Dissertation

IONIC LIQUID TECHNOLOGIES FOR VALUABLE INGREDIENT ISOLATION FROM BIOMASS

Ausgeführt zum Zwecke der Erlangung des akademischen Grades eines
Doktors der technischen Wissenschaften unter der Leitung von

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DEDICATED TO RUDI

Thank you for being there for me.

Thank you for everything.

I wish you were here.

In life, as in football
you won't go far
unless you know where the goalposts are.

ARNOLD H. GLASOW

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Abstract

The present work focuses on alternative extraction or isolation processes of valuable ingredients from biomaterials using novel ionic liquid-based technologies.

In the first part of this thesis a set of ionic liquids, including protic, hydrophilic, biodegradable and surface-active species was successfully synthesized and characterized. Tailor-made ionic liquids were further applied for different extraction/isolation problems by dissolution of various kinds of biomass.

The valuable ingredient piperine that was extracted from black pepper using aqueous solutions of surface-active ionic liquids. Compared to pure water extraction yields were tremendously increased. A simple scale-up strategy was developed allowing the recovery of the ionic liquid solution for several times without any loss of efficiency. The use of ionic liquid-based micellar solutions was expanded to the isolation of eugenol from cloves. Again, those systems were suitable for the enhanced extraction of the active ingredient compared to aqueous systems. However the combination of extraction and an *in situ* derivatization towards isoeugenol in micellar systems was not possible so far.

For the isolation of betulin birch bark was dissolved in hydrophilic ionic liquids. After the formation of a two-phase system *via* the addition of water and an environmentally benign organic solvent betulin was successfully isolated in enhanced yields in short time at room temperature. In this case, the *in situ* functionalization of the crude extract was possible, allowing to directly convert betulin to the value-added betulinic acid in high yields.

Ionic liquids were also investigated for the *in situ* extraction and biocatalytic transformation of limonene from orange peels towards a valuable chiral polymer building block. Some ionic liquids were compatible with two different bacterial strains and biocatalysis was successfully performed in the presence of ionic liquids. However the combined extraction and conversion is still under investigations.

In the last part of this thesis a simple, fast and efficient strategy based on an ionic liquid/buffer system was developed for a rapid determination of genetically modified maize *via* the extraction of DNA and analysis with qPCR. The developed method was superior to conventional systems, since not only time, but also costs were saved by reducing heating and centrifugation steps while isolating a comparable amount of DNA. The versatility of this method was further demonstrated by expanding ionic liquid/buffer systems to different meats.

In summary a suitable ionic liquid was found for every extraction or isolation problem for valuable ingredients derived from a variety of different types of biomass.

Kurzfassung

Die vorliegende Arbeit befasst sich mit alternativen Extraktions- bzw. Isolationsstrategien von Wertstoffen aus biologischen Materialien durch Verwendung unterschiedlicher auf ionischen Flüssigkeiten basierenden Techniken.

Im ersten Teil dieser Dissertation wurde eine Vielzahl an ionischen Flüssigkeiten mit mannigfaltigen Eigenschaften hergestellt, um für jedes Extraktions- oder Isolationsproblem eine maßgeschneiderte ionische Flüssigkeit zu finden.

Für die Isolation von Piperin aus schwarzem Pfeffer wurden wässrige Lösungen von oberflächenaktiven ionischen Flüssigkeiten eingesetzt. Dabei konnte mithilfe der mizellaren Systeme nicht nur deutlich höhere Ausbeuten als mit rein wässrigen Systemen erzielt werden, sondern es konnte auch in einem simplen Prozess eine Durchführung im Großmaßstab erzielt werden. Die ionische Flüssigkeit konnte erfolgreich zurückgewonnen und 4-mal ohne an Extraktionseffizienz zu verlieren wieder verwendet werden. Die mizellaren Systeme wurden anschließend auch für die Extraktion von Eugenol aus Nelken erfolgreich getestet. Allerdings konnten keine geeigneten Bedingungen für die direkte Umwandlung zu Isoeugenol gefunden werden.

Für die Isolation von Betulin aus Birkenrinde wurden hydrophile ionische Flüssigkeiten zum Auflösen der Biomasse verwendet, welche nach Zugabe von Wasser und organischem Lösungsmittel ein Zweiphasen-Gemisch bildeten. Der bei Raumtemperatur in kurzer Zeit in der organischen Phase angereicherte Wertstoff wurde in hohen Ausbeuten direkt zur pharmazeutisch wertvollen Betulinsäure oxidiert.

Für die direkte biokatalytische Kaskadenreaktion von Limonen aus Orangenschalen zu einem Ausgangsmaterial für chirale Polymere wurde die Kompatibilität von ionischen Flüssigkeiten mit zwei Bakterienstämmen getestet. Limonen konnte erfolgreich in hohen Ausbeuten mithilfe von ionischen Flüssigkeiten extrahiert werden. Die direkte Umsetzung von Limonen mithilfe der Kaskadenreaktion muss jedoch noch optimiert werden.

