture of analytes is separated in a gas chromatograph and detected in a mass spectrometer.
In gas chromatography, the separation of analytes takes place between a mobile gaseous and a stationary phase. The analytes have to be volatile or vaporizable, which is the limitation factor of this method. Important parts of the gas chromatograph are the liner, the mobile phase and the column. In the liner the sample is vaporized which is influenced by the liner temperature, the boiling point of analytes and the molecular weight of the analyte. The mobile phase which is usually an inert or unreactive gas transports the analytes from the liner, through the column to the detector. The column includes the stationary phase where the analytes are separated because of different interactions influenced mainly by polarity of analyte and column. This factors result into the separation of the sample and into different retention times of the analytes which then could be detected individually by the mass spectrometer. The mass spectrometer detects ions that could be generated in different ways by their mass-to-charge ratio. In this work electron impact ionisation was used for GC-MS. The analytes that come from the column are ionized by an electron stream which is generated by a filament because of an electric current. The produced ions are then sorted by their mass-to-charge ration and detected by e.g. electron multipliers.
Electron impact is considered to be a hard ionisation technique because it results in high fragmentation of the analyte molecules. It gives a more or less characteristic spectrum for every analyte which helps to identify them. A big advantage of electron impact ionisation is the comparability of the mass spectra that were detected with 70 eV with databases which makes identification sometimes very easy. Besides the mass spectrum the retention time of the GC could be an important information about the analyte. However, retention time changes from system to system and a general index, the retention index, is used to compare data.

1.3.1.1. Theory of Retention Indices

Gas chromatography is the most popular method for the analysis of volatile substances. Often it is necessary to separate and identify substances in small concentrations that may be very similar which is not always possible through their mass spectrum. In such cases it is possible to determine retention indices and compare them with data in the literature to

identify the unknown compounds. Retention indices are indices with which it is possible to compare data recorded on different measurement instruments and with different methods. The indices are summarised in databases were they could be compared to each other to identify substances through their retention indices.

To calculate retention indices a mix of n-alkanes has to be measured with the same method as the samples. With the retention times of the n-alkanes that are in the immediate surrounding of the unknown substance the retention index is calculated. Calculation is possible using different methods. In this work the method of Dool and Kratz was used, which uses the formula

$$RI = 100 * \frac{T_x - T_z}{T_{z+1} - T_z} + 100 * z$$

for the calculation of the retention indices. In this formular T_x is the retention time of the unknown compound, T_z and T_{z+1} the retention times of the n-alkanes that enclose the unknown compound and z the number of the C-atoms of the smaller n-alkane (65). The calculated retention indices are compared to indices given in literature.

1.3.2. Liquid-Chromatography Mass-Spectrometry

Liquid-chromatography with mass-spectrometry is the counterpart of gas-chromatography. In this method the separation of the sample takes place between a mobile liquid phase and a stationary phase. This means the limit of this method is the solubility of the analytes in an appropriate solvent. Separation of the analyte mixture again depends on the properties of analyte and column, which results in different retention times. In this work the eluted analytes were ionized by electron spray ionisation, where the liquid analyte stream is dispersed by electrospray into an aerosol from which the ions are emitted because of electrostatic processes. The generated ions are again sorted and detected.

Electron spray ionisation is a soft ionisation method. This means that fragmentation hardly takes place and the molecule ion or quasimolecule ions (addition of cations like H^+, Na^+ to the molecule ion) could be detected instead of fragments. For electron spray ionisation comparison of detected data and identification of analytes is more difficult.

1.3.3. Two-Dimensional Comprehensive GC×GC-MS

Two-dimensional comprehensive GC×GC-MS or short comprehensive GC-MS or GC×GC-MS is a method used when a complex mixture of analytes has not be separated efficiently in one-dimensional GC-MS (66). The comprehensive GC-MS uses two columns with different selectivity to separate the analytes efficiently. The name comprehensive refers to a method that is able to separate the entire sample eluting from a primary column on a secondary column, rather than separating just a specific fraction as in "heart-cut" multidimensional GC. The components separated in the first dimension must remain separated in the second one (67) (68).

The comprehensive GC×GC-MS is build-up of an injection port, a primary column, a resampling device (modulator), the secondary column and the detector. The two columns are be placed in the same or in different ovens resulting in the same or in different temperature programs (69). The first column is a conventional, typically nonpolar GC-column and separates the analytes by their boiling point or rather volatility. The second column has opposite polarity to the first, is much shorter and uses the polarity of the analytes to separate them. This results into the three dimensions: the first column gives the separation through boiling point, the second column the separation through polarity and the detector gives the mass spectrum or intensity as the third dimension (70 p. 96).

The two columns are connected in series by a modulator. The modulator is the most important unit of the comprehensive GC-MS since he controls the flow from the first column into the second. The analytes that leave the first column are trapped in the modulator for a defined time before they are focused and injected onto the second column. The time needed to complete these steps are called modulation time or frequency. Modulation frequency is normally between three and six seconds. In this time, the analytes that come from the first column are trapped, focused and reinjected onto the second column. This means that separation on the second column has to be fulfilled between two modulations, otherwise there will be a phenomenon called wrap-around leading to peak broadening Therefore, the dimensions of the second column are usually between 0.5 and 1.5 meters' length.

Modulators are divided into three classes: heat-based modulators, cryogenic modulators and flow modulators. In this work, a cryogenic two-stage loop modulator was used, which traps the analytes using a continuous cold jet creating two cold spots in the modulation loop at a temperature below that of the GC oven. The first cold spot traps the analytes during one modulation time, the second one focuses the analytes stream during a second modulation

and reinjection and separation is done by a third modulation. This modulation cycle prevents interferences by analytes eluting from the first column during reinjection and peak broadening in the second dimension. The cold jet was produced by heat exchange using liquid nitrogen. Reinjection was made using a hot jet impulse, which is blown against the modulation tube. (70) (71).

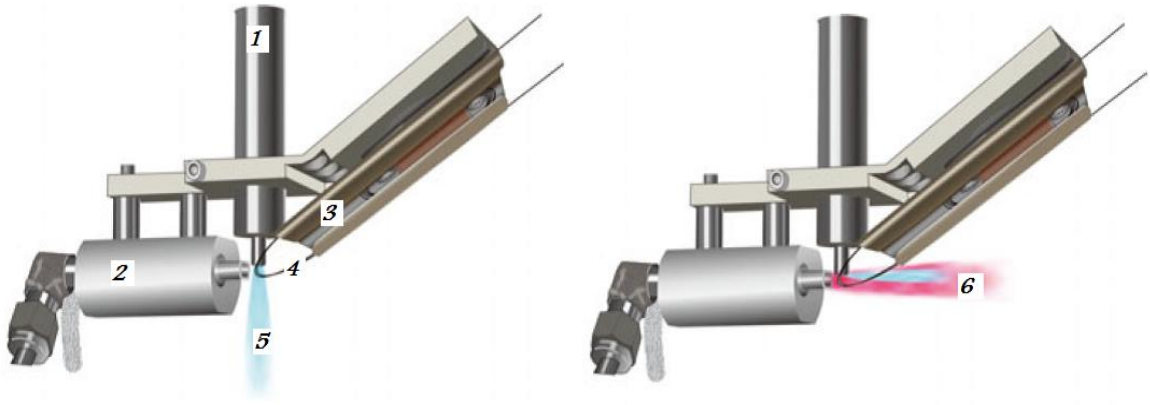


Figure 8: two-stage loop modulator showing (1) cold jet assembly, (2) hot jet assembly, (3) column holder, (4) modulation loop/tube, (5) cold jet, (6) hot jet; image derived from (71).

The result of a two dimensional comprehensive GC×GC-MS analysis are series of two-dimensional chromatograms derived from modulation of the one-dimensional separation (Step 1; Figure 9). These series are transformed to a second-dimensional chromatogram with the axis intensity, retention time on first column and retention time on second column (Step 2; Figure 9). The second-dimensional chromatogram is transformed into colour, contour or 3D plots that visualise the peak intensity by different colours or shades (Step 3; Figure 9). To visualise peaks with different intensities it is often important to change the contrast settings when working with visualisation through colour or shading. Specially designed software programs are used to create the two-dimensional image (72).

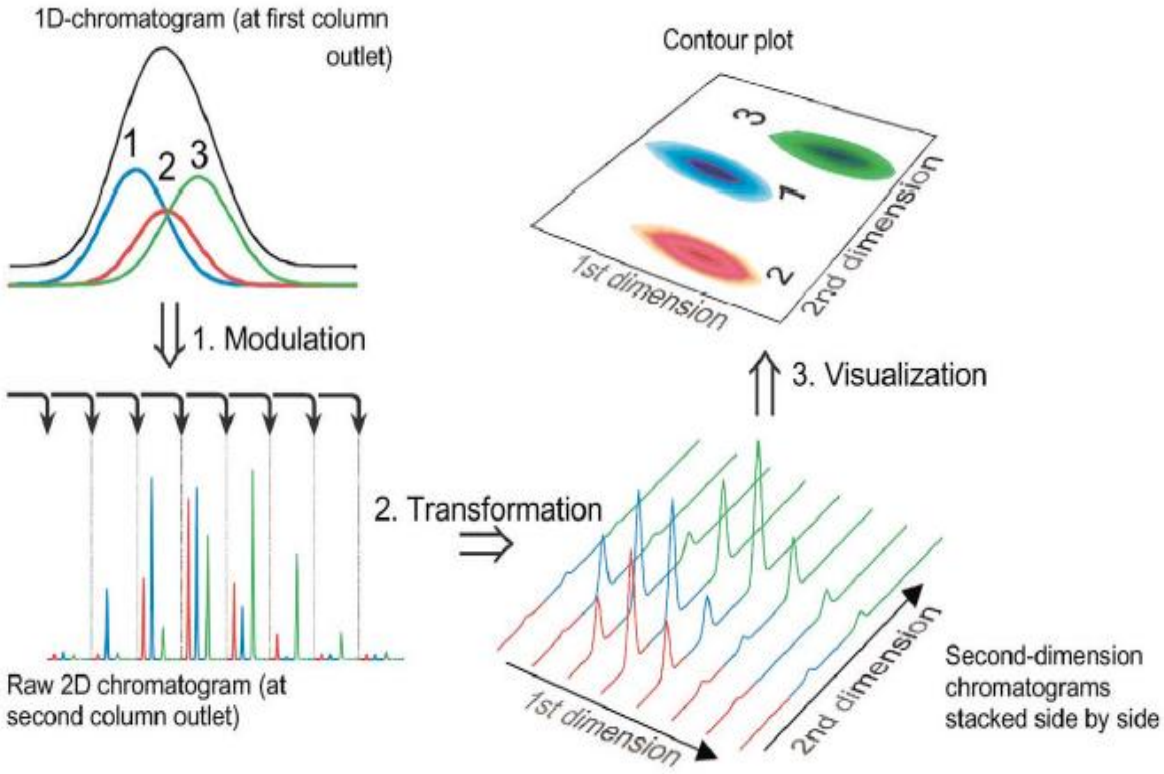


Figure 9: Creating a two dimensional comprehensive GC×GC-MS image. Figure derived from (72)

The advantages of comprehensive GC-MS are considered to be excellent selectivity, high sensitivity, good separation power, speed and structured chromatograms (70 p. 8).

2. Experimental

2.1. Solvents, Standards and Internal Standard

The solvents used for GC-MS analysis were acetone (ROTISOLV® ≥99.9 %, UV/IR-Grade; Carl Roth GmbH + Co. KG, Karlsruhe, Germany), ethyl acetate (Picograde® for Residue Analysis; LGC Promochem GmbH, Wesel, Germany), methanol (ROTISOLV® HPLC Gradient Grade; Carl Roth GmbH + Co. KG, Karlsruhe, Germany). For derivatisation BSTFA + TMCS (99:1; Sylon BFT; Supelco, Bellefonte, USA) and pyridine (puriss. p.a., ACS; ⩾99.8%; Fluka, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) were used.
For LC-MS the used solvents and reagents were methanol (HiPerSolv CHROMANORM for HPLC, LC-MS grade; VWR Chemicals, Radnor, Pennsylvania), acetonitrile (ROTISOLV® ⩾99.95 %, LC-MS Grade; Carl Roth GmbH + Co. KG, Karlsruhe, Germany), ultrapure water (18 mΩ), ammonium acetate (puriss. p.a. for HPLC; ⩾ 99.0%; %; Fluka, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and acetic acid (glaciale, 100% p.a.; Merck KGaA, Darmstadt, Germany).

As internal standard palmitic acid-d_{31} (98 atom % D; Aldrich Chem. Co.) has been used. An internal standard solution of 10 g L^{-1} in methanol has been made and 100 µL were added into the slurry. For the paper samples 10 µL of the 10 g L^{-1} standard in methanol were added. This results in a final concentration of internal standard of 100 ng absolute in 1 µL injected sample for GC-MS analysis.

For LC-MS analysis, a 1 g L^{-1} standard solution of palmitic acid-d_{31} and a 1 g L^{-1} standard solution of abietic acid in methanol (LC-MS grade) were prepared. From these standard solutions, a mix of both substances was prepared in concentrations of 1 and 5 mg L^{-1} in the used mobile phase.
The 1 g L^{-1} standard of palmitic acid d_{31} was also used for quantification in the product analysis. 10 µL were added to the samples prior extraction, resulting in 10 ng absolute in 1 µL injected sample.

2.2. Instrumentation

2.2.1. Gas Chromatography-Mass Spectrometry

Gas chromatographic separation was done using a Hewlett Packard HP 6890 Series GC system equipped with an Agilent 7683 series injector and an Agilent HP5-MS capillary column ((5%-phenyl)-methylpolysiloxane; 30 m × 250 µm; 0.25 µm). Injection was done in split-less mode and injector port temperature was 250°C. Helium was used as carrier gas with a constant flow of 36 cm sec^{-1}. Initial oven temperature was 90°C and was raised to 310°C with a ramp of 15°C min^{-1}. Detection was done with a Hewlett Packard 5973 mass selective detector. Ions were generated with electron ionisation (70 eV), EMVolts were set at 1200, mass spectrometer scanned from m/z 50 to 450 with a scan rate of 3.59 scans sec^{-1}. The software used was the Agilent Technologies "Enhanced ChemStation".

2.2.2. Liquid-Chromatography-Mass Spectrometry

The HPLC system used was a Shimadzu Nexera X2 and consisted of two DGU-$20A_{5R}$ degassing units, two LC-30AD pumps, a SIL-$30AC_{MP}$ autosampler, a SPD-M30A diode array detector, a CTO-20AC column oven (set at 40°C) and a CBM-20A communications module. Chromatographic separation was done using a Phenomenex Kinetex® C18 column (100 x 2.1 mm; 1.7 µm particle size; 100 Å pore size). Different mobile phases were tested as described in chapter 2.5.1. Mobile Phase. The best results were achieved using methanol and water with 0,05% (v:v) acetic acid starting at 70:30 rising to 100% water with 0,05% (v:v) acetic acid within 10 min, holding for two minutes and getting back to 70:30 within three minutes. The flow rate was 0,4 ml min^{-1}.

The MS system used was a Shimadzu LCMS-8050 liquid chromatograph mass spectrometer with electron spray ionization. Heating and drying gas flow was 10 L min^{-1}, nebulizing gas flow 3 L min^{-1}. Interface temperature was set a 300°C, heating block temperature 400°C and DL temperature 250°C. Detection was performed using positive (m/z 303 and 301 for abietic acid) and negative (m/z 299 for dehydroabietic acid and 286 for palmitic acid d_{31}) SIM-mode. Detection in positive and negative scan-mode was also tried, scanning from m/z 50 to 500. The software used was "LabSolutions".

2.2.1. Two-Dimensional Comprehensive GC×GC-MS

Comprehensive GC-MS was built up from following units: an OPTIC-4 Multimode GC Inlet and a AOC-5000 Plus auto sampler, a Shimadzu gas chromatograph for mass spectrometer GC-2010 Plus equipped with a HT1 column (30 m × 0.25 mm; 0.25 µm) and a BPX5 column (2.5 m × 15 mm; 0.15 µm) placed in the same oven. Columns were connected directly by using a push fit connector and gluing with polyimide resin. Injection was done in split-less mode, split was opened after 1 min with a split ratio of 30 and injector port temperature was 270°C. Helium was used as carrier gas in linear velocity flow control mode. Pressure was set at 150 kPa, purge flow was 1.0 ml min^{-1} and split ratio was 10. Initial oven temperature was 60°C, was raised to 200°C with a ramp of 20°C min^{-1} and further to 300°C with a ramp of 3°C min^{-1}. The modulator used was a ZOEX corporation modulator model 186 liquid level controller. Modulation frequency was 4 sec with hotjet impulse at 280°C for 350 ms. Detection was done using the mass spectrometer GCMS-QP2010 Ultra. Ions were generated with electron ionisation (70 eV), ion source temperature was set at 200°C, interface temperature at 290°C, mass spectrometer was scanned with a scan speed of 2000 amu sec^{-1} resulting in 33 full scan spectra recorded from m/z 50 to 500. Data were acquired between 7 and 45 min runtime.