Im letzten Teil dieser Arbeit wurde ein einfaches, schnelles und effizientes Verfahren für die Detektion von DNA aus genetisch modifiziertem Mais entwickelt. Besonders dafür geeignet waren konventionelle Puffersysteme in Kombination mit ionischen Flüssigkeiten. Die hierbei entwickelte Methode war effizienter als herkömmliche Methoden und erwies sich als vielseitig, da sie auch erfolgreich für die Extraktion und Analyse von DNA aus unterschiedlichen Fleischsorten angewendet werden konnte.

Es konnte für jeden Wertstoff eine geeignete maßgeschneiderte ionische Flüssigkeit gefunden werden und eine geeignete Methode für die Extraktion oder Isolation entwickelt werden.

1 Introduction

1.1 Biomass as sustainable source for active ingredients

Natural products obtained from biomass are of considerable importance for the pharmaceutical industry, as they provide a diverse and unique source of bioactive compounds for drug manufacturing (Figure 1).[1] The use of naturally derived drugs dates back to the years 2900-2600 BC: Already more than 4000 years ago old Egyptian pharmaceutical records describe the use of over 700 drugs originating from plant material. Oils from *Cupressus sempervirens* and *Commiphora* species were applied 2600 B.C. in Mesopotamia to treat coughs, colds and inflammation and are still used nowadays.[2] More than 200 years ago the first biologically active pure compound was isolated from a plant. Morphine was found in the seed pods of *Papaver somniferum* (poppy flower).[3] Probably the most prominent example of a drug obtained from a natural product is salicin that was isolated from the bark of the willow tree *Salix alba* L.[4] Its main component acetylsalicylic acid that is better known under the brand name Aspirin is used to relieve minor pains, to reduce fever and serves as an anti-inflammatory drug. One milestone in the history of naturally derived products was the discovery of the antibiotic penicillin by Alexander Fleming in 1929, thus isolated from the fungus *Penicillium notatum*.[5] For breast cancer treatment Paclitaxel (Taxol®) was isolated from the bark of Pacific yew *Taxus brevifolia*. Due to rising demand it can now be synthesized from Baccatin III that is present in higher quantities in the needles of the Pacific yew or from 10-deacetyl-baccatin III present in the European yew, *Taxus baccata*.[6]

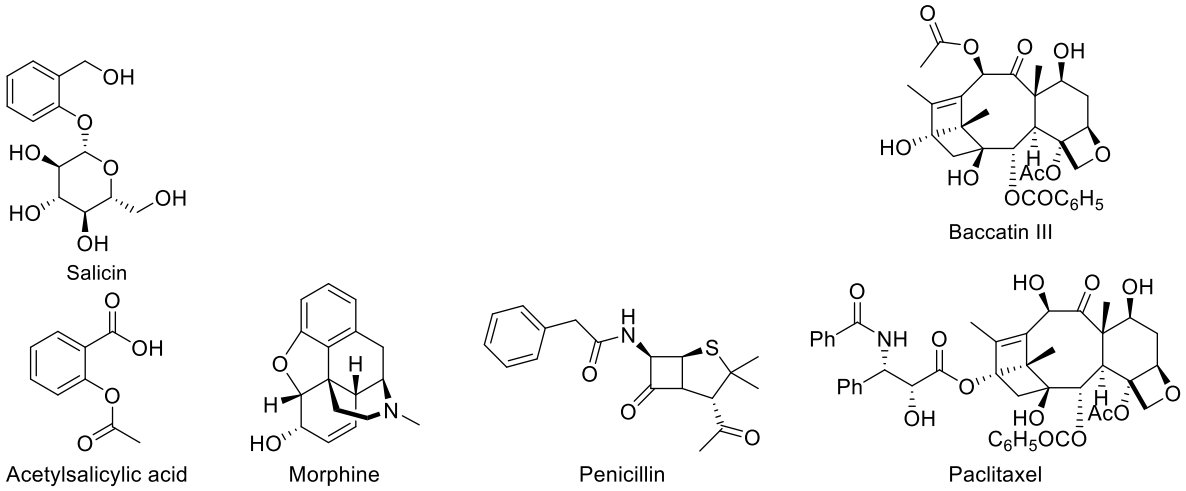


Figure 1: Historically important drugs and their precursors derived from natural products

According to the worldwide sell in the early 2000, natural product-derived drugs are frequently found in top position, and 8 out of 29 small molecule drugs launched in the 2000s owe their origins to a natural source.[7, 8,9] Figure 2 shows the number of approved drugs in the United States from 1981 to 2007. As it can be seen a major part of released drugs owes its origin to nature.[10]

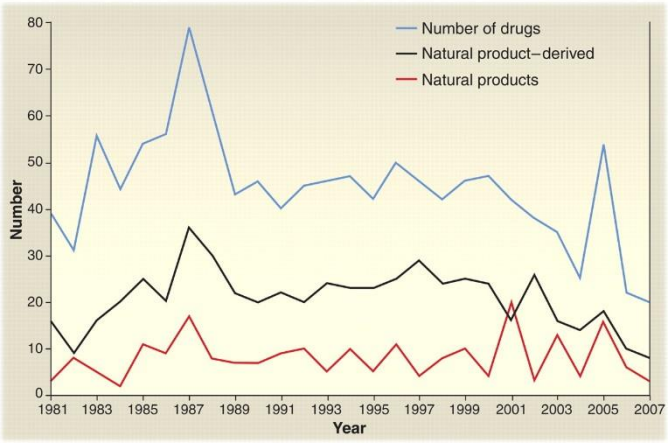


Figure 2: Number of drugs approved in the US from 1981-2007[10]

Current estimation of the pharmaceutical market indicate that between 25% and 50% of drugs are derived from natural products, and the conventional extraction/isolation of a drug or its precursor can be the bottleneck in the manufacturing process.[11] Table 1 shows a small selection of naturally derived drugs on the market in the last decade.

Table 1: Selected drugs derived from natural products in the world market (WW = worldwide)

Entry	Drug	Treatment	Rank	Year
1	Atorvastatin	blood cholesterol	1 WW	2002[8]
2	Simvastatin	blood cholesterol	2 WW	2002[8]
3	Tamiflu	influenza	26 in USA	2014[1]
4	Paclitaxel	breast cancer	25 WW	2000[8]

The isolation of natural product is still challenging nowadays, despite of the development of extraction and separation techniques as well as analytical devices, e.g. LC-NMR or LC-MS allowing an identification of natural products prior to isolation.[12] The typical commercial extraction process for pharmaceutical active ingredients from plant materials is based on the use of volatile organic compounds (VOCs) as extraction media. A standard procedure starts with the identification, collection and preparation of biomass, which usually includes a drying step. This is followed by the extraction using various solvents with different polarities. After long periods of refluxing and separation of by-products such as waxes, essential oils, and chlorophylls from the raw extract, the purification of active ingredients typically requires several additional stages for the removal of unwanted matrix, e.g. crystallization. (Semi-)preparative HPLC or liquid-liquid extraction techniques can be used for the isolation after performing a variety of purification steps.[13] (Figure 3)

1 http://www.drugs.com/stats/top100/units, last acceess 1.7.2015

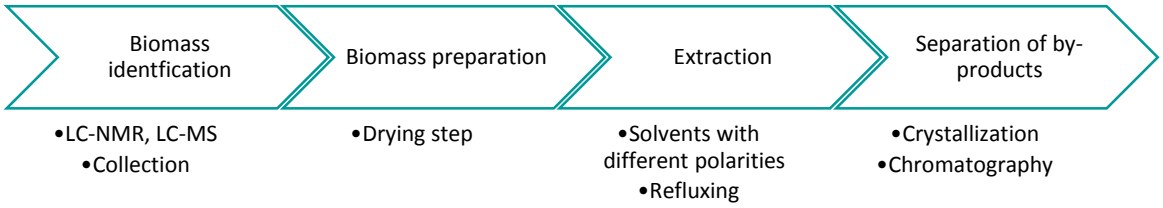


Figure 3: Procedure for the isolation of a newly discovered natural product

Conventional extraction is usually performed with a set of organic solvent with different polarities, water or mixtures thereof. Different techniques are applied including the highly solvent consuming maceration method, *i.e.* the soaking of plant material in solvents with low extraction times. Classical Soxhlet extraction requires long hours of refluxing and might degrade thermo-labile natural products. Despite the risk of chemical modification when working at elevated temperatures, distillation methods are used for the isolation of essential oils. However all these processes suffer often from low overall yields due to several purification steps. The large amounts of VOCs may often cause a threat to the environment and a risk of exposure to hazardous chemical compounds. As a consequence, modern extraction methods and media for the isolation of active ingredients from plant materials have become more popular than the conventional reflux methods.[14-18]

As an alternative method ultrasound-assisted extraction (UAE) can shorten extraction times and improve the extraction yield due to mechanical stress induced cavitations and cellular breakdown. Another technique that can also be combined with ultrasound strategies is microwave-assisted extraction (MAE).[19] Advantages of this technique are shorter extraction times, the requirement of less solvents and better extraction efficiency with lower cost. Several advanced MAE instrumentations and methodologies have become available, such as pressurized microwave-assisted extraction and solvent-free microwave-assisted extraction, a combination of microwave heating and dry distillation without solvents.[20] Methods at elevated temperature and pressure as well as supercritical fluid extraction were also established. This techniques gained attention due to their nondestructive extraction of sensitive natural compound. Especially the use of supercritical carbon dioxide ($scCO_2$) was promising for the isolation of pharmaceutical products due to its low toxicity.[21] The use of $scCO_2$ in combination with organic solvent to increase solubility of organic compounds has attracted attention as well.

However, only recently research started to focus on the use of ionic liquids (ILs, salts melting below 100 °C) as leaching media for pharmaceutically valuable compounds from plant components, as they can be benign and efficient solvents for the isolation of active ingredients. Table 2 shows a brief comparison of organic solvents and ionic liquids. The infinite possible combination of different anions and cations leads access to an unlimited number of ionic liquids.

Table 2: Comparison of organic solvents and ionic liquids, adapted from Plechkova et al. from 2008[22]

Property	**Organic solvents**	**Ionic liquids**
Number of solvents	>1000	>1 000 000
Applicability	Single function	Multifunction
Catalytic ability	Rare	Common and tuneable
Chirality	Rare	Common and tuneable
Vapour pressure	Obeys the Clausius-Clapeyron equation	Negligible vapor pressure under normal conditions
Flammability	Usually flammable	Usually nonflammable
Solvation	Weakly solvating	Strongly solvating
Tuneability	Limited range of solvents available	Virtually unlimited range means "designer solvents"
Cost	Normally cheap	Typically between 2 and 100 times the cost of organic solvents
Recyclability	Green imperative	Economic imperative

The use of non-volatile ionic liquids may enable a safer and environmentally benign approach in industrial processes, as problems exciting with highly volatile and often toxic organic solvents can be circumvented.