The software used for data evaluation was Version 2.5 of “GC Image” from Zoex Corporation (compare Figure 9).

2.3. Samples

For the development of a method to identify and quantify abietic acid, other resin acids and degradation product an aqueous slurry of abietic acid, which is used for the coating of paper, and three paper samples were received. According to the material safety data sheet the slurry consists of 10-20% tall oil colophonium fumarate, 1-5% colophonium that has been treated with fumarate, <10% aluminium sulfate and <15 ppm of a mixture of 5-chloro-2-methyl-2H-isothiazol-3-on and 2-methyl-2H-isothiazol-3-on. The slurry was used either as received or after drying. Different drying methods has been tested which are described in chapter 2.4.2.1. Drying.

Three paper samples with the sample codes 2016_0383 (grammage: 75 g m^{-2}), 2016_0506 (grammage: 35 g m^{-2}) and 2016_0511 (grammage: 110 g m^{-2}) were received as samples for method development. This paper samples have been treated with the slurry in an application amount of 10 liter slurry per ton of paper. The paper samples were cut into small pieces of about 5x5 mm and extracts were made in 20 ml glass vials with screw cap using an ultrasonic bath at 40°C and different solvents and extraction times that have been described in literature (45; 46; 47). For GC-MS analysis, the extracts were decanted into 50 ml turbovap vials and evaporated to a volume of 0.5 ml using a TurboVap® II (Biotage, Uppsala, Sweden). Then the samples were transferred to 1.5 ml glass vials with screw caps and the turbovap tubes were washed with 0.5 ml of the solvent used for extraction. The samples were further evaporated to dryness under a stream of N_2 prior derivatisation. Derivatisation of samples for GC-MS analysis was made using BSTFA + TMCS and pyridine at room temperature or at 60°C for 15 or 30 min as described in literature (29; 49; 52). Ethyl acetate was added to yield a volume of 1 ml after derivatisation. Prior to analysis with GC-MS the slurry samples were diluted 1:10 with ethyl acetate (100 µL sample + 900 µL ethyl acetate), the paper samples were measured non-diluted. Alternatively, aliquots of the extracts were taken instead of all of the extract and evaporated under a stream of N_2.

For analysis with LC-MS 1 ml of the extracts were taken, evaporated to dryness, resolved in mobile phase (methanol/water 70:30) and measured without derivatisation.

For market analysis, different hygiene products were purchased. They differ in brand, producer and country of purchase and are summarised in Table 5. Brand and producer names were coded with numbers.

Sample preparation for the hygiene products was made as described in 2.6. Market Analysis. Extraction was done as described above, using methanol/ acetone 1:1 as solvent and derivatisation was made using 50 µL BSFTA and 50 µL pyridine for 30 min at room temperature prior GC-MS analysis.

Table 5: hygiene product samples

Products	Brand Code	Producer Code	Purchase Country	Purchase Date	Production Date
Tampons	T1	1	Germany	11.10.2016	06.2012
	T2	2	Germany	11.10.2016	-
	T3	3	Czech Republic	-	28.07.2013
	T4	4	Belgium	28.09.2016	-
	T5	4	Romania	15.10.2016	-
	T6	3	Belgium	28.09.2016	19.07.2016
	T7	5	Romania	15.10.2016	21.07.2015
Panty Liner	PL1	3	Belgium	28.09.2016	14.08.2016
	PL2	4	Romania	15.10.2016	08.08.2016
	PL3	6	-	-	28.08.2016
	PL4	3	Czech Republic	-	18.07.2016
	PL5	3	Romania	15.10.2016	24.03.2015
	PL6	2	Germany	11.10.2016	-
	PL7	7	Germany	11.10.2016	07.05.2016
	PL8	1	Germany	11.10.2016	15.06.2015
	PL9	8	Czech Republic	-	11.2015
	PL10	5	Romania	15.10.2016	26.07.2016
Nursing pads	NP1	8	Czech Republic	-	11.2015
	NP2	5	Romania	15.10.2016	20.06.2016
	NP3	4	Romania	15.10.2016	06.2015
	NP4	9	Belgium	28.09.2016	29.12.2015
Diapers	D1	4	Romania	15.10.2016	-
	D2	3	Romania	15.10.2016	17.09.2019
	D3	5	Romania	15.10.2016	02.03.2016

2.4. Experiments GC-MS

2.4.1. Derivatisation

For testing different derivatisation methods extracts of 500 mg of the paper sample 2015_0511 were prepared using 10 ml methanol and extraction for 30 min at 40°C in ultrasonic bath two time. The extracts were combined afterwards and all of it or aliquots were taken and evaporated to dryness prior derivatisation.

First, extracts of 500 mg of the paper sample 2015_0511 were made (n=2) and aliquots of 0.5 ml were taken (n=6 from each extract). These aliquots were evaporated to dryness under a stream of N_2 and derivatised at room temperature for 15 or 30 min with amounts of BSTFA and pyridine of 100 + 100 µL (n=4), 100 + 50 µL (n=4) or 50 + 50 µL (n=4).

Second, extracts of the paper sample 2015_0511 were made (n=8) and the solvent was evaporated to dryness. Derivatisation was made at room temperature or at 60°C for 30 min with 100 µL BSTFA + 100 µL pyridine.

Third, derivatisation was tested using vacuum dried slurry. 10 mg were weight into 2 ml glass vials with screw caps, internal standard was added and the solvent dried under a stream of N_2 (n=10). Derivatisation was made using amounts of BSTFA and pyridine of 100 + 100 µL (n=2; derivatisation only at room temperature), 50 + 50 µL (n=4) or with only 50 µL BSTFA (n=4) at room temperature or at 60°C for 30 min.

2.4.2. Slurry

2.4.2.1. Drying

Different methods for the drying of the slurry were tested:

First, 20 mg of slurry were weight into a 1.5 ml glass vial, 100 µL internal standard were added and drying was made in a drying oven at 100°C for about 30 min. Second, 20 mg of slurry were weight into a 1.5 ml glass vial, 100 µL internal standard were added and drying was made under a stream of N_2. Third, 5 g of slurry were weighted into a crystallizing dish and placed in a drying oven at 100°C over night. Thereby the slurry got oxidized and a black powder was received as product after homogenization. 10 mg were weight into a 1.5 ml glass vial, 100 µL internal standard were added and the solvent evaporated under a stream of N_2. Fourth, 20 g of slurry were weighted into a crystallizing dish and dried in a vacuum drying oven (VDL115; Binder; Tüttlingen, Germany) at 40°C for 2h. The yield of dry slurry was

6,31 g (30.99%). 10 mg of dried slurry were weighted into 1.5 ml glass vials with screw cap after homogenization and 100 µL of internal standard were added. The solvent was evaporated under a stream of N_2.

Derivatisation of all samples was made using 50 µL BSTFA and 50 µL for 30 min at room temperature. 900µL ethyl acetate were added afterwards to yield a final volume of 1 ml. The samples were diluted 1:10 with ethyl acetate (100 µL samples + 900 µL ethyl acetate) prior analysis with GC-MS.

2.4.2.2. Extraction of Slurry

10 mg of slurry dried in vacuum drying oven were weighted into 2 ml glass vials with screw cap (n=6). Then 500 µL hexane (n=2), methanol (n=2) or ethanol/hexane 1:1 (n=2) were added and the samples were extracted for 30 min at 50°C in an ultrasonic bath. Afterwards the samples were allowed to stand until all of the fine powder was settled and the clear supernatant could be decanted. The decanted solvent was evaporated to dryness under N_2 and the samples derivatised with 100 µL BSTFA and 100 µL pyridine for 30 min at room temperature. 800 µL ethyl acetate were added to a final volume of 1 ml and the samples were diluted 1:10 with ethyl acetate prior analysis with GC-MS in SCAN mode.

2.4.3. Paper

Paper samples were cut into small pieces of about 5x5 mm and extracted with a solvent in an ultrasonic bath. Different solvents and extraction times were tried that are described in literature (45; 46; 47) and adapted to the problem.

1 g paper sample 2015_0511 was weighed into 20 ml glass vials with screw cap (n=5). Extraction was done with 10 ml of methanol (n=1), acetone (n=1) or ethyl acetate (n=1) for 30 min at 40°C in an ultrasonic bath. A second and third extraction with methanol has been done for 45 min (n=1) and 60 min (n=1), respectively.

Second, 500 mg paper sample 2016_0511 were weighed into glass vials with screw cap (n=8). They were extracted with 5 ml methanol (n=4) or acetone (n=4) for 30 min at 40°C in an ultrasonic bath and the solvent was decanted. Four of the paper samples were extracted a second time with 5 ml of methanol (n=2) or acetone (n=2) for 30 min at 50°C in an ultrasonic bath. The extracts were combined afterwards.

Third, 500 mg paper sample 2015_0511 were weighed into 20 ml glass vials with screw cap and 10 µL of internal standard were added. Extraction was done with 5 ml of a methanol and acetone 1:1 solution for 30 min at 50°C in an ultrasonic bath. After 30 min the solvent was

decanted and the paper was extracted a second time with 5-10 ml of methanol/acetone 1:1 for 30 min.

After the extraction, the solvents were decanted into 50 ml TurboVap tubes and the solvent evaporated to a volume of 0.5 ml using a TurboVap® II. The samples were transferred to 2 ml glass vials with screw cap and the tubes were washed with 0.5 mL of the solvent used for the extraction. Then the samples were evaporated further to dryness under a stream of N_2 and derivatised with 100 µL of BSTFA and 100 µL pyridine for 30 min at room temperature. Afterwards 800 µL ethyl acetate were added to give a final volume of 1 ml prior analysis with GC-MS.

2.4.4. Validation

For validation of method the levels 0, 5, 10 and 15 mg kg^{-1} and 0, 50, 100 and 150 mg kg^{-1} were chosen to do a standard addition because values of abietic acid varied between the low range in the market products and the high range in the received paper samples. As samples the tampons T2 from producer 2 were used for the lower range, the tampons with the product name T7 from producer 5 were used for the higher range. These two products were chosen because they had the highest initial weight. This was important because triplicates were done and for one replicate of the calibration range one tampon was used (n=4 from one tampon). 500 mg of the cotton of one tampon were weight into 30 ml glass vials four times. 20 ml of methanol/acetone 1:1, 10 µL of a 1 g L^{-1} internal standard solution of palmitic acid-d_{31} in methanol and from a 1 g L^{-1} abietic acid standard in methanol the appropriate amount was added. Extraction was done in an ultrasonic bath for 30 min at 40°C two times. After the extraction, the solvents were decanted into 50 ml TurboVap tubes and the solvent evaporated to a volume of 0.5 ml using a TurboVap® II. The samples were transferred to 2 ml glass vials with screw cap and the tubes were washed with 0.5 mL methanol/acetone 1:1. Then the samples were evaporated further to dryness under a stream of N_2 and derivatised with 50 µL BSTFA and 50 µL pyridine for 30 min at room temperature. 900 µL ethyl acetate were added to give a final volume of 1 ml prior analysis with GC-MS.

Further, two external calibration with the levels 0,5,10 and 15 mg kg^{-1} and 0, 50, 100, 150, 200 and 250 mg kg^{-1} were made to determine limit of detection and limit of quantification. Therefore, 10 µL of a 1 g L^{-1} internal standard solution of palmitic acid-d_{31} in methanol and the appropriate amount of a 1 g L^{-1} abietic acid standard in methanol were added into 1.5 ml glass vials with screw caps (n=3). The solvent was evaporated to dryness under a stream of

N_2 and derivatised with 50 µL of BSTFA and 50 µL pyridine for 30 min at room temperature. Afterwards, 900 µL ethyl acetate were added to give a final volume of 1 ml prior analysis with GC-MS.
The received results were validated using "ValiData" Version 3.02.48, an excel macro for method validation.

2.4.5. Sample Preparation for Two-Dimensional Comprehensive GC×GC-MS

For analysis of the slurry with comprehensive GC-MS 100 µL of the 10 g L^{-1} were added into a 1.5 ml glass vial with screw cap and the solvent was evaporated under a stream of N_2. Into this vail, 10 mg of vacuum dried slurry were weight in and the samples derivatised with 50 µL BSTFA and 50 µL pyridine for 30 min at room temperature. Afterwards 900 µL ethyl acetate were added and the samples diluted 1:20 with ethyl acetate prior analysis with comprehensive GC-MS.

One sample of panty liner was also analysed with comprehensive GC-MS. Therefore, the release liner and the pad were separately cut into small pieces of about 5x5 mm and 200 mg liner and 500 mg padding were weight into 30 ml glass vials with screw cap. 10 µL of a 1 g L^{-1} internal standard solution were added and 20 ml of methanol/acetone 1:1 were added. Extraction was done in ultrasonic bath at 40°C for 30 min twice. Afterwards the two extracts were combined and evaporated to dryness using a TurboVap and a stream of N_2. Derivatisation was done with 50 µL BSTFA and 50 µL pyridine for 30 min at room temperature. 900 µL ethyl acetate were added and the samples diluted 1:2 with ethyl acetate prior analysis with comprehensive GC-MS.

2.5. Experiments LC-MS

2.5.1. Mobile Phase

As mobile phase acetonitrile/water or methanol/water with different additives were described in literature (see chapter 1.1.4.3. Analysis Parameter for LC and GC). The described mobile phases were tested and compared using the 1 and 5 mg L^{-1} standard mix and an injection volume of 1 µL. The LC conditions used were isocratic flow of water/organic phase 70:30 and a run time of 20 min. First of all, 70:30 acetonitrile/water was tested, followed by acetonitrile/water with 0.05% (v:v) acetic acid and acetonitrile/water with 50 mM ammonium acetate. Afterwards the solvents were changed to methanol/water 70:30, methanol/water with 0.05% (v:v) acetic acid and methanol/water with 50 mM ammonium acetate. Using methanol results into higher retention times (above 20 min) and the isocratic method was replaced by gradient elution (starting with 70:30 rising to 100% methanol within 15 min).

2.5.2. Paper Samples

The paper samples 2016_0511 and 2016_0506 were cut into pieces of about 5x5 mm and extracts were made out of 300 mg using methanol as solvent in an ultrasonic bath at 40°C for 30 min twice. The two fractions were combined afterwards and 1 ml of these extracts were taken and evaporated to dryness. 50 µL of a 100 mg L^{-1} internal standard solution (prepared from 1 g L^{-1} standard solution) and 950 µL methanol/water 70:30 were added. 1 µL of this samples were injected into HPLC-MS.

2.6. Market Analysis

2.6.1. Tampons

Two tampons with high packaging contact area (from the corner of the cardboard packaging) were taken for the determination of abietic acid and degradation products to get worst-case concentrations. The initial weight of the tampons was determined prior opening them, then the plastic jackets were weighted and rejected. The tampons T6 include retractable applicators, which were also rejected. The mat of fibers that surrounds the cotton batting was separated and weighted into a 20 ml glass vials (n=4; duplicates of two tampons). Then 500 mg of cotton were taken and weight into 20 ml glass vials (n=4; duplicates of two tampons). The string was also weighted and rejected afterwards. To the mat of fibers 10 ml of a methanol: acetone mixture 1:1 were added, for the cotton 20 ml were necessary to cover all of the sample. As internal standard 10 µL of 1 g L^{-1} standard solution were added to the samples. The samples were extracted 30 min at 40°C in an ultrasonic bath. Afterwards the extracts were decanted into 50 ml TurboVap tubes and the cotton squashed with glass pipettes to retrieve as much solvent as possible. The extraction was repeated using 10 ml of methanol: acetone 1:1 for all samples. Afterwards the extracts were combined in the TurboVap tubes and the solvent evaporated to a volume of 0.5 ml using a TurboVap® II. The samples were transferred to 1.5 ml glass vials with screw cap and the tubes were washed with 0.5 mL of methanol: acetone 1:1. Then the samples were evaporated further to dryness under a stream of N_2 and derivatised with 50 µL of BSTFA and 50 µL pyridine for 30 min at room temperature. Afterwards 900 µL ethyl acetate were added to give a final volume of 1 ml prior analysis with GC-MS.

2.6.2. Nursing pads

Two nursing pads with high packaging contact area were taken to determine worst-case concentrations. The initial weight of the products was determined, then the release liner was removed and the weight of both, liner and padding was determined. Afterwards liner and padding were cut into small pieces and weight into 30 ml glass vials with screw cap separately (n=4; from two panty liners). The whole liner of one nursing pad was used (200-300 mg), from the pad 500 mg were weight in. Sample preparation and derivatisation was made as described above under 2.6.1. Tampons.

2.6.3. Diapers

Diapers were not packed into cardboard, so two diapers were taken randomly, and initial weight was determined. One part from the middle of the diaper was cut out; the filling that consists of cotton and the fleece that will have the most skin contact during use were separated. The fleece was cut into small pieces and 500 mg were weight into 30 ml glass vials, from the cotton 500 mg were weight in (n=4; from two diapers). Sample preparation and derivatisation was made as described above under 2.6.1. Tampons.