2 State of the art

2.1 Ionic liquids

Ionic liquids (ILs) are defined as salts with a melting point below 100 °C. They usually consist of an organic cation, such as pyrrolidinium, imidazolium, pyridinium, quaternary ammonium or phosphonium cations. Counterions are typically halides, bis(trifluoromethane)sulfonimides (bistriflimides), carboxylates, alkylsulfates or the fluorinated tetrafluoroborate and hexafluorophosphate. (Figure 4)

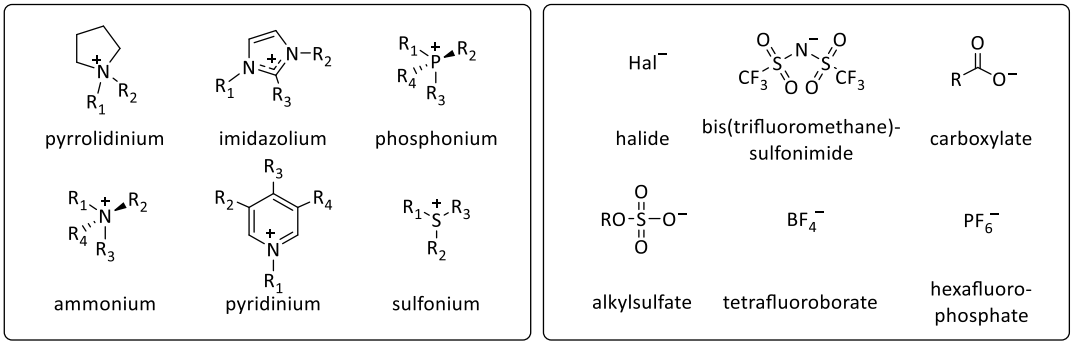


Figure 4: Examples of commonly used cations and anions

The first ionic liquid reported in literature dates back to 1888: Ethylammonium nitrate that has a melting point of 52-55 °C was mentioned by Gabriel *et al*.[23] In 1914 Paul Walden described the ionic liquid ethylammonium nitrate, which is often considered as the hour of birth of modern ionic liquids.[24] Responsible for the unusually low melting points of these salts is the bulky and asymmetric structure of the cations in combination with a good charge distribution in the anions.[25]

So far more than 1500 different ionic liquids are reported in literature. However an almost unlimited number of ionic liquids is theoretically possible, due to the variable combination of cations and anions. Their outstanding properties made them not only interesting for academic research, but also for industrial applications. Briefly, ionic liquids have an insignificant vapour pressure, are usually non-flammable and show high thermal stability. Ionic liquids possess powerful dissolution properties and are able to dissolve biomass. However their physical, chemical and biological properties can be fine-tuned by switching anions or cations or designing specific functionalities into the cations and/or anions.[25] These attractive properties of ionic liquids resulted in considerable interest from both academia and industry and the constant development of novel applications even on industrial scale that have been intensively reviewed.[22] Figure 5 provides a selection of commercialized processes featuring ionic liquids.

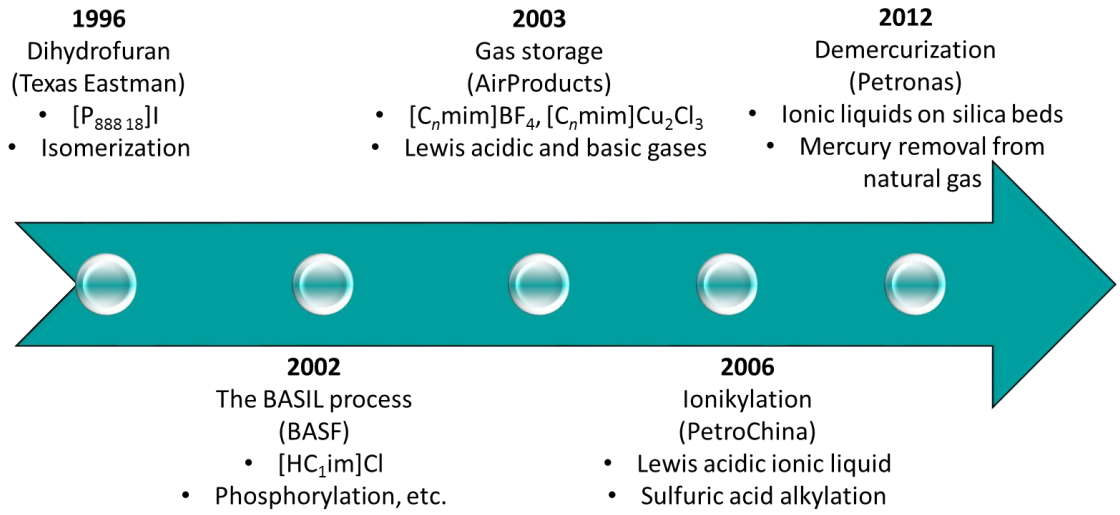


Figure 5: Selection of commercialized processes using ionic liquids

On the other hand, the economic feasibility of replacing conventional solvents with ionic liquids can be questioned and needs to be justified. The non-volatility makes isolation processes and the recovery of ionic liquids sometimes even more complex. Ionic liquids cannot be regained by a simple distillation like organic solvents although distillable ionic liquids were already reported in literature.[26] Another drawback associated with ionic liquids is their high price. Table 3 shows a comparison of prices of organic solvents and ionic liquids. Remarkably is that in the last years processes were developed for the relative inexpensive synthesis of some ionic liquids.[27] However the commonly used imidazolium based ionic liquids still suffer from high prices compared to organic solvents.