2.6.4. Panty liner

Two panty liners with high packaging contact area (from the front side and the backside of the cardboard packaging) were taken to determine worst-case concentrations. The initial weight of the products was determined, then the release liner was removed and the weight of both, liner and padding was determined. Afterwards liner and padding were cut into small pieces and weight into 30 ml glass vials with screw cap (n=4; from two panty liners). The whole liner of one panty liner was used; from the padding, 500 mg (or the whole padding if initial weight is below 500 mg) were weight in.

From the panty liners in which the highest concentrations of resin acids were detected the packaging was also extracted. Therefore, the cardboard packaging was cut into small pieces of 5x5 mm and 500 mg were weight into 30 ml glass vials with screw cap.

Sample preparation and derivatisation for panty liners and packaging was made as described above under 2.6.1. Tampons.

3. Results

3.1. GC-MS

3.1.1. Derivatisation

Derivatisation with BSTFA and pyridine was tested using one extract of a paper sample 2016_0511 and taking aliquots of it (0.5 ml from 10 ml extract). Derivatisation was done with different amounts of reagents and 15 or 30 min reaction time. The results are that derivatisation with amounts of BSTFA + pyridine of 100 + 100 µL for 30 min works best, followed by 100 + 50 µL for 30 min which is comparable with 100 + 100 µL for 15 min. Using 50 + 50 µL gave for both reaction times the worst result. Figure 10 shows how different amount of derivatization reagents influence the detected amount of abietic acid in the samples.

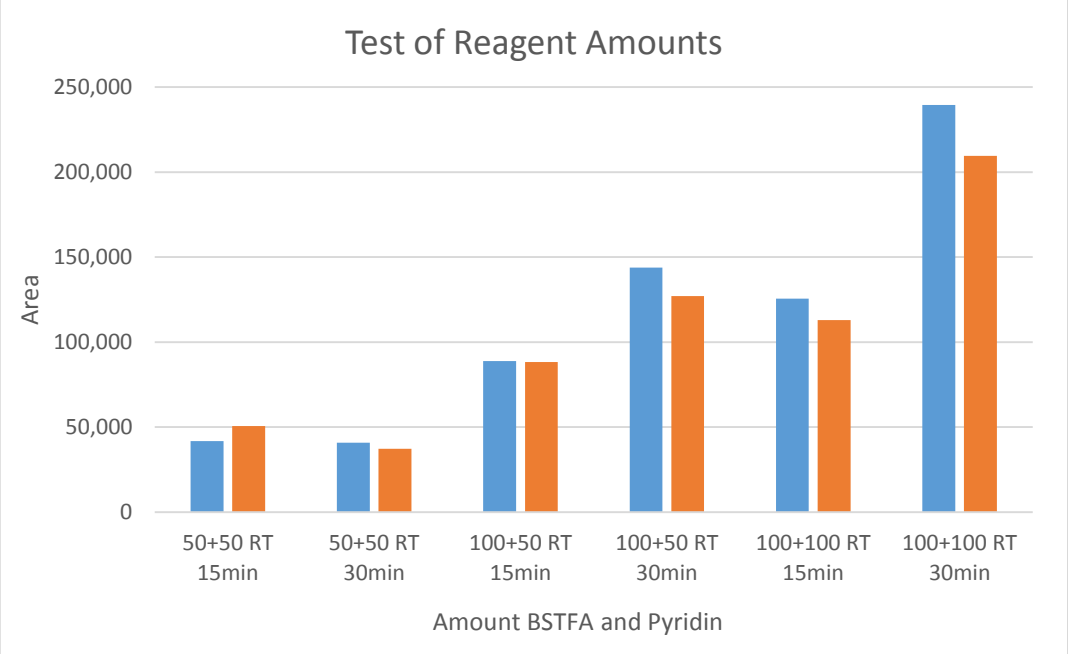


Figure 10: Comparison of derivatisation with different reagent amounts at room temperature for 15 min or 30 min (n=2)

In the next step it was tested if derivatisation at 60°C works better than at room temperature when using 100 µL BSTFA and 100 µL pyridine and 15 min derivatisation time. The results are that there were no differences between the two temperatures and derivatisation could be made at room temperature. For this test, extracts of the paper sample 2016_0511 were made and all of extract (10 ml) was evaporated to dryness. This results into the higher area of

abietic acid of factor 20 in Figure 11 in comparison with Figure 10 and into the bigger deviations between the replicates.

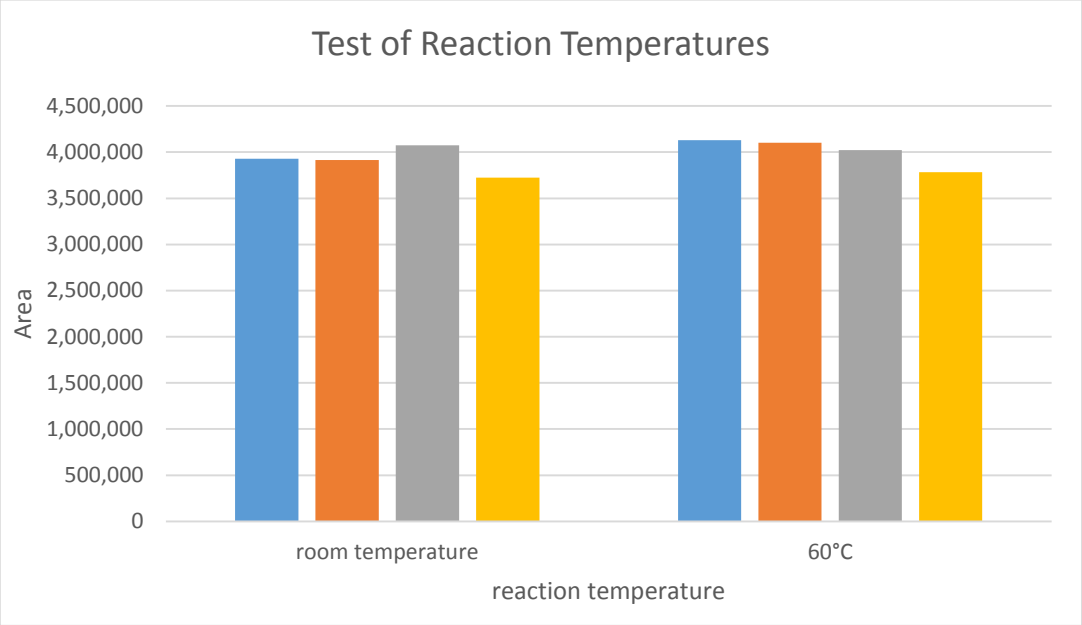


Figure 11: Comparison of derivatisation at room temperature and at 60°C (n=4)

When using an internal standard for quantification it is possible to correct deviations in the derivatisation step. The internal standard is derivatised in the same amount than the analytes in the sample and could be used to correct losses of analyte. Tests in which the derivatisation of the slurry with 100 µL BSTFA and 100 µL pyridine were compared to 50 µL BSTFA and 50 µL pyridine show that quantification using the internal standard gave similar results. Therefore, it was decided to save resources and use 50 µL BSTFA and 50 µL pyridine for derivatisation when using an internal standard for quantification.

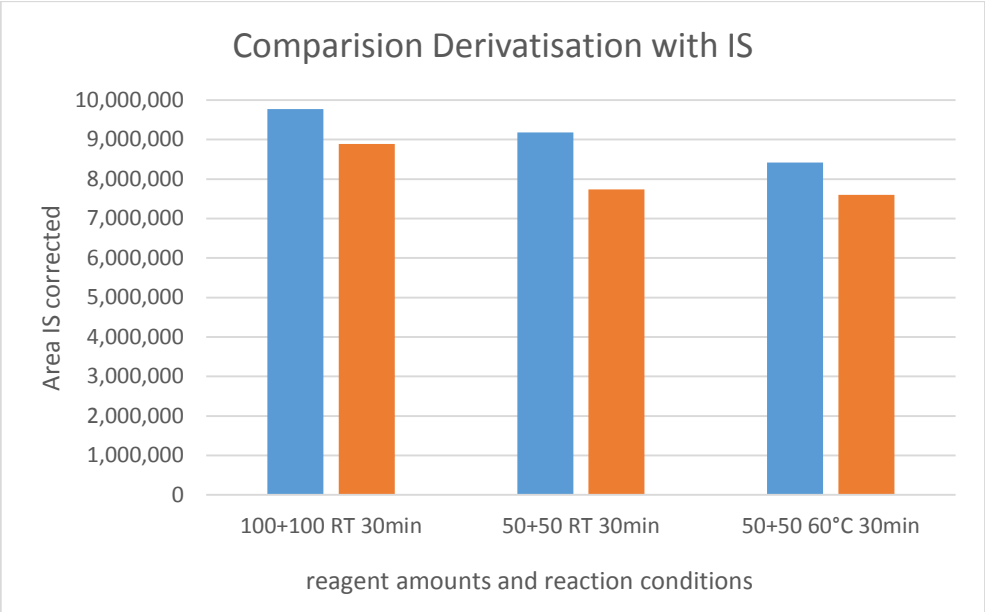


Figure 12: Comparison of derivatisation methods for slurry with internal standard correction (n=2)

The last thing tried was the derivatisation without pyridine at room temperature and at 60°C. Again, there are no differences during the derivatisation at room temperature and at 60°C. However, the derivatisation with pyridine gave slightly higher results as shown in Figure 13.

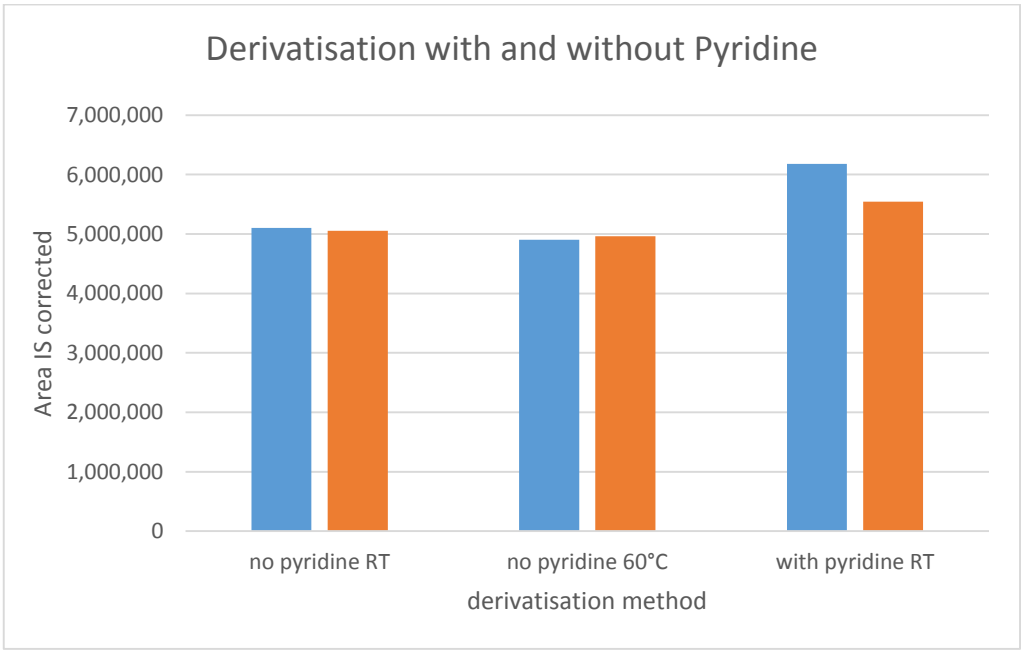


Figure 13: Comparison of derivatisation with and without pyridine (n=2)

3.1.2. Slurry

The slurry has to be dried for analysis with GC-MS because derivatisation is not possible with a wet sample. Prior to sampling, the vessel in which the slurry is stored should always be vigorously shaken to avoid settling of particles.

The easiest and quickest way for drying a small amount of slurry (20 mg), would be drying in a vacuum oven at 100°C for 30 min. However, the results show that the ratio between abietic acid and dehydroabietic acid changes in comparison to samples that have not been dried at 100°C due to degradation (see 3.1.5. Identified Substances and Concentrations). Therefore, it is also not possible to dry bigger amounts of slurry (~5 g) because the time needed for drying is too long and the slurry is degraded. However, drying a bigger amount of slurry would be preferred due to the homogeneity of the sample. Analysis of the degraded slurry show lower amounts of abietic acid and bigger amounts of dehydroabietic acid and higher degradation products.

Also very easy but more time consuming is to dry the slurry under a stream of N_2. No oxidation or degradation was seen with this method. The disadvantage is again, that only small amount of samples were dried and no homogenization is possible.

More complicated and not for everyone available is to dry the slurry in a vacuum drying oven at 40°C. The low temperature during the drying process allows it to dry any amounts of slurry without degradation. For the drying of 20 g wet slurry 2 h were needed to get ~6 g of dry sample (30.99% dry mass), which is in comparison to drying under N_2 a huge gain of time. The sample was homogenized afterwards and was used for different tests and to identify and determine the concentration of resin acids.

In conclusion, it would be preferred to dry the slurry in a vacuum drying oven, because it is quick, any amount of sample could be dried and therefore homogenization is possible. However, a vacuum drying oven is not always available, so as alternative drying small amounts of slurry under N_2 could be used. No differences between the identified and determined concentrations of resin acids could be found between these two methods.

The dried slurry was derivatised with BSTFA and pyridine and diluted 1:10 prior analysis with GC-MS. A second method tried was the extraction of dried slurry in ultrasonic bath with hexane, methanol or a mixture of hexane and ethanol. Besides higher amount of work and time this method does not improve the results that are given in chapter 3.1.5. Identified Substances and Concentrations.

3.1.3. Paper

The extraction of the paper samples was tested using the solvents methanol for 30, 45 and 60 min, acetone for 30 min and ethyl acetate for 30 min. Figure 14 shows the results based on the areas of abietic acid The extraction with methanol for 60 min works best, which is similar to methanol for 45 min. Methanol and acetone for 30 min gave similar results and the extraction with ethyl acetate the worst.

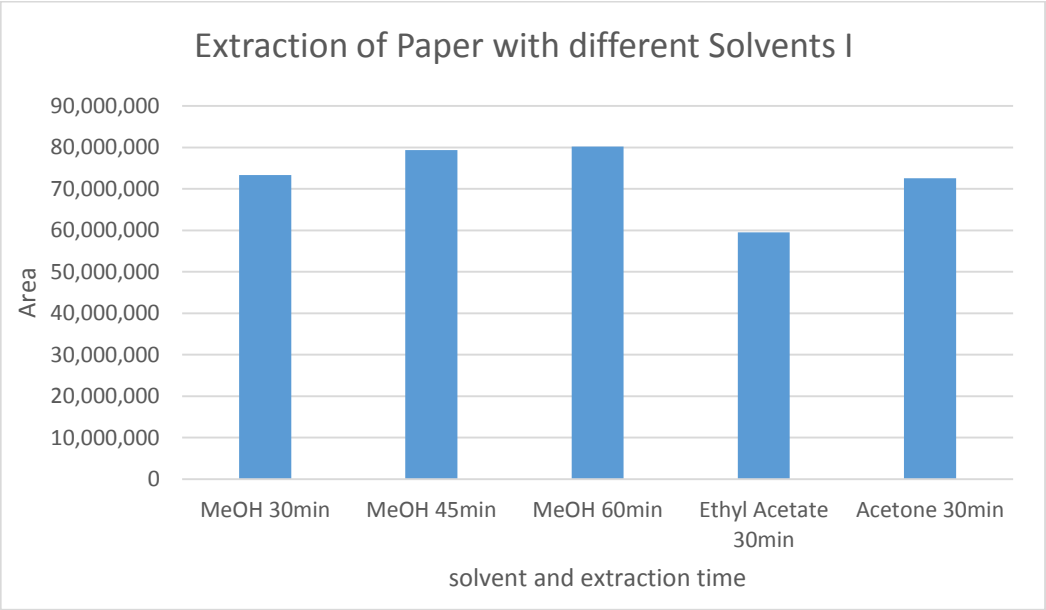


Figure 14: Comparison of solvents for the extraction of paper (n=1)

In the next step the extraction strength of acetone and methanol with 30 min and two times 30 min extraction time were compared, with the results that the extraction with methanol for two times 30 min works best, methanol for 30 min and acetone for two times 30 min are similar and acetone for 30 min is the worst one. Figure 14 and Figure 15 show a big difference between the areas because the initial weight of paper was reduced from 1 g to 0.5 g, derivatization method was changed and the samples were diluted 1:10 prior analysis in Figure 15.