Table 3: Prices of industrial production of organic solvents and ionic liquids[27-29]

Organic solvents per $US		**Ionic liquids per $US**	
Toluene	0.74-1.05 L^{-1}	[$C_n$mim]-based	14-34 kg^{-1}
Ethanol	0.82-0.98 L^{-1}	$[HC_1im]HSO_4$	2.96-5.88 kg^{-1}
Methanol	0.25-0.54 L^{-1}	$[HN(Et)_3]HSO_4$	1.24 kg^{-1}
Acetonitrile	1.06-1.12 kg^{-1}	[chol]Cl	1.21 kg^{-1}
n-Butyl acetate	1.48-1.59 kg^{-1}	$[N_{11(10)(10)}]Cl$[a]	8.71 kg^{-1}
Chloroform	0.53-1.04 kg^{-1}	Cocoalkonium chloride	5.28 kg^{-1}
Ethylene glycols	1.43 kg^{-1}		
n-Methyl pyrolidone	4.08-4.19 kg^{-1}		
Sulfolane	~ 3.7 kg^{-1}		

[a] Didecyl-dimethylammonium chloride

Generally it can be said that ammonium, e.g. choline ionic liquids and phosphonium ionic liquids are cheaper than the imidazolium derivatives and represents a good alternative for biomass dissolution.[30] (Figure 6) Among the anions chloride anions are considered as cheap especially compared to e.g. ionic liquids containing a hexafluorophosphate anion, since their synthesis requires an additional step. Therefore the choice of ionic liquids as leaching media might not only rely on the ideal properties, but on a compromise between functionality and price.

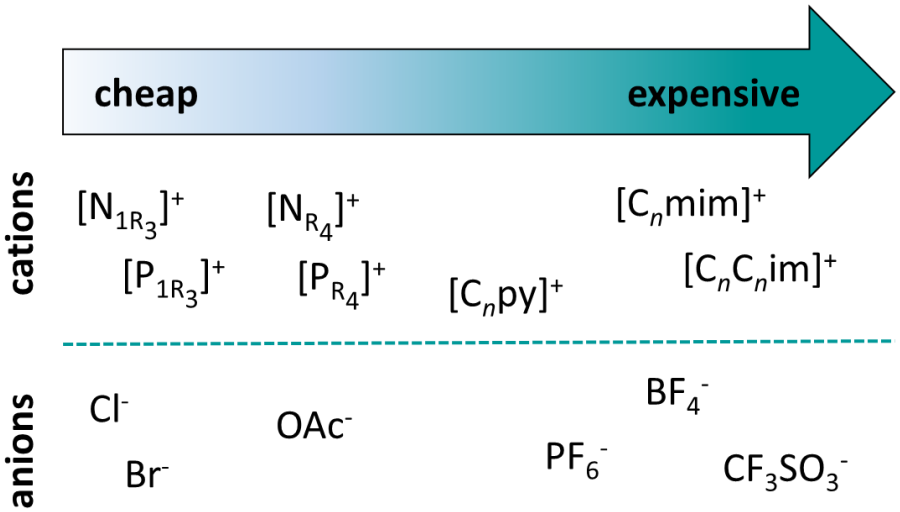


Figure 6: Comparison of prices of different ionic liquids[31]

The role of ionic liquids in the pharmaceutical industry can be widespread: Apart from the use as extracting solvent, e.g. for the extraction of valuable building blocks or pharmaceutical intermediates from fermentation broths,[32] ionic liquids forms of formerly solid drugs are attracting increasingly attention.

Although ionic liquids possess environmentally benign properties such as a low vapour pressure and may, if chosen carefully, prevent or reduce pollution, their association as "green solvents" has been questioned due to recent toxicity studies about ionic liquids.[33] In addition, imidazolium based cations are synthesized from imidazoles and alkyl halides which are obtained from non-renewable petroleum feedstock, and organic solvents are often used for the synthesis of ionic liquids. However, renewable resources for ionic liquids, e.g. nicotinic acid based anions are available to improve this issue. Furthermore, some typically anions such as PF_6^- salts can be quite toxic when in contact with water, and thus a responsible handling of the term "green solvents" is necessary when dealing with ionic liquids.

2.2 Dissolution of biomass with ionic liquids

The dissolution of biomass in ionic liquids has become an intense and constantly growing area of research in the past years. Biomass feedstocks have become a unique and indispensable resource for various fields: Apart from the use of biomass as an alternative energy production e.g. bioethanol or HMF, biomass feedstocks including food waste can be used for the manufacturing of high-value fine chemicals.[34,35,36]

Biomass dissolution in molten salts dates back to 1934 when Graenacher partially dissolved cellulose using the molten salt *N*-ethylpyridinium chloride.[37] Almost 50 years later, Seddon *et al*. published that chloroaluminate ionic liquids could dissolve kerogen, a fossilized organic material present in sedimentary rocks which had been insoluble in all known solvents except for hydrofluoric acid.[31] Several years later, the group of Rogers reported a groundbreaking paper on the dissolution of cellulose in the imidazolium-based ionic liquids 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$) without derivatization in high concentrations, thereby opening a novel and attractive opportunity for the direct processing of cellulose and other biopolymers.[38]

Cellulose is present in the primary cell wall of green plants with an average content about 33%, but can reach up to 90% in cotton. In a cellulose polysaccharide between hundreds and thousands β-1,4-linked glucose units are available. The β configuration at the anomeric carbon results in a stretched chain conformation, which is in contrast to the helical shape of starch due to anomeric α configuration. Crystalline fibrils can be built by the packing of numerous linear cellulose strands. In native cellulose two intramolecular hydrogen bonds and one intermolecular hydrogen bond can occur for each glucose unit (Figure 7).[39] These inter- and intramolecular interactions present in cellulose result in considerable difficulties for dissolution in conventional organic solvents or water.

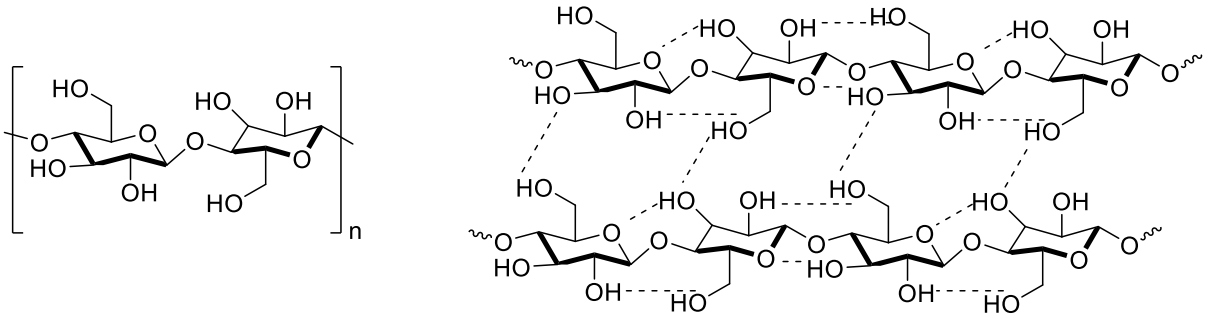


Figure 7: Inter and intramolecular hydrogen bonding in cellulose

As a consequence cellulose hardly dissolves in conventional solvents due to its strong hydrogen bonding between the polymer chains. Current dissolution processes are performed in large excess of aggressive chemicals, e.g. aqueous solutions of NaOH resulting in derivatization or degradation or require expensive reagents, such as *N*-methylmorpholine-*N*-oxide monohydrate (NMO)[40] or the use of concentrated phosphoric acid[41] to avoid these side reactions. Another strategy converts the hydroxyl groups of cellulose into xanthogenate esters using carbon disulfides to make the derivatized cellulose soluble in organic solvents.[42] This strategy also known as Viscose® process is currently applied for the production of cellulose fibers. However this process suffers from using hazardous chemicals such as CS_2 for the derivatization of cellulose and sulfuric acid for the spinning batch, as well as treatment of the fibers with hypochlorite or hydrogen peroxide. Novel ionic liquid-based technologies for improved processing of cellulose that take advantage of the direct dissolution and non-derivatization of biomass are therefore of considerable interest.[33, 43] Since Swatloski's pioneering publication in 2002 a number of papers has been published not only on the dissolution of cellulose in ionic liquids, but also on the role of ionic liquids as reaction medium for the functionalization of cellulose, the preparation of cellulose fibers, films, beads and cellulose composite materials.[33, 43, 44] Cations based on methylimidazolium and methylpyridinium including allyl-, ethyl-, or butyl-side chains are all suitable for the dissolution of biomass, and the best dissolution results were obtained with the C_4 side chain. Considering the anions, chloride, acetate and formate are all anions with good prospects.[33] A selection of ionic liquids used for the dissolution of cellulose is shown in Table 4. The topic dissolution of cellulose in ionic liquids has been reviewed lately.[33, 43]

Table 4: Selection of ionic liquids used for the dissolution of cellulose

Entry	Ionic liquid	Cellulose	Conditions	Solubility [wt%]	Reference
1	[C_4mim]Cl	pulp cellulose	MW	25	Swatlovski, 2002[38]
2	[C_2mim]OAc	Cellulose	-*	>20	Sun, 2011[45]
3	[C_2mim]OAc	Avicel	110 °C	15	Zhao, 2008[46]
4	[C_4mim]fom	micro crystalline	70 °C	13	Xu, 2010[47]
5	[amim]fom	micro crystalline	85 °C	22	Fukaya, 2006[48]
6	[amim]Cl	micro crystalline	sonication	27	Mikkola, 2007[49]
7	[C_4Pyr]Cl	Avicel (286)	105 °C	39	Heinze, 2005[50]

* not available. [amim] = 1-allyl-3-methylimidazolium, fom = formate, [C_4Pyr] = *N*-butylpyridinium

The dissolution process of cellulose has even been licensed to the company BASF and 5 wt% solutions of cellulose in 1-ethyl-3-methylimidazolium acetate ([C_2mim]OAc) are commercially available through Sigma-Aldrich under the trade name CELLIONIC™.