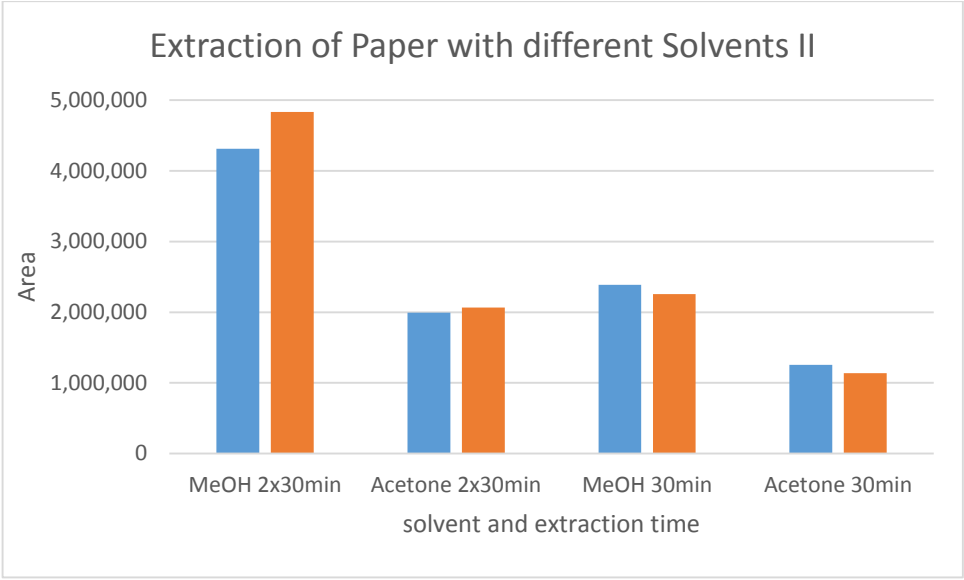


Figure 15: Comparison of Acetone and Methanol for the extraction of paper (n=2)

In the last step of testing solvents methanol was compared to a mixture of methanol and acetone 1:1 with extraction time of two times 30 min. As shown in Figure 16 the mixture of methanol/acetone works even better than pure methanol. So it was decided to use the mixture of methanol and acetone instead of pure methanol because acetone is more volatile which saves time during the evaporation of solvent prior derivatisation.

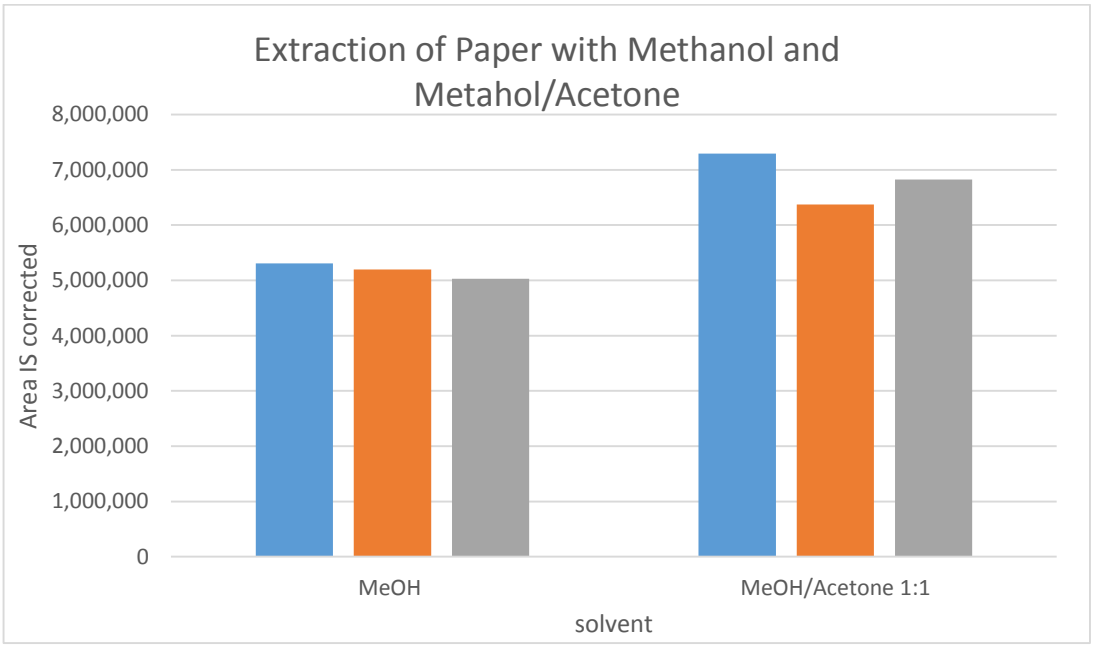


Figure 16: Comparison of Methanol and Methanol/Acetone 1:1 for the extraction of paper (n=3)

3.1.4. Validation

The developed GC-MS method was validated using "ValiData" Version 3.02.48 which is an excel macro for method validation. All tests carried out show that the developed method is conforming to standards.

With the external calibration with the levels 0, 5, 10 and 15 mg kg^{-1} limit of detection was determined as 1.07 mg kg^{-1} and limit of quantification as 3.25 mg kg^{-1} by using the calibration method. The variance test and the linearity test show no significant differences at a level of 95 and 99%. Correlation coefficient was above 0.99, y-intercept was -0.18 area $area^{-1}$, slope 0.07 area kg mg^{-1}, residual standard deviation 0.04 area $area^{-1}$, standard deviation of the procedure 0.54 mg kg^{-1}, relative standard deviation of the procedure 5.7% (n=12).

The external calibration with the levels 50, 100, 150, 200 and 250 mg kg^{-1} is also conforming to standards. Variance test and linearity test show no significant differences at a level of 99%. Correlation coefficient was above 0.99, y-intercept was -0.54 area $area^{-1}$, slope 0.04 area kg mg^{-1}, residual standard deviation 0.28 area $area^{-1}$, standard deviation of the procedure 6.89 mg kg^{-1}, relative standard deviation of the procedure 4.9 % (n=15).

Both standard additions with tampons T2 in the levels 0, 5, 10 and 15 mg kg^{-1} and tampons T7 in range of 0, 50, 100 and 150 mg kg^{-1} gave the results that the method is conforming to standards. A variance test shows no significant differences at a level of 95%, linearity test shows no significant differences at a level of 99% for both ranges. For both samples, the amount of abietic acid was determined to be under limit of detection, which is in accordance to the developed method.

The validation has been made for abietic acid only, because no other standards were available. However, it could be assumed, that the other resin acids, which are isomers of abietic acid and also dehydroabietic acid, which is very similar to abietic acid, behave in the same way.

"ValiData" results are given in the appendix (page 83-88).

3.1.5. Identified Substances and Concentrations in Paper and Slurry

An example of a chromatogram of a slurry sample is shown in Figure 17.

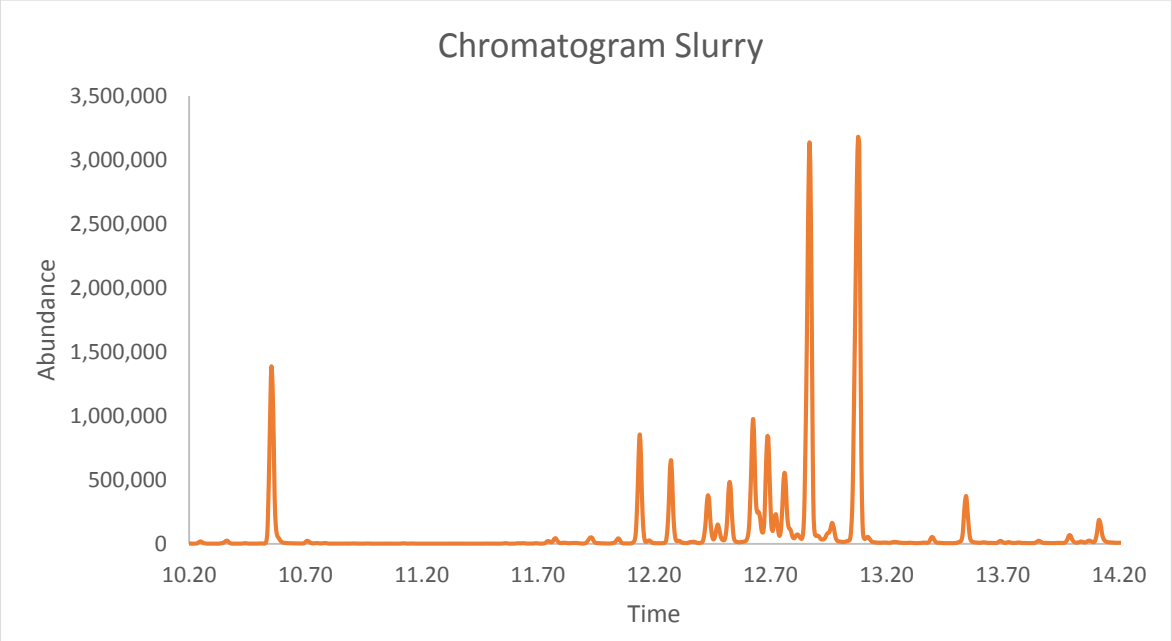


Figure 17: Example for chromatogram slurry

The found substances in the papers and the slurry and their retention times, calculated retention indices, retention indices given in literature, the characteristic ions and sources of mass spectra in literature are summarized in Table 6. The calculated retention indices were compared with the NIST Mass Spec Data Center (73) and different sources in literature (74) (75) (76).

Table 6: Found substances in slurry and paper samples with GC-MS

t_R	RI calc.	RI Lit	Substance	m/z 1	m/z 2	m/z 3	m/z 4	m/z 5	m/z 6	Source
10.556	2019	-	Internal standard	344 (100%)	73 (42%)	135 (33%)	120 (28%)	149 (23%)	359 (9%)	-
11.008	2089	2064	Dehydroabietane	255 (100%)	159 (62%)	173 (62%)	185 (44%)	270 (34%)		(77)
12.142	2278	2329	Isomer of Isopimaric Acid	241 (100%)	73 (42%)	256 (24%)	257 (23%)	359 (17%)		(34)
12.278	2301	2329	Isomer of Isopimaric Acid	241 (100%)	73 (36%)	256 (22%)	257 (20%)	359 (19%)		(34)
12.312	2307	2263	Dehydroabietic acid-aldehyde	269 (100%)	173 (82%)	284 (77%)	241 (67%)	159 (67%)	298 (52%)	(77)
12.437	2330	2301	Isomer of Pimaric Acid	73 (100%)	121 (92%)	120 (47%)	257 (32%)	359 (25%)	374 (14%)	(34)
12.476	2337	2329	Isomer of Isopimaric Acid	256 (100%)	241 (69%)	73 (69%)	257 (31%)	359 (23%)	374 (6%)	(34)
12.530	2346	2301	Isomer of Pimaric Acid	73 (100%)	121 (92%)	120 (43%)	257 (32%)	359 (31%)	374 (18%)	(34)

12.629	2364	2329	Isomer of Isopimaric Acid	241 (100%)	256 (75%)	73 (59%)	257 (32%)	259 (23%)	374 (6%)	(34)
12.670	2372	2338	Palustric Acid	241 (100%)	73 (53%)	359 (30%)	374 (26%)	242 (21%)		(34)
12.724	2381	-	Hydroxy-dehydroabietic acid	237 (100%)	73 (22%)	238 (21%)	252 (18%)	195 (11%)	256 (10%)	(78)
12.76	2388	-	Not identified	241 (100%)	256 (71%)	201 (63%)	73 (55%)	185 (34%)	359 (19%)	-
12.871	2408		Dehydroabietic acid	239 (100%)	240 (20%)	73 (13%)	357 (9%)	173 (8%)	372 (7%)	(77)
13.077	2445	2412	Abietic Acid	256 (100%)	241 (53%)	73 (25%)	185 (24%)	213 (22%)	359 (11%)	(77)
13.540	2532	2556	Neoabietic acid	135 (100%)	73 (43%)	121 (41%)	374 (38%)	148 (33%)	359 (12%)	(34)
13.692	2561	-	β7-Hydroxy-dehydroabietic acid	254 (100%)	73 (56%)	239 (41%)	297 (19%)	372 (14%)	357 (11%)	(77)
13.860	2593	2521	15-Hydroxy-dehydroabietic acid	445 (100%)	73 (53%)	446 (38%)	237 (7%)	255 (7%)	327 (6 %)	(52)
13.985	2619	-	7-Oxo-dehydroabietic acid	253 (100%)	268 (68%)	73 (66%)	397 (40%)	187 (35%)	327 (24%)	(52)

The retention indices have to be considered carefully because of the different isomers that were present. In literature no information about the different isomers was given so the calculated retention indices may vary a lot from them given in literature.

The concentration of resin acids in the slurry has been determined twice: once with wet slurry which is dried under a stream of N_2, once with dry mass after drying in vacuum drying oven. The results are 164.44 ± 6 g kg^{-1} for dry mass (n=3) and 175 and 176 g kg^{-1} for wet mass (n=2). Different resin acids were identified as their trimethylsilyl esters for example abietic acid, dehydroabietic acid, different isomers of isopimaric acid and pimaric acid and some higher degradation products. Retention times t_R, retention indices RI and the concentration of the substances are given in Table 10 and Table 11 that could be seen in the appendix.

The paper samples 2015_0383, 2015_0506 and 2015_0511 were treated with the slurry in an application amount of 10 L t^{-1}. The found resin acids and their concentrations in the samples are given in Table 12 to Table 14. Table 12 shows the results for paper sample 2016_0383 with a concentration of resin acids of 2.55 ± 0.08 g kg^{-1} (n=4). Table 13 shows the results for sample 2016_0506 with a concentration of resin acids of 1.04 ± 0.04 g kg^{-1} (n=4) and Table 14 the results for paper sample 2016_0511 with a concentration of 894 and 927 g kg^{-1} (n=2).

3.1.1. Two-Dimensional Comprehensive GC×GC-MS

One sample of slurry and one panty liner sample were measured on comprehensive GC-MS. The image of the slurry and the pad of the panty liner are given in Figure 18 and Figure 19. In this images the characteristic ions m/z 237, 239, 241, 253, 256 and 344 (see chapter 3.1.5.) were extracted from the scan to make illustration easier and to eliminate column bleeding and especially for the panty liner interfering peaks. The original spectra are given in the appendix (Figure 21 to Figure 23).

The comprehensive GC-MS results verify the results found in normal GC-MS at the one hand, at the other hand they show that there are some substances that could not be detected with one-dimensional GC-MS:

Figure 18 shows in accordance with GC-MS structured chromatograms of the four isomers of isopimaric acid and the two isomers of pimaric acid. Looking at other peaks that show group-type patterns made it possible to identify an isomer of 7-Hydroxydehydroabietic acid that was not detectable in one-dimensional GC-MS and an isomer of neoabietic acid that was overlaid by abietic acid in one-dimensional GC-MS. The peak with m/z 256, 241 and 201 that was detected and not identified in the one-dimensional GC-MS could also be detected in comprehensive GC-MS in two isomeric forms. Several other compounds that show mass to charge ratios that are similar to identified substances were seen in the image, but identification of new substances was not possible using comparison with spectra given in literature.

The results for the panty liner sample are equal (Figure 19). The only differences are that the concentrations of resin acids in the samples are smaller and not as much substances as in the slurry were detected. However, the image shows several other peaks that were not resin acids or degradation products of it.

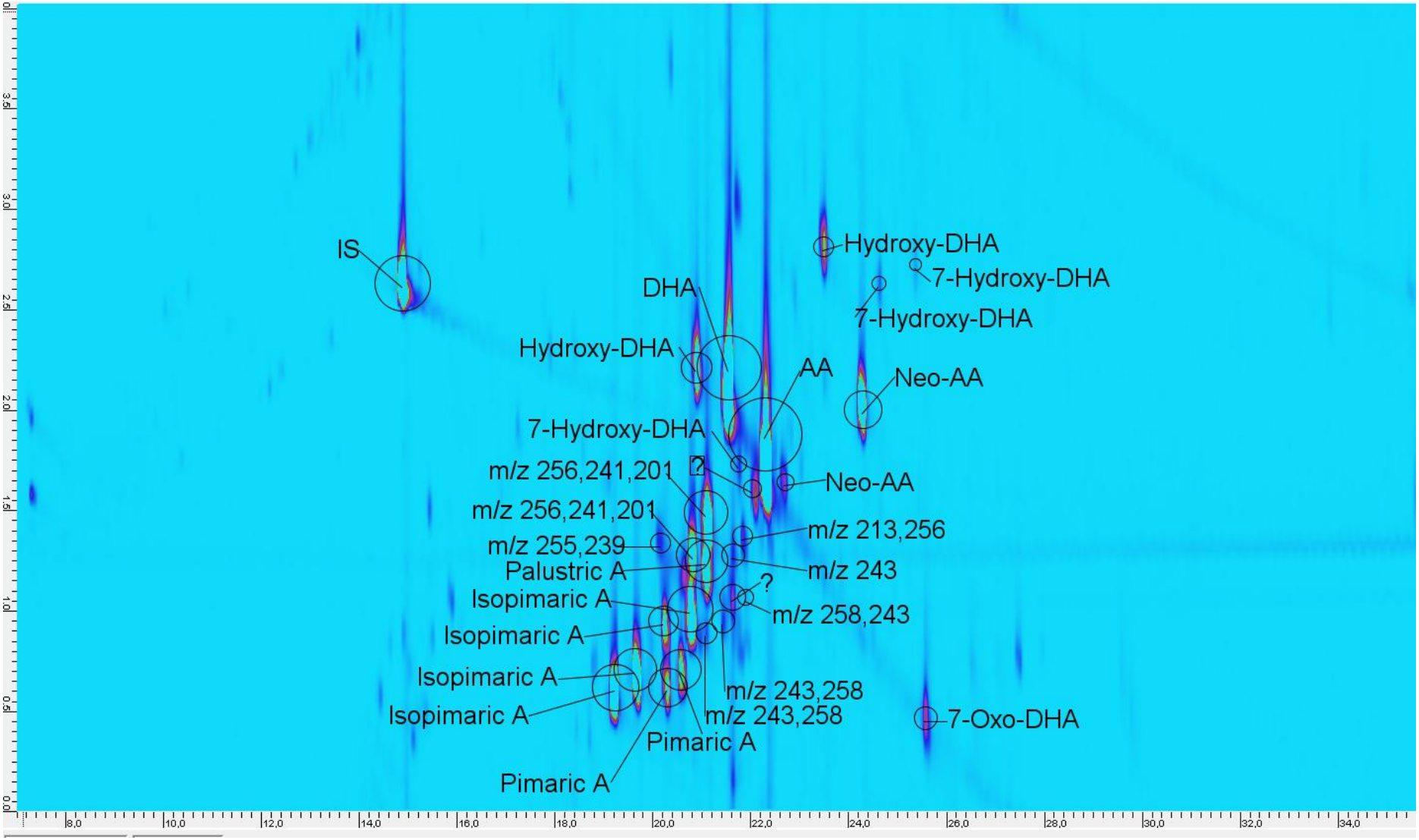


Figure 18: Comprehensive GC-MS image of slurry showing m/z 237, 239, 241, 253, 256, 344 extracted from scan

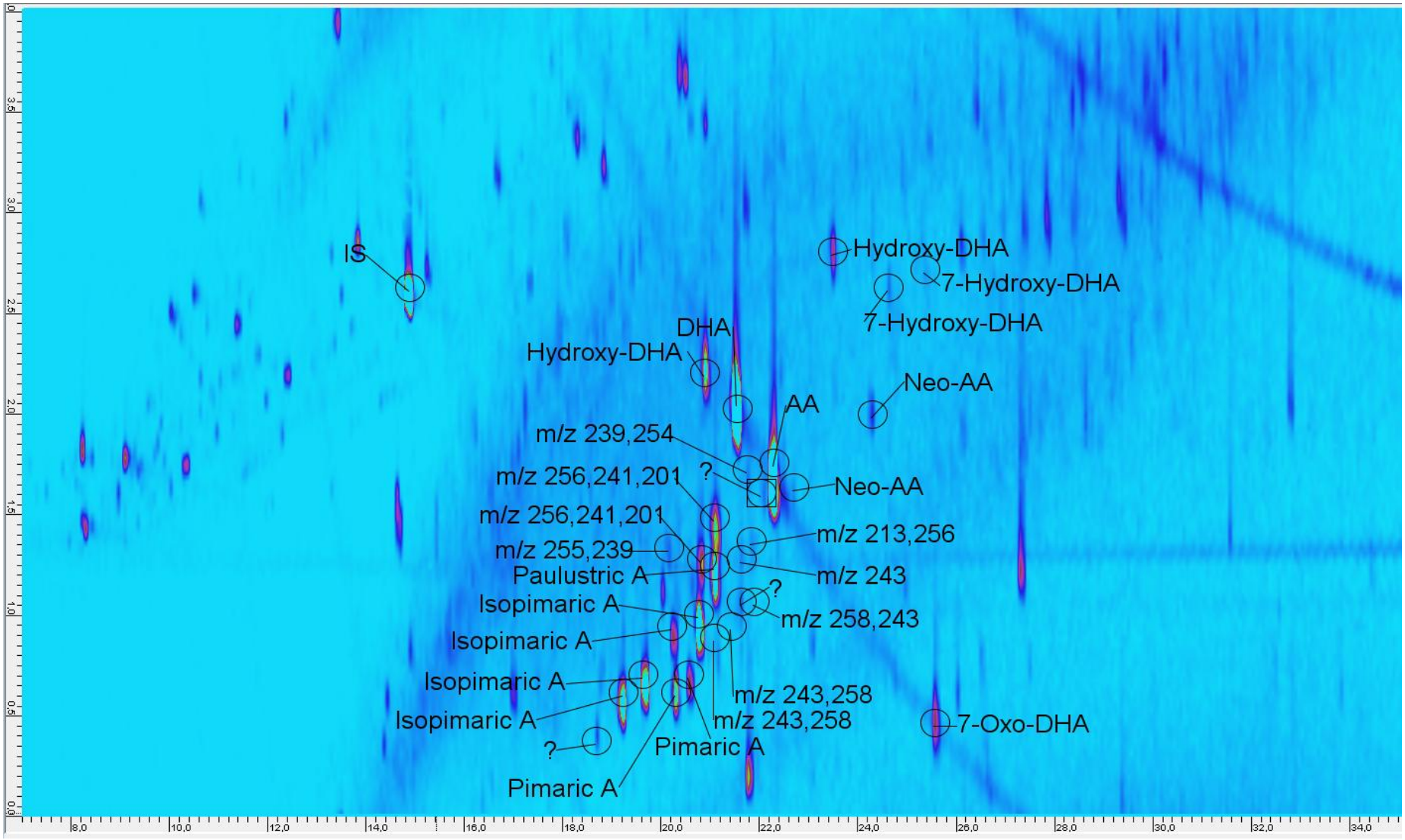


Figure 19: Comprehensive GC-MS image of panty liner pad showing m/z 237, 239, 241, 253, 256, 344 extracted from scan

3.2. LC-MS

First of all, it was tried to develop a method for screening of samples in scan mode, similar to GC-MS. However, measurements in scan mode show to less sensitivity and no target substances could be seen. So it was decided to use detection in sim-mode. In positive mode m/z 303 for abietic acid and m/z 315 for 7-Oxodehydroabietic acid were used. In negative mode m/z 299 for dehydroabietic acid, m/z 301 for abietic acid and m/z 286 for the internal standard were used. Measuring in SIM-mode has the advantage of better sensitivity, but the disadvantages that identification and quantification are only possible using corresponding standards. Since only abietic acid is available as standard it was not possible to quantify any other substance. Identification was also possible for dehydroabietic acid since this is the second common compound and often described in literature.

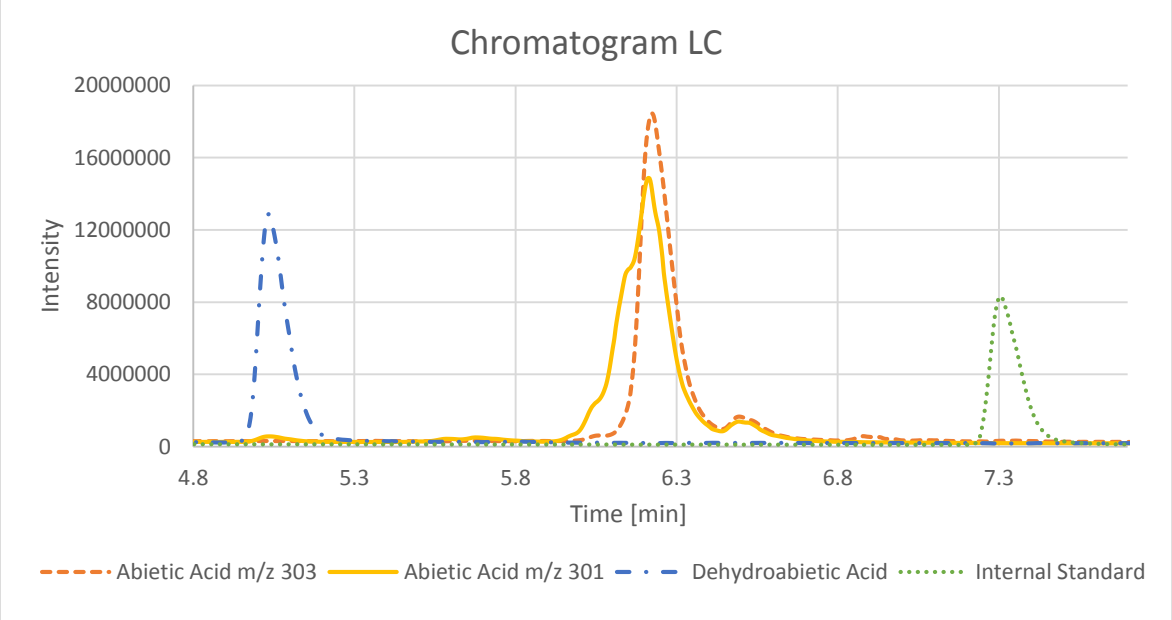


Figure 20: example for LC chromatogram of paper sample

3.2.1. Mobile Phases

In general, adding ammonium acetate into the mobile phase decreased the sensitivity, adding acetic acid increased sensitivity. Using methanol gave better sensitivity than using acetonitrile. So it was decided to use methanol/water with 0.05% acetic acid as mobile phase. In general, changing the solvent and the additives also changes the retention times of the analytes and the elution mode and the time program has to be changed when using methanol.

This makes the comparison of the different methods more difficult, but still it could be seen that using methanol/water with 0.05% acetic acid gives far the best sensitivity.

3.2.2. Paper Samples

For extraction of paper samples, the method developed for GC-MS could be used. However, instead of evaporating all of the solvent aliquots of 1 ml extract were taken and resolved in the mobile phase. Resolving the solvent in mobile phase brings better sensitivity and shapelier peaks. The determined concentration of abietic acid in the paper samples 2016_0506 and 2016_0511 are given in Table 7.

Table 7: Concentration of abietic acid in paper samples; values in mg kg^{-1} paper

Sample	m/z 301	m/z 303	sample	m/z 301	m/z 303
2016_506_1	528	391	2016_0511_1	427	315
2016_506_2	501	374	2016_0511_2	426	327
2016_506_3	516	403	2016_0511_3	421	309
mean	514	389	mean	425	317
std deviation	± 11	± 12	std deviation	± 3	± 8

3.3. Market Analysis

3.3.1. Tampons

In the tampons only abietic acid and dehydroabietic acid could be detected. No other resin acids or degradation products were present. The highest concentration of abietic acid and dehydroabietic acid were found in the matt of fibers of the T2 tampons. However, these concentrations were still under the limit of quantification of 3.25 mg kg^{-1} for abietic acid, for dehydroabietic acid the concentrations were 4.69 and 5.63 mg kg^{-1}. In general, in the matt of fibers the concentration of analytes were higher than the concentration in the cotton pad, which were under the limit of detection for every sample.

3.3.2. Nursing Pads

In the nursing pads of NP1 no abietic acid or dehydroabietic acid could be detected. In the NP2 and NP3 product dehydroabietic acid was detected, but under the limit of quantification. In the liner of the NP4 product abietic acid was detected in a concentration of 27.9 and 27.5 mg kg^{-1} (1.14 µg and 1.13 µg per liner) and dehydroabietic acid in a concentration of 16.6 and 16.2 mg kg^{-1} (0.68 µg and 0.67 µg per liner). It was also possible to identify isopimaric acid in this liner sample. In the cotton pad the concentrations were again under limit of quantification.

3.3.3. Diapers

The detected concentrations of abietic acid and dehydroabietic acid were below limit of detection for all diaper samples.

3.3.4. Panty Liner

In the panty liners the found concentrations for abietic acid and dehydroabietic acid were the highest for all products. Especially in the release liners of the products the concentrations were extremely high and also other resin acids like isopimaric acid, pimaric acid could be detected in relevant concentrations. The results are given in Table 8.

Table 8: Results panty liner in mg kg^{-1} product and µg piece^{-1} (n=2 from two pieces); LOD= 1.07 mg kg^{-1}, LOQ= 3.25 mg kg^{-1}

	mg kg^{-1}								**µg unit^{-1}**							
	Padding		***Liner***		***Padding***		***Liner***		***Padding***		***Liner***		***Padding***		***Liner***	
	No. 1	***No.2***	***No. 1***	***No.2***	***No. 1***	***No.2***	***No. 1***	***No.2***	***No. 1***	***No. 2***	***No. 1***	***No. 2***	***No. 1***	***No. 2***	***No. 1***	***No. 2***
Substance	**PL9**				**PL4**				**PL9**				**PL4**			
AA	<LOQ	<LOQ	162	149	<LOQ	<LOQ	76.8	77.4	<LOQ	<LOQ	329	310	<LOD	<LOD	78.9	79.2
DHA	6.37	5.48	114	100	5.8	6.0	156	158	12.55	10.95	231	208	11.4	11.8	161	162
Isopimaric	-	-	14.3	13.8	-	-	23.6	23.9	-	-	29.1	28.7	-	-	24.3	24.5
Pimaric	-	-	<LOD	<LOD	-	-	<LOD	<LOD	-	-	<LOD	<LOD	-	-	<LOD	<LOD
	PL1				**PL2**				**PL1**				**PL2**			
AA	<LOD	<LOD	69.0	72.9	19.7	17.1	483	501	<LOD	<LOD	124	130	42.1	37.7	1034	1102
DHA	3.84	4.50	156	171	17.7	16.1	398	415	7.66	8.87	279	305	37.8	35.4	851	914
Isopimaric	-	-	23.3	24.8	-	-	67.0	67.7	-	-	41.7	44.3	-	-	143	149
Pimaric	-	-	<LOD	<LOD	-	-	8.31	8.57	-	-	<LOD	<LOD	-	-	17.8	18.9
	PL8				**PL5**				**PL8**				**PL5**			
AA	7.17	6.45	295	274	<LOQ	<LOQ	59.3	61.3	14.23	12.67	506	474	<LOD	<LOD	70	71.7
DHA	8.49	9.59	245	248	6.40	5.98	110	108	14.53	16.6	419	430	12.8	11.9	130	126
Isopimaric	-	-	35.6	36.8	-	-	15.7	16.2	-	-	60.9	63.9	-	-	18.5	18.9
Pimaric	-	-	24.3	25.7	-	-	<LOQ	<LOQ	-	-	41.7	44.6	-	-	<LOD	<LOD
	PL6				**PL3**				**PL6**				**PL3**			
AA	2.26	3.02	99.1	86.0	9.13	17.6	476	355	4.46	5.95	171	151	22.6	41.5	1180	838
DHA	4.14	3.36	149	130	10.4	9.90	336	313	8.18	6.61	257	229	25.9	23.3	831	738
Isopimaric	-	-	26.8	24.5	-	-	75.5	71.8	-	-	46.1	43.0	-	-	187	169
Pimaric	-	-	<LOQ	<LOQ	-	-	5.0	4.8	-	-	<LOD	<LOD	-	-	9.97	9.63
	PL10				**PL7**				**PL10**				**PL7**			
AA	<LOD	<LOD	61.5	61.4	<LOD	<LOD	86.0	113.5	<LOD	<LOD	138	145	<LOD	<LOD	424	558
DHA	3.97	3.92	50.0	51.7	3.39	3.54	87.1	93.2	7.93	7.82	113	122	<LOQ	<LOQ	429	458
Isopimaric	-	-	8.39	8.34	-	-	9.62	10.1	-	-	18.9	19.7	-	-	47.4	50.0
Pimaric	-	-	<LOQ	<LOQ	-	-	<LOQ	<LOQ	-	-	<LOD	<LOD	-	-	<LOD	<LOD

Besides the above-mentioned substances, it was possible to detect the resin acids palustric acid and neoabietic acid in concentration below limit of quantification in the paddings and liners of the samples. The degradation products 7-Oxodehydroabietic acid, 7-Hydroxydehydroabietic acid and 15-Hydroxydehydroabietic acid were also detected in concentrations lower the limit of quantification in the liners.
In general, the concentrations are higher in the release liners than in the paddings but it has to be mentioned that the liners are not considered to have skin contact.

As could be seen above in Table 8 the products with the code name PL2, PL3 and PL8 had the highest concentrations of resin acids in their release liners. For this samples also the concentration of resin acids in the cardboard packaging were analysed. The results are given in Table 9. In comparison with the results in Table 8 it could be seen that the concentrations in the release liners are 8 to 30-fold higher for abietic acid and about 3-fold higher for dehydroabietic acid. These low concentrations in the packaging show that the found concentrations in the liner are not derived from the packaging, but rather came from the panty liners.