Several tentative mechanisms for the dissolution process of cellulose in ionic liquids have been proposed: NMR-experiments showed that the high chloride concentration and activity in [C_4mim]Cl breaks the extensive hydrogen bonding network, thus allowing the dissolution of higher concentrations of cellulose.[38] Furthermore it was claimed that dissolution of cellulose in ionic liquids involves hydrogen-bonding between the carbohydrate hydroxyl protons and the ionic liquid anion in ratios of 3:4-1:1 and is therefore those interactions play an important role for the dissolution process.[51-53]

Simulation experiments proved that the intermolecular hydrogen bond between hydrogen and oxygen in cellobiose is broken and new bonds are formed between hydroxyl hydrogen atoms in cellobiose and anion.[54] Other studies agree that the dissolution process is governed by the interactions between the anions of the ionic liquids and the hydroxyls of cellulose.[55-57] However, the detailed mechanism at the molecular level still remains unclear.

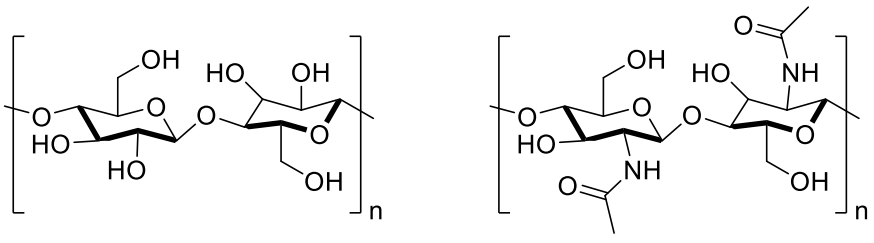


Figure 8: Structure of cellulose (left) and chitin (right)

A very similar structured biopolymer is chitin with an additional acetamide group compared to cellulose (Figure 8), which was partially dissolved at elevated temperatures by Xie *et al.* in [C_4mim]Cl[58] and by Yamazaki in [amim]Br.[59] Native chitin was dissolved in [C_4mim]OAc with 3-7% solubility at 110 °C.[60] Sun *et al.* developed a process for the extraction of chitin from raw crustacean shells with [C_2mim]OAc. The consecutive precipitation provides chitin with high purity and high molecular weight in one step only, whereas a multi-step procedure is currently done in industry. The basic acetate anion seems to improve the solubility of chitin and shrimp shells in comparison to the halide anion.[61]

The processing of biomass is however not limited to the dissolution of cellulose and chitin (Figure 9):

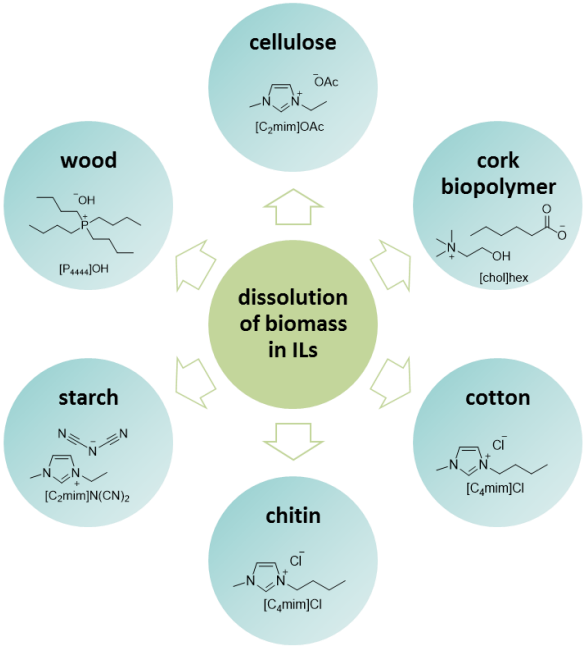


Figure 9: Biomass dissolution in ionic liquids - state of the art

The number of dissolved biopolymers seems to be unlimited: Apart from the biopolymers mentioned above, silk fibroin,[62] wool keratin,[63] starch and zein protein[64] were successfully dissolved in chloride imidazolium ionic liquids. Chitosan[58] was dissolved in $[C_4mim]OAc$, whereas $[C_4mim]BF_4$ was used for the dissolution of the polysaccharide Konjac glucomannane.[65] Biocompatible choline ionic liquids such as [chol]hex were successfully applied for the dissolution of cork biopolymers.[30] The dissolution of wood will be explained in more details later on in this chapter.