Table 9: Results of selected panty liner packaging compared with results of hygiene products (n=2); results are given in mg kg^{-1}

Sample		Replicate	AA	DHA	Isopimaric	Pimaric	Palustric
PL2	***Packaging***	***No. 1***	52.3	123	19.0	<LOD	-
		No. 2	49.7	127	19.4	<LOD	-
	Padding	***No. 1***	19.7	17.7	-	-	-
		No. 2	17.1	16.1	-	-	-
	Liner	***No. 1***	483	398	67.0	8.31	-
		No. 2	501	415	67.7	8.57	-
PL3	***Packaging***	***No. 1***	43.8	142	25.0	<LOD	<LOD
		No. 2	50.0	162	28.4	<LOD	<LOD
	Padding	***No. 1***	9.13	10.4	-	-	-
		No. 2	17.6	9.90	-	-	-
	Liner	***No. 1***	476	336	75.5	5.0	-
		No. 2	355	313	71.8	4.8	-
PL8	***Packaging***	***No. 1***	9.34	89.3	13.1	<LOD	-
		No. 2	9.76	94.0	13.4	<LOD	-
	Padding	***No. 1***	7.17	8.49	-	-	-
		No. 2	6.45	9.59	-	-	-
	Liner	***No. 1***	295	245	35.6	24.3	-
		No. 2	274	248	36.8	25.7	-

4. Conclusion

4.1. Developed Methods

Below the developed methods for analyzing slurry, papers and other cellulose based products with GC-MS and the found substances and concentrations are summarized:

For analyzing the slurry 20 mg of wet slurry are weight in and internal standard is added. Afterwards the samples are dried under a stream of N_2 and derivatised with 50 µL BSTFA and 50 µL pyridine at room temperature for 30 min. Ethyl acetate was added to a volume of 1 ml and the samples diluted 1:10 with ethyl acetate prior analysis with GC-MS. The found concentration of resin acids in the slurry are 169 ± 8 g kg^{-1}. The main acids found are abietic acid (50 ± 2.8 g kg^{-1}) and dehydroabietic acid (45.4 ± 3.3 g kg^{-1}), other resin acids like isopimaric acid and pimaric acid could be found in different isomeric forms. Also some degradation products like 7-Oxodehydroabietic acid (0.24 ± 0.07 g kg^{-1}), 15-Hydroxydehydroabietic acid (0 .98 ± 0.13 g kg^{-1}) and β-7-Hydroxydehydroabietic acid (0.30 ± 0.12 g kg^{-1}) could be found in smaller concentrations.

Paper samples and other cellulose based products are cut into pieces of about 5x5 mm, 500 mg were weight in and internal standard was added. Extraction was done with a mixture of methanol and acetone 1:1 in an ultrasonic bath at 40°C for 30 min twice. The two extracts were combined afterwards and evaporated to dryness. Derivatisation was made using 50 µL BSTFA and 50 µL pyridine at room temperature for 30 min. Ethyl acetate was added to a volume of 1 ml prior analysis with GC-MS. For the paper samples total resin acid concentration is between 0.91 and 2.55 ± 0.08 g kg^{-1}. Considering an application amount of slurry of 10 liter slurry per ton paper the resin acid concentration in the paper should be about 1.7 g kg^{-1}. So the found concentrations of resin acids in the papers is within the expected range. In paper samples the main resin acid is dehydroabietic acid followed by abietic acid. This means that some of the abietic acid was degraded to dehydroabietic acid. Furthermore, the amount of higher degradation products increases in comparison to the slurry.

Validation of the developed method with "ValiData" gave the results that the method is conforming to standards. Limit of detection was determined as 1.07 mg kg^{-1} and limit of quantification as 3.25 mg kg^{-1}. The variance test and the linearity test show no significant differences at a level of 99%.

For LC-MS the papers were extracted in the same way as for GC-MS. Afterwards 1 ml of extract was taken, evaporated to dryness and resolved in methanol/water 70:30. Analysis was done using methanol and water with 0.05% acetic acid as mobile phase with gradient elution (starting with 70:30 and rising to 100% methanol within 10 min).

In comparison with GC-MS the results for LC-MS are much higher. The concentration for abietic acid in the paper samples 2016_0506 and 2016_0511 were determined as 514 ± 11 and 425 ± 3 mg kg^{-1} for m/z 301 and 389 ± 12 and 317 ± 8 mg kg^{-1} for m/z 303. However, looking at Figure 20 it could be seen that the peak for m/z 301 shows shoulders. This is an indication that other substances co-elute with the abietic acid. McMartin et al. described that the isomers abietic acid, pimaric acid and isopimaric acid are not separated under various conditions using an C_8 or C_{18} column (79). Latorre et al. also reported that the seven non-aromatic resin acids with the same molecular mass show a common ion of m/z 301 using negative ionization and an identification and separation is impossible using LC-MS (34). Considering the information that the peak of m/z 301 may include all isomers of the abietic acid and comparing the result of LC-MS with the sum of isomers in GC-MS the found concentrations are in good accordance. Axelsson et al. described for measuring m/z 303 in positive mode that abietic acid co-eluted with levopimaric and isopimaric acid (36). So also the accuracy of the measurement of m/z 303 is questionable.

In comparison to data found in literature the concentrations found in the paper sample 2016_0506, 2016_0511 and 2016_0383 are higher but still in the same range of mg kg^{-1}. For example, Karlberg et al. analyzed newspaper samples onto resin acids and found concentrations of 1-87 mg kg^{-1} for abietic acid, 2-191 mg kg^{-1} for dehydroabietic acid and 9-63 mg kg^{-1} for 7-Oxodehydroabietic acid (46).

4.2. Comparison of GC and LC

In conclusion the biggest advantage of GC-MS is the comparability of mass spectra that were recorded in EI-mode with 70 eV. With this big advantage it was possible to identify most of the peaks present in the gas chromatogram via their mass spectrum. Further advantages are the better separation of the resin acids and the easier use of GC. The disadvantages are the derivatisation step that is another source of error and consume some more time in sample preparation (about 45 min). The run time of GC-MS for one sample is 25 min (plus time to getting back to initial settings) and higher than the 15 min of one LC run (no time to getting back to initial settings). However, for LC it has to be considered that the system needs about 30 min for stabilizing measurement parameters (pressure, flow) after start running. Another disadvantage of LC-MS is that it was to less sensitive for measuring in scan mode and no untargeted screening was possible. Measuring in SIM-mode is more sensitive, but quantification is only possible for substances where a standard is available, identification is also possible for substances that has been well described in literature. So for LC-MS the only resin acid that could be identified was abietic acid, the only degradation product was dehydroabietic acid. Quantification was made for abietic acid only because no other standard substances were available, but abietic acid was not separated from the other resin acids efficiently so this information is defective. In comparison with GC-MS the obtained information about the samples is poor and it was decided to do the product studies with GC-MS only.

4.3. Market Analysis

In general, the detected concentrations of abietic acid and dehydroabietic acid in the products were between not detected and 501 mg kg^{-1} for abietic acid and 476 mg kg^{-1} for dehydroabietic acid. Isopimaric acid was detected in concentrations up to 75.5 mg kg^{-1} and pimaric acid in a concentration of 25.7 mg kg^{-1} in the release liners of the panty liners. Other resin acids or degradation products were detected in concentrations below limit of quantification only.

In the tampon samples concentrations of abietic and dehydroabietic acid were below limit of quantification for all samples except T2, which has a concentration of dehydroabietic acid in the matt of fibres of 4.69 and 5.63 mg kg^{-1} (n=2).

For one nursing pad no abietic acid or dehydroabietic acid could be detected (NP1), for two the detected amounts were under limit of quantification (NP2 and NP3). In the product NP4 abietic acid was detected in a concentration of 27.9 and 27.5 mg kg^{-1} (n=2), dehydroabietic acid in a concentration of 16.6 and 16.2 mg kg^{-1} (n=2).

The highest amounts of resin acids and degradation products were found in panty liners, especially in the release liners of the products where the concentrations of abietic acid are up to 501 mg kg^{-1}. In the samples it was possible to detect all resin acids in different isomeric forms and also degradation products. However, the release liners are not considered to have skin contact and the substances that are considered to be allergenic were found in concentrations below limit of quantification. From the three samples that have the highest concentration of resin acids also the packaging (cardboard) was analysed. The found concentrations in the cardboard were 8 to 30-fold lower for abietic acid and about 3-fold lower for dehydroabietic acid in comparison to the liners of the products. So it is unlikely that all of the found resin acids are migrated from the packaging.

Karlberg et al. described in literature that she analyzed diapers and found concentrations of abietic and dehydroabietic acid of 104 and 225 mg kg^{-1} (16). In the diapers analyzed during this work no abietic or dehydroabietic acid could be detected.

Considering the information found in literature abietic acid has no or low sensitizing potential, dehydroabiteic acid is non sensitizing. None of the highly sensitizing peroxides could be detected in the product samples. The higher degradation products 15-Hydroxydehydroabietic acid and 7- Oxodehydroabietic acid that are considered to be highly allergenic could only be detected in the release liners of the panty liners that do not have skin contact and in concentrations below limit of quantification. Karlberg et al. stated in

1996 that there were no studies demonstrating the lowest concentration of rosin to sensitize in humans and therefore it is difficult to say which amount of rosin cause sensitization (16). Therefore, it is also difficult to say whether the found concentrations in the tested products are relevant or not to cause allergenic reactions.

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6. Appendix

Results for slurry and paper samples

Table 10: Substances and concentrations in slurry from dry mass; values in g kg^{-1}

t_R	t_I	Substance	Slurry 1	Slurry 2	Slurry 3	mean	Std deviation
12.142	2278	Isomer of Isopimaric Acid	9.54	10.5	9.93	9.98	± 0.38
12.278	2301	Isomer of Isopimaric Acid	7.20	7.84	7.51	7.51	± 0.26
12.312	2307	dehydroabietic acid-aldehyde	0.31	0.33	0.31	0.32	± 0.01
12.437	2330	Isomer of Pimaric Acid	4.53	4.93	4.75	4.73	± 0.16
12.476	2337	Isomer of Isopimaric Acid	1.81	2.04	1.91	1.92	± 0.10
12.530	2346	Isomer of Pimaric Acid	5.70	6.17	5.91	5.93	± 0.19
12.629	2364	Isomer of Isopimaric Acid	13.9	15.4	14.3	14.5	±0.6
12.670	2372	Isomer of Palustric Acid	9.79	10.9	10.5	10.4	± 0.4
12.724	2381	Hydroxy-dehydroabietic acid	2.48	2.49	2.53	2.50	± 0.02
12.76	2388	Not identified	8.79	9.69	9.26	9.25	± 0.36
12.871	2408	dehydroabietic acid	41.6	45.5	41.7	43.0	± 1.8
13.077	2445	Abietic Acid	47.0	51.2	46.3	48.2	± 2.2
13.540	2532	Neoabietic acid	4.61	5.02	4.59	4.74	± 0.20
13.692	2561	β7-Hydroxy-dehydroabietic acid	0.19	0.52	0.19	0.30	± 0.16
13.860	2593	15-Hydroxy-dehydroabietic acid	0.28	0.33	0.27	0.29	± 0.03
13.985	2619	7-Oxo-dehydroabietic acid	0.99	0.98	0.79	0.92	± 0.10
Sum [g kg^{-1}]			156	174	161	164	± 6

Table 11: Substances and concentrations in slurry from wet mass; values in g kg^{-1}

t_R	t_I	Substance	Slurry 1	Slurry 2	mean
12.142	2277	Isomer of Isopimaric Acid	10.3	10.3	10.3
12.278	2301	Isomer of Isopimaric Acid	7.68	7.57	7.63
12.312	2307	dehydroabietic acid-aldehyde	0.35	0.31	0.33
12.437	2329	Isomer of Pimaric Acid	5.09	4.98	5.03
12.476	2336	Isomer of Isopimaric Acid	1.94	1.92	1.93
12.530	2346	Isomer of Pimaric Acid	5.99	6.01	6.00
12.629	2364	Isomer of Isopimaric Acid	14.5	14.8	14.7

12.670	2371	Isomer of Palustric Acid	10.6	11.7	11.2
12.724	2381	Hydroxy-dehydroabietic acid	2.70	2.82	2.76
12.760	2387	Not identified	8.96	9.27	9.11
12.871	2407	dehydroabietic acid	50.0	48.1	49.0
13.077	2445	Abietic Acid	52.4	53.0	52.7
13.540	2531	Neoabietic acid	4.37	4.30	4.34
13.692	2561	β7-Hydroxy-dehydroabietic acid	0.34	0.25	0.30
13.860	2593	15-Hydroxy-dehydroabietic acid	0.17	0.15	0.16
13.985	2618	7-Oxo-dehydroabietic acid	1.20	0.93	1.07
Sum [g kg^{-1}]			176	175	176

Table 12: Substances and concentrations in paper 2015_0383; values in mg kg^{-1}

t_R	t_I	Substance	0383_1	0383_2	0383_3	0383_4	mean	std deviation
11.008	2089	Dehydroabietane	5.31	6.54	5.50	4.95	5.57	± 0.59
12.142	2278	Isopimaric Acid	202	210	219	214	211	± 6
12.278	2301	Isopimaric Acid	128	133	133	134	132	± 2
12.312	2307	dehydroabietic acid-aldehyde	36.2	36.8	39.4	37.0	37.3	± 1.2
12.437	2330	Pimaric Acid	73.6	75.3	73.9	74.4	74.3	± 0.7
12.476	2337	Isopimaric Acid	33.4	34.8	31.9	32.8	33.2	± 1.0
12.530	2346	Pimaric Acid	92.8	94.3	110	112	102	± 9
12.629	2364	Isopimaric Acid	247	244	253	212	239	± 16
12.670	2372	Palustric Acid	136	150	108	87.9	120	± 24.1
12.724	2381	Hydroxy-dehydroabietic acid	43.1	43.0	41.0	42.4	42.4	± 0.8
12.760	2388	Not identified	163	169	166	168	166	± 2
12.871	2408	dehydroabietic acid	553	564	657	670	611	± 53
13.077	2445	Abietic Acid	532	518	632	625	577	± 52
13.540	2532	Neoabietic acid	75.6	81.0	69.1	71.2	74.2	± 4.6
13.692	2561	β7-Hydroxy-dehydroabietic acid	23.3	19.3	12.3	19.6	18.6	± 4.0
13.860	2593	15-Hydroxy-dehydroabietic acid	32.0	36.0	30.3	35.8	33.5	± 2.4
13.985	2619	7-Oxo-dehydroabietic acid	67.3	71.2	65.0	69.0	68.1	± 2.3
Sum [mg kg^{-1}]			2444	2485	2645	2609	2546	± 84
Sum [g kg^{-1}]			2.44	2.49	2.64	2.61	2.55	± 0.08

Table 13: Substances and concentrations in paper 2015_0506; values in mg kg^{-1}

t_R	t_I	Substance	0506_1	0506_2	0506_3	0506_4	mean	std deviation
11.008	2089	Dehydroabietane	5.74	5.54	5.19	5.13	5.40	± 0.25
12.142	2278	Isopimaric Acid	90.7	90.4	93.1	91.0	91.3	± 1.1
12.278	2301	Isopimaric Acid	50.5	49.6	50.0	49.2	49.8	± 0.5

12.312	2307	dehydroabietic acid-aldehyde	35.0	33.8	34.6	34.0	34.3	± 0.5
12.437	2330	Pimaric Acid	34.9	34.4	36.5	35.1	35.2	± 0.8
12.476	2337	Isopimaric Acid	13.6	13.1	12.9	12.7	13.0	± 0.4
12.530	2346	Pimaric Acid	52.6	50.5	51.6	51.0	51.4	± 0.8
12.629	2364	Isopimaric Acid	72.6	70.9	73.0	72.1	72.2	± 0.8
12.670	2372	Palustric Acid	30.3	30.2	67.0	64.9	48.1	± 17.8
12.724	2381	Hydroxy-dehydroabietic acid	15.9	15.2	15.7	15.4	15.6	± 0.3
12.760	2388	Not identified	64.3	61.4	62.5	60.3	62.1	± 1.5
12.871	2408	dehydroabietic acid	252	243	275	271	260	± 13
13.077	2445	Abietic Acid	222	219	243	238	231	± 10
13.540	2532	Neoabietic acid	27.0	26.0	22.8	22.1	24.5	± 2.1
13.692	2561	β7-Hydroxy-dehydroabietic acid	9.68	12.4	9.60		10.6	± 1.3
13.860	2593	15-Hydroxy-dehydroabietic acid	32.7	30.9	8.27	7.98	20.0	± 11.9
13.985	2619	7-Oxo-dehydroabietic acid	9.89	8.9	28.7	28.2	19.0	± 9.6
Sum [mg kg^{-1}]			1019	996	1089	1058	1040	± 36
Sum [g kg^{-1}]			1.02	1.00	1.09	1.06	1.04	± 0.04

Table 14: Substances and concentrations in paper 2015_0511; values in mg kg^{-1}

t_R	t_I	Substance	2016_0511_1	2016_0511_2	mean
11.008	2089	Dehydroabietane	2.52	2.92	2.72
12.142	2278	Isopimaric Acid	72.7	76.2	74.4
12.278	2301	Isopimaric Acid	41.1	43.7	42.4
12.312	2307	dehydroabietic acid-aldehyde	26.9	28.0	27.5
12.437	2330	Pimaric Acid	29.6	30.8	30.2
12.476	2337	Isopimaric Acid	9.30	9.89	9.59
12.530	2346	Pimaric Acid	38.1	40.4	39.3
12.629	2364	Isopimaric Acid	60.0	61.3	60.7
12.670	2372	Palustric Acid	58.1	59.5	58.8
12.724	2381	Hydroxy-dehydroabietic acid	13.1	13.5	13.3
12.760	2388	Not identified	53.7	56.7	55.2
12.871	2408	dehydroabietic acid	233	239	236
13.077	2445	Abietic Acid	201	208	204
13.540	2532	Neoabietic acid	20.2	22.3	21.2
13.860	2593	15-Hydroxy-dehydroabietic acid	9.59	7.43	8.51
13.985	2619	7-Oxo-dehydroabietic acid	26.0	27.6	26.8
Sum [mg kg^{-1}]			894	927	910
Sum [g kg^{-1}]			0.89	0.93	0.91

„ValiData“ results for external calibration 0, 5, 10, 15 mg kg^{-1}

3/13/2017		SOP		Bearbeiter								Validata 3.02
Beschreibung	Beschreibung						VKALA10.xls[Komponente1]					
Verfahren	Verfahren											
Kalibrationskurve												
			NORMGERECHT VALIDIERT									
# Messung	# Rep.	# Konzstufen	Profil		Arbeitsbereich							
9	3	3	Standard		4.7	14.1						
x	y1	y2	y3	y4	y5	y6	y7	y8	y9	y10	y_varianz	y_i_quer
Konzeinheit	Inf.-einheit											
4.7	0.201003574	0.180823895	0.153777269								0.000561511	0.178534913
9.4	0.433667396	0.528831877	0.50851424								0.002511856	0.490337838
14.1	0.845720914	0.910429044	0.877290682								0.001046991	0.877813546

Datenblatt (Linear (normgerecht))

Konzentration	Meßwerte	geschätzte Werte	Residuen	Vertrauens-intervall (-)	Vertrauens-intervall (+)	Prognose-intervall (-)	Prognose-intervall (+)	Gewichte	berechnete Konzentration	% Abweichung
4.7	0.201003574	0.165922782	0.035080792	4.032767468	5.367232532	3.710334222	5.689665778		5.171570883	10.03342304
4.7	0.180823895	0.165922782	0.014901113	4.032767468	5.367232532	3.710334222	5.689665778		4.900307085	4.261852869
4.7	0.153777269	0.165922782	-0.012145513	4.032767468	5.367232532	3.710334222	5.689665778		4.53673486	-3.473726389
9.4	0.433667396	0.515562099	-0.081894703	8.978005094	9.821994906	8.556010188	10.24398981		8.299136673	-11.71131199
9.4	0.528831877	0.515562099	0.013269778	8.978005094	9.821994906	8.556010188	10.24398981		9.578377986	1.89763815
9.4	0.50851424	0.515562099	-0.007047859	8.978005094	9.821994906	8.556010188	10.24398981		9.305259686	-1.007875678
14.1	0.845720914	0.865201416	-0.019480502	13.43276747	14.76723253	13.11033422	15.08966578		13.83813483	-1.857199777
14.1	0.910429044	0.865201416	0.045227628	13.43276747	14.76723253	13.11033422	15.08966578		14.70796896	4.31183657
14.1	0.877290682	0.865201416	0.012089266	13.43276747	14.76723253	13.11033422	15.08966578		14.26250904	1.15254638

Test der Varianzen

	unten	oben
s(rel)	13.27259388	3.686116886
Freiheitsgrade	2	2
Varianz	0.000561511	0.001046991
Prüfwert	1.864596395	
F_95Var	19	
F_99Var	99	
Ok, kein signifikanter Unterschied auf Niveau 95%		
Ok, kein signifikanter Unterschied auf Niveau 99%		

Linearitätstest

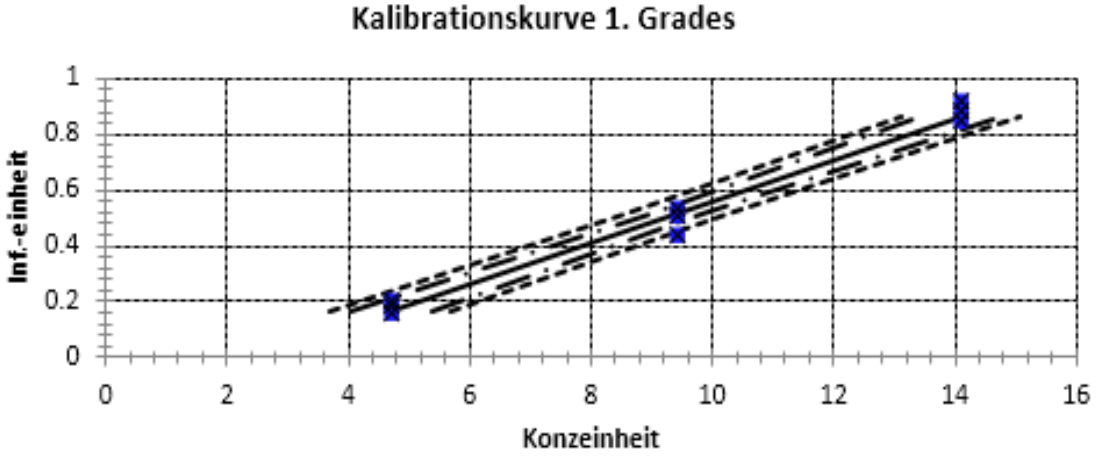
Prüfwert	2.084662933
F_99	13.74502253
Ok, kein signifikanter Unterschied (99% Niveau)	

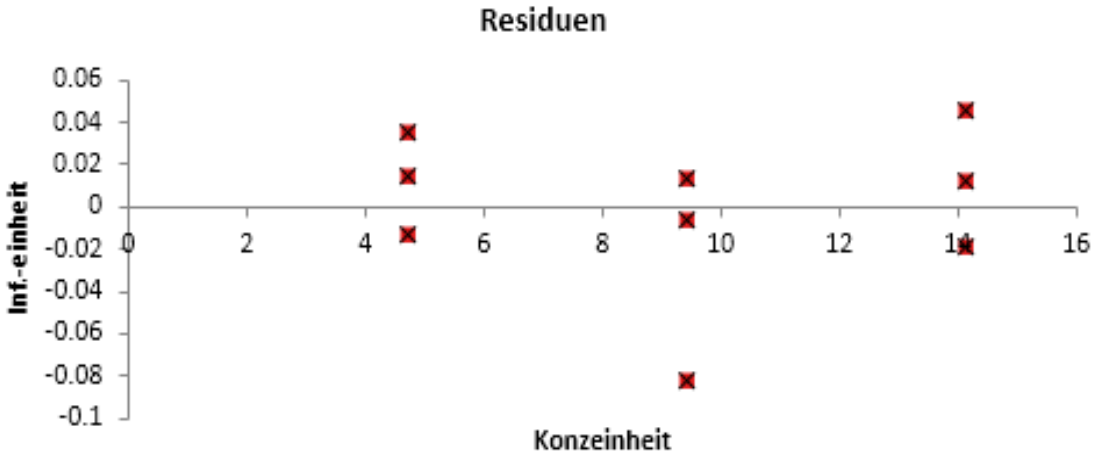
Kalibrierfunktion 1. Grades (y=a+b*x)			
Steigung	0.074391344		Inf.-einheit/(Konzeinheit)
VB(Steigung)	0.06621089	0.082571798	Inf.-einheit/(Konzeinheit)
Achsenabschnitt	-0.183716535		Inf.-einheit
VB(Achsenabschnitt)	-0.266773993	-0.100659077	Inf.-einheit
Mittelwert(x)	9.4		Konzeinheit
Mittelwert(y)	0.515562099		Inf.-einheit
Reststandardabweichung	0.039828021		Inf.-einheit
Verfahrensstd.abweichung	0.535385153		Konzeinheit
Rel. Verfahrensstd.abweich.	5.695586736		%
t-Wert (95%)	2.364624252		
Qx	132.54		(Konzeinheit)^1

Kalibrierfunktion 2. Grades (y=a+b*x+c*x^2)		
a	-0.057595229	Inf.-einheit
b	0.04219016	Inf.-einheit/Konzeinheit
c	0.001712829	
Empfindlichkeit	0.074391344	Inf.-einheit/(Konzeinheit)
Mittelwert(x)	9.4	Konzeinheit
Mittelwert(y)	0.515562099	Inf.-einheit
Reststandardabweichung	0.037060117	Inf.-einheit
Verfahrensstd.abweichung	0.498177816	Konzeinheit
Rel. Verfahrensstd.abweich.	5.299764003	%
t-Wert (95%)	2.446911851	
Prüfwert (Lösung)	-12.31592891	Konzeinheit
Ok, kein Extremwert innerhalb des Arbeitsbereiches.		

Nachweisgrenze - Kalibrationsmethode			
Ergebnisunsicherheit (k)	3		
Konzentration (0)	0		
Geschätzter Meßwert (0)	-0.183716535		Inf.-einheit
Wiederholungen (Meßprobe)	3		
Entscheidungsniveau NWG	0.95		
t-Werte (1/2-seitig)	1.894578605	2.364624252	
Entscheidungsniveau VB	0.95		
Faktoren VB	0.661174145	2.035271928	
Kritischer Wert	-0.104177539		Inf.-einheit
Nachweisgrenze	1.069196916		Konzeinheit
Schnellschätzung NWG	1.284924368		Konzeinheit
VB Nachweisgrenze	0.706925357	2.176106469	Konzeinheit
Erfassungsgrenze	2.138393833		Konzeinheit
Schnellschätzung EG	2.569848735		Konzeinheit
VB Erfassungsgrenze	1.413850713	4.352212937	Konzeinheit
Bestimmungsgrenze	3.253320447		Konzeinheit
Schnellschätzung BG	3.854773103		Konzeinheit
VB Bestimmungsgrenze	2.151011364	6.621391778	Konzeinheit
Ok, Arbeitsbereich abgesichert.			

ANOVA für Lineare Regression					
Quelle	FG	QS	QS/FG	F-Verhältnis	Wahrsch.
Modell	1	0.733485912	0.733485912	462.3962668	1.18555E-07
Residuen	7	0.011103899	0.001586271		
LOF	1	0.002863185	0.002863185	2.084662933	0.198897311
PE	6	0.008240714	0.001373452		


Kalibrationskurve 1. Grades
Inf.-einheit
Konzeinheit
0
0.2
0.4
0.6
0.8
1
0
2
4
6
8
10
12
14
16


Residuen
Inf.-einheit
Konzeinheit
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
-0.1
0
2
4
6
8
10
12
14
16

„Validata“ results for external calibration 50, 100, 200, 250 mg kg^{-1}

3/13/2017		SOP		Bearbeiter								Validata 3.02
Beschreibung	Beschreibung						3.xls[Komponente1]					
Verfahren	Verfahren											
Kalibrationskurve												
			NORMGERECHT VALIDIERT									
# Messung	# Rep.	# Konzstufen	Profil		Arbeitsbereich							
15	3	5	Standard		47	235						
x	y1	y2	y3	y4	y5	y6	y7	y8	y9	y10	y_varianz	y_i_quer
Konzeinheit	Inf.-einheit											
47	1.344200675	1.334698789	1.449152151								0.004034112	1.376017205
94	3.388326575	3.307691117	3.148719162								0.014864312	3.281578951
141	4.945178934	5.303035223	5.435244714								0.064284157	5.227819624
188	6.713634788	7.391600217	7.215192771								0.12371952	7.106809259
235	8.405142539	9.318954543	9.399548732								0.305065246	9.041215271

Datenblatt (Linear (normgerecht))

Konzentration	Meßwerte	geschätzte Werte	Residuen	Vertrauens-intervall (-)	Vertrauens-intervall (+)	Prognose-intervall (-)	Prognose-intervall (+)	Gewichte	berechnete Konzentration	% Abweichung
47	1.344200675	1.375562774	-0.031362099	40.33945894	53.66054106	36.12338199	57.87661801		46.23050355	-1.637226494
47	1.334698789	1.375562774	-0.040863985	40.33945894	53.66054106	36.12338199	57.87661801		45.99736649	-2.133262783
47	1.449152151	1.375562774	0.073589377	40.33945894	53.66054106	36.12338199	57.87661801		48.80557954	3.841658597
94	3.388326575	3.291125418	0.097201157	89.29028625	98.70971375	84.19594902	103.804051		96.38491515	2.537143778
94	3.307691117	3.291125418	0.016565699	89.29028625	98.70971375	84.19594902	103.804051		94.40645387	0.432397731
94	3.148719162	3.291125418	-0.142406256	89.29028625	98.70971375	84.19594902	103.804051		90.50593833	-3.717086887
141	4.945178934	5.206688062	-0.261509128	137.1545348	144.8454652	131.5805725	150.4194275		134.5836455	-4.55060604
141	5.303035223	5.206688062	0.096347161	137.1545348	144.8454652	131.5805725	150.4194275		143.3639616	1.67656852
141	5.435244714	5.206688062	0.228556652	137.1545348	144.8454652	131.5805725	150.4194275		146.6078368	3.977189209
188	6.713634788	7.122250706	-0.408615918	183.2902862	192.7097138	178.195949	197.804051		177.9742521	-5.332844621
188	7.391600217	7.122250706	0.269349511	183.2902862	192.7097138	178.195949	197.804051		194.6087252	3.51527934
188	7.215192771	7.122250706	0.092942065	183.2902862	192.7097138	178.195949	197.804051		190.2804146	1.212986493
235	8.405142539	9.03781335	-0.632670811	228.3394589	241.6605411	224.123382	245.876618		219.4768699	-6.605587268
235	9.318954543	9.03781335	0.281141193	228.3394589	241.6605411	224.123382	245.876618		241.8980443	2.935338018
235	9.399548732	9.03781335	0.361735382	228.3394589	241.6605411	224.123382	245.876618		243.8754931	3.776805554

Test der Varianzen		
	unten	oben
s(rel)	4.615833087	6.108992012
Freiheitsgrade	2	2
Varianz	0.004034112	0.305065246
Prüfwert	75.6214176	
F_95Var	19	
F_99Var	99	
WARNUNG: Signifikanter Unterschied auf Niveau 95%		
Ok, kein signifikanter Unterschied auf Niveau 99%		

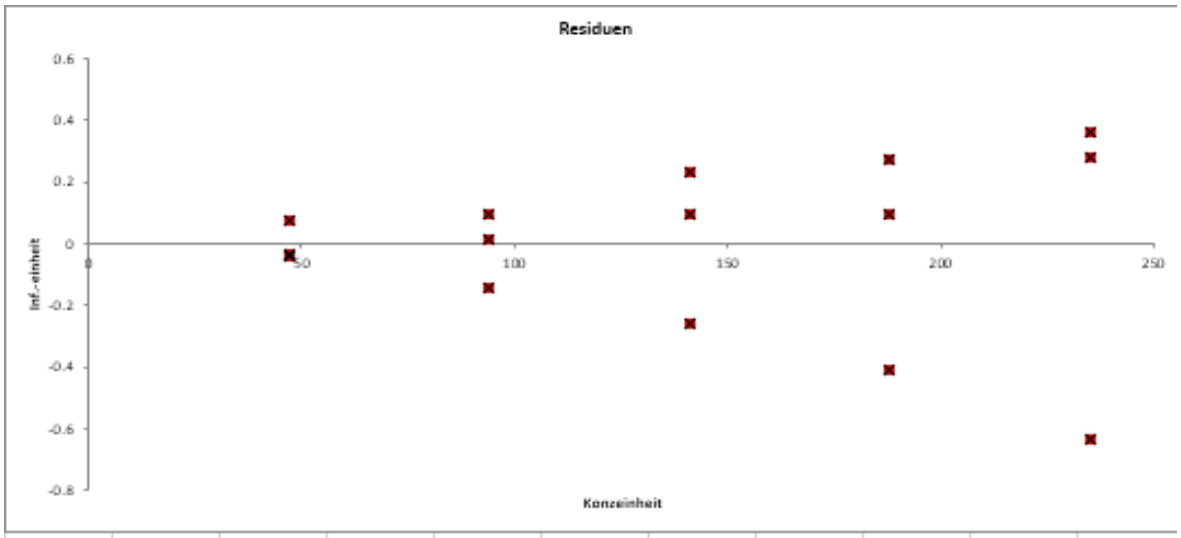
Linearitätstest	
Prüfwert	0.000229114
F_99	9.330212103
Ok, kein signifikanter Unterschied (99% Niveau)	

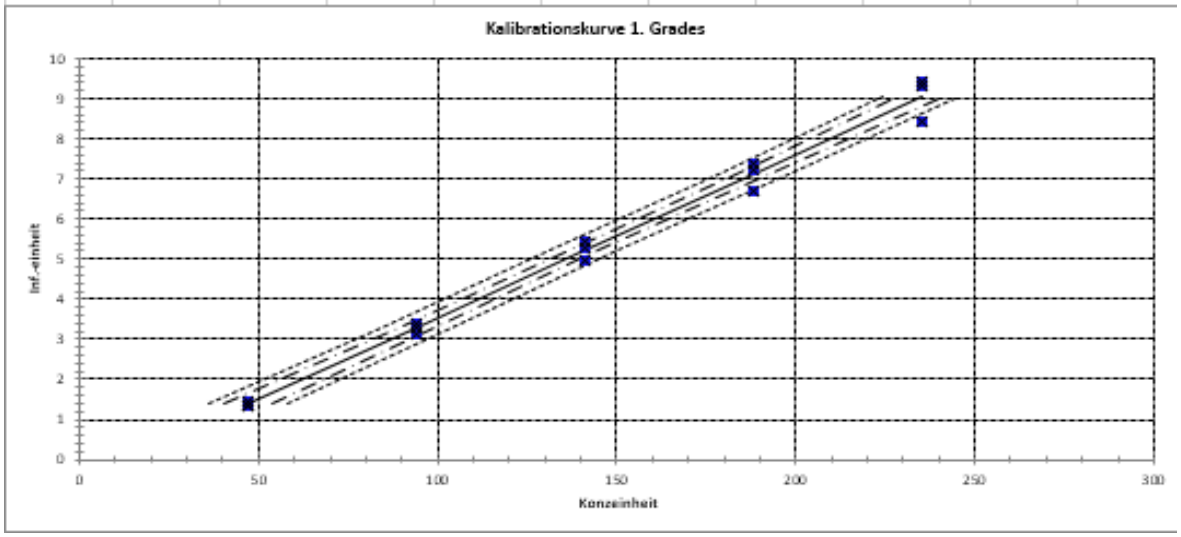
Kalibrierfunktion 1. Grades (y=a+b*x)			
Steigung	0.040756652		Inf.-einheit/(Konzeinheit)
VB(Steigung)	0.038398702	0.043114602	Inf.-einheit/(Konzeinheit)
Achsenabschnitt	-0.53999987		Inf.-einheit
VB(Achsenabschnitt)	-0.907560281	-0.172439459	Inf.-einheit
Mittelwert(x)	141		Konzeinheit
Mittelwert(y)	5.206688062		Inf.-einheit
Reststandardabweichung	0.28097336		Inf.-einheit
Verfahrensstd.abweichung	6.893926431		Konzeinheit
Rel. Verfahrensstd.abweich.	4.889309526		%
t-Wert (95%)	2.160368656		
Qx	66270		(Konzeinheit)^2

Kalibrierfunktion 2. Grades (y=a+b*x+c*x^2)		
a	-0.544781123	Inf.-einheit
b	0.040843848	Inf.-einheit/Konzeinheit
c	-3.09206E-07	
Empfindlichkeit	0.040756652	Inf.-einheit/(Konzeinheit)
Mittelwert(x)	141	Konzeinheit
Mittelwert(y)	5.206688062	Inf.-einheit
Reststandardabweichung	0.292443554	Inf.-einheit
Verfahrensstd.abweichung	7.175357628	Konzeinheit
Rel. Verfahrensstd.abweich.	5.088906119	%
t-Wert (95%)	2.17881283	
Prüfwert (Lösung)	66046.33635	Konzeinheit
Ok, kein Extremwert innerhalb des Arbeitsbereiches.		

Nachweisgrenze - Kalibrationsmethode			
Ergebnisunsicherheit (k)	3		
Konzentration (0)	0		
Geschätzter Meßwert (0)	-0.53999987		Inf.-einheit
Wiederholungen (Meßprobe)	3		
Entscheidungsniveau NWG	0.95		
t-Werte (1/2-seitig)	1.770933396	2.160368656	
Entscheidungsniveau VB	0.95		
Faktoren VB	0.724953932	1.611042417	
Kritischer Wert	-0.123690301		Inf.-einheit
Nachweisgrenze	10.21451834		Konzeinheit
Schnellschätzung NWG	14.89088109		Konzeinheit
VB Nachweisgrenze	7.405055235	16.45602231	Konzeinheit
Erfassungsgrenze	20.42903667		Konzeinheit
Schnellschätzung EG	29.78176218		Konzeinheit
VB Erfassungsgrenze	14.81011047	32.91204462	Konzeinheit
Bestimmungsgrenze	34.13793556		Konzeinheit
Schnellschätzung BG	44.67264327		Konzeinheit
VB Bestimmungsgrenze	24.74843062	54.99766221	Konzeinheit
Ok, Arbeitsbereich abgesichert.			

ANOVA für Lineare Regression					
Quelle	FG	QS	QS/FG	F-Verhältnis	Wahrsch.
Modell	1	110.0814073	110.0814073	1394.388144	1.3011E-14
Residuen	13	1.026298381	0.078946029		
LOF	3	0.002363687	0.000787896	0.007694786	0.999008152
PE	10	1.023934693	0.102393469		


Residuen
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
Inf.-einheit
0
50
100
150
200
250
Konzeinheit


Kalibrationskurve 1. Grades
10
9
8
7
6
5
4
3
2
1
0
Inf.-einheit
0
50
100
150
200
250
300
Konzeinheit

Images of Two-Dimensional Comprehensive GC×GC MS

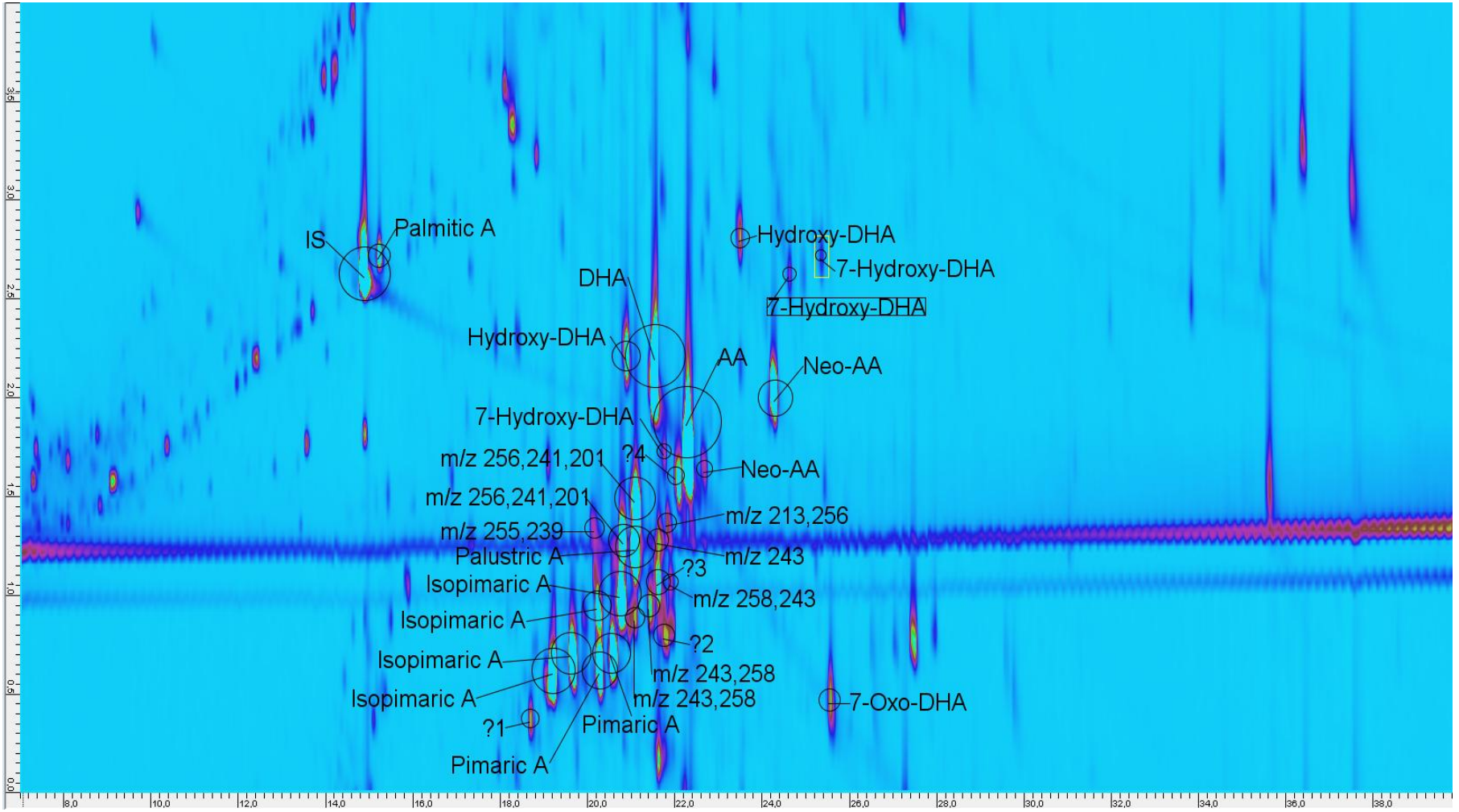


Figure 21: Comprehensive GC-MS image of slurry (scan-mode)

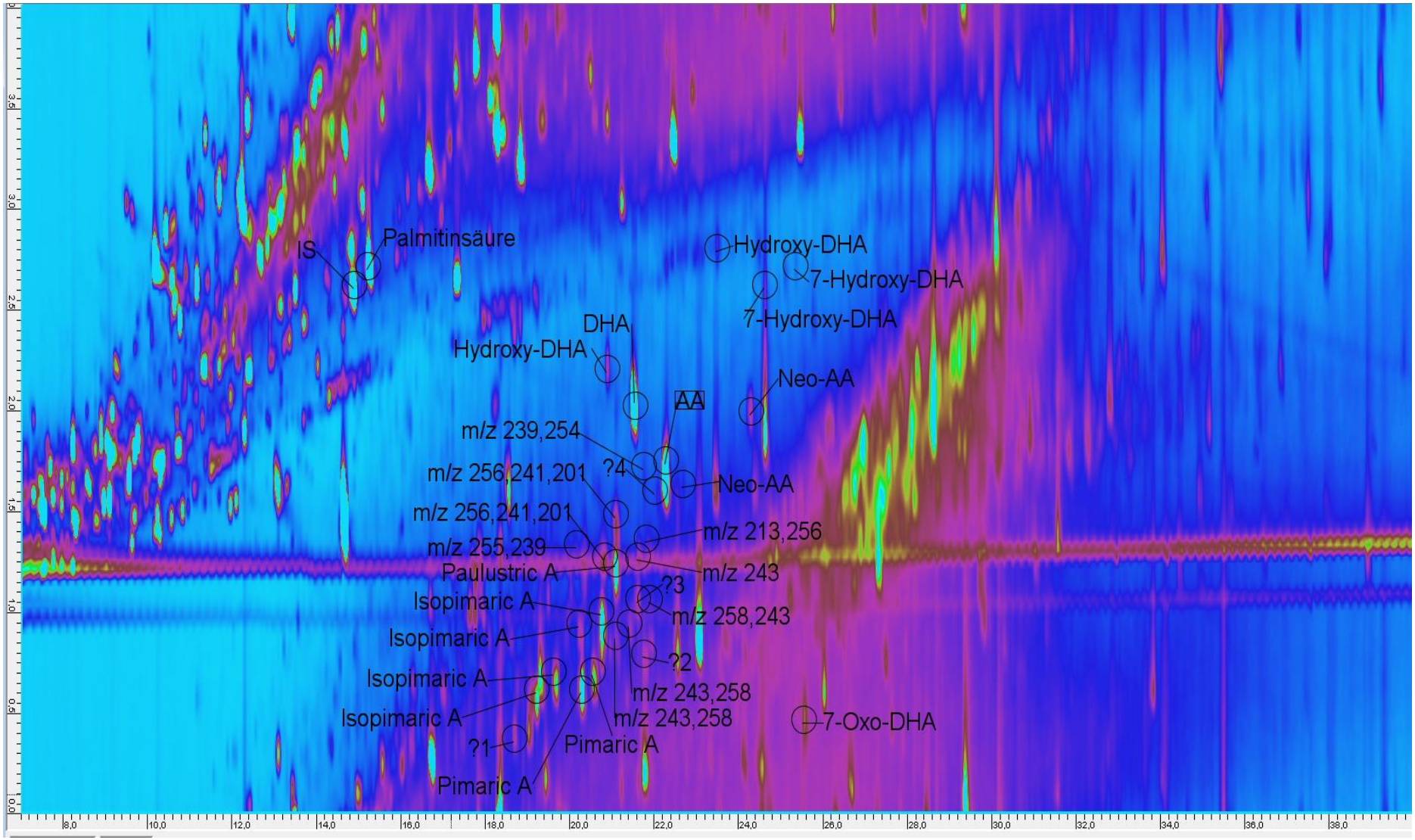


Figure 22: Comprehensive GC-MS image of panty liner pad (scan-mode)

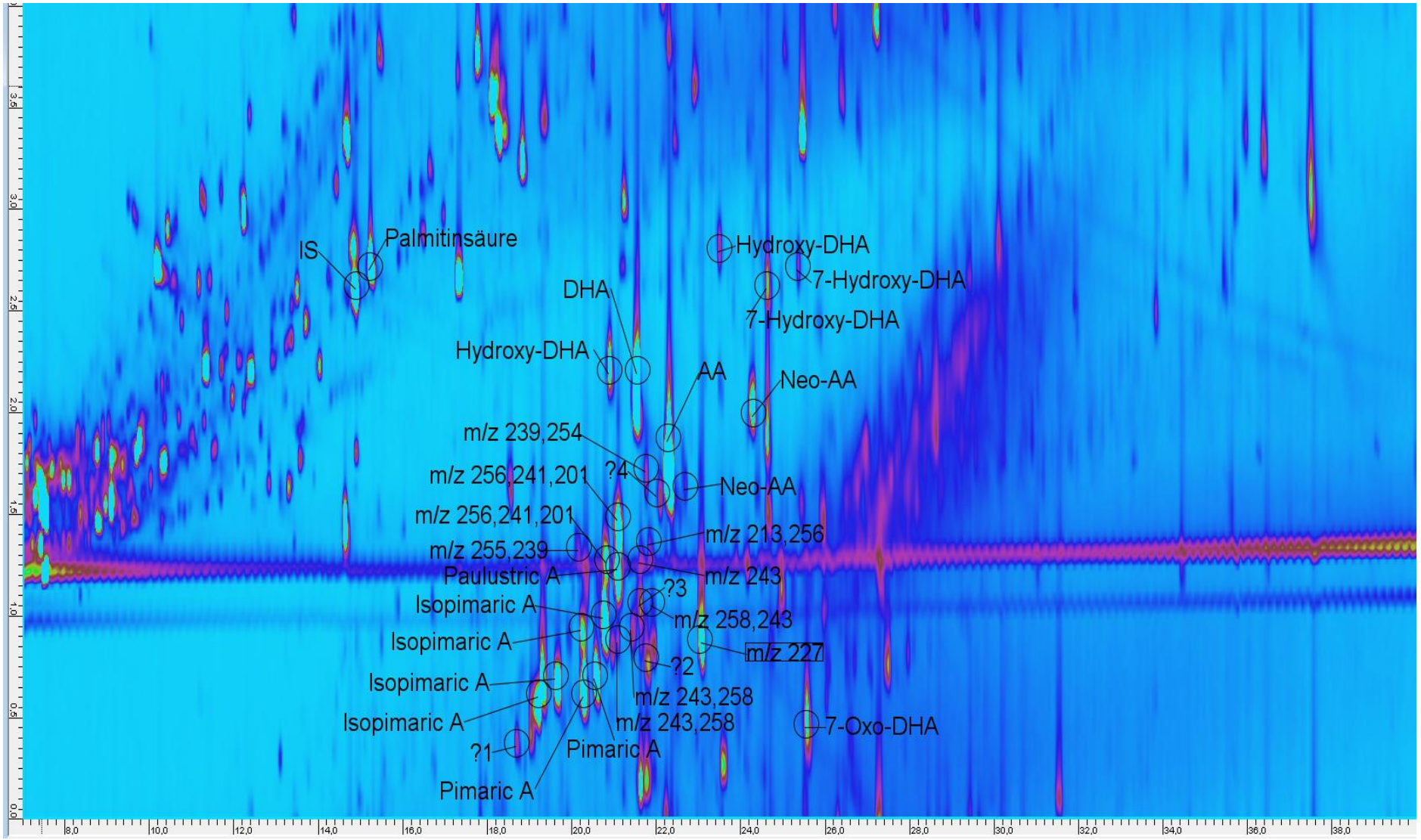


Figure 23: Comprehensive GC-MS image of the release liner of the panty liner sample (scan mode)