Among all different types of biomass that can and have been dissolved in ionic liquids, lignocellulosic biomass has particularly attracted attention as the trend towards energy production from biomass feedstocks is continuously increasing. However, current biofuels are sometimes derived from edible components of food crops, e.g. sucrose, starch and vegetable oils. A direct competition between food and fuel production is feared. Other concerns are raised due to questionable reduction of CO_2 emission.[66]

Lignocellulosic biomass is the most abundant plant material worldwide and available in high quantities.[67] Approximately 90% of lignocellulosic biomass is represented by cellulose, hemicellulose and lignin. Lignocellulose forms the cell wall of plant matter. (Figure 10)

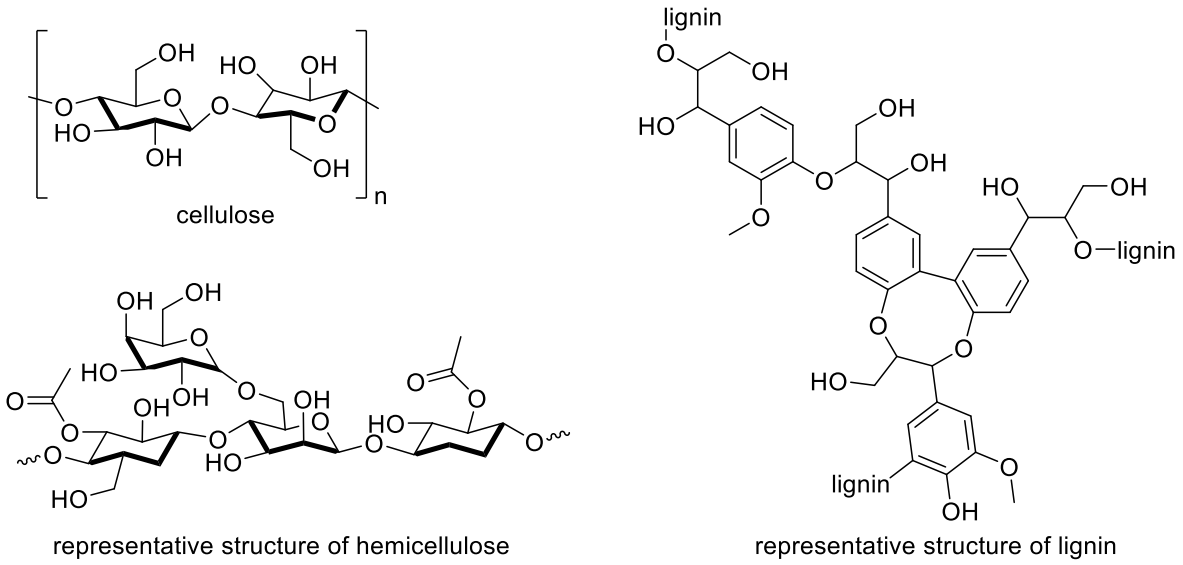


Figure 10: Structures of the main constituents of lignocellulose. For hemicellulose galactoglucomannan, the major hemicellulose in softwood is shown.

Cellulose is available in a content of 35-50 wt%, hemicellulose up to 25 wt% and the complex aromatic polymer lignin between 20 and 30 wt%. Compared to cellulose whose structure has already been discussed above, hemicellulose consists of different carbohydrates polymers with a lower polymerization degree. Pentoses such as xylose and arabinose and hexoses such as glucose, mannose and galctose are used for its backbone. (Figure 11) Additionally, the polymers can contain functional groups e.g. acetyl and methyl groups.[68]

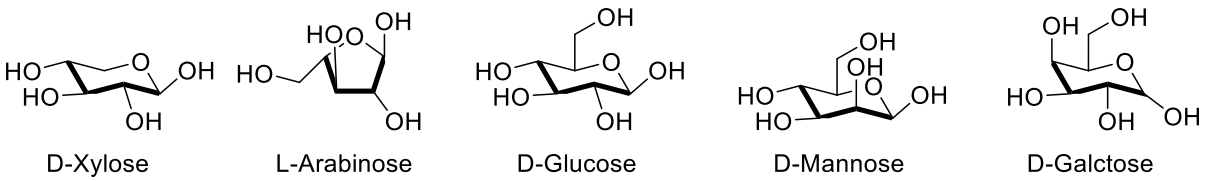


Figure 11: Sugars present in hemicellulose

Hemicellulose binds non-covalently to the surface of cellulose fibrils. Since its structure is non-crystalline, depolymerisation is more likely to occur than for cellulose. Lignin consists of different aromatic units and is water-insoluble. The three aromatic moieties lignin is built of namely coniferyl, sinapyl and *p*-coumaryl alcohols are shown later. (Figure 80) An energy efficient deconstruction process is still limited by the lignin crust.[69] Furthermore lignin prevents access of enzymes e.g. for polysaccharide hydrolases.[70]

Apart from the three main constituents of lignocellulose small amounts of e.g. waxes, lipids, pectins, proteins or inorganic compounds are also present depending on the plant source and can be potentially valuable. However, despite the high carbohydrate content and the wide availability of lignocellulosic biomass it is not used for biofuel production since its processing is cost effective due to the complexity of the structure.[69]

Conventional dissolution/deconstruction processes of lignocellulosic materials e.g. for paper production require aqueous mixtures of sodium hydroxide or sodium hydrogen sulfide and temperatures of 130-180 °C including long processing times of several hours. This process is called

Kraft pulping and dissolves nearly completely lignin and partially hemicellulose, which can be used for energy production by burning. Other pulping processes such as sulfite pulping or thermomechanical pulping are suitable methods for high cellulose yield. Figure 12 shows the deconstruction of lignocellulosic biomass.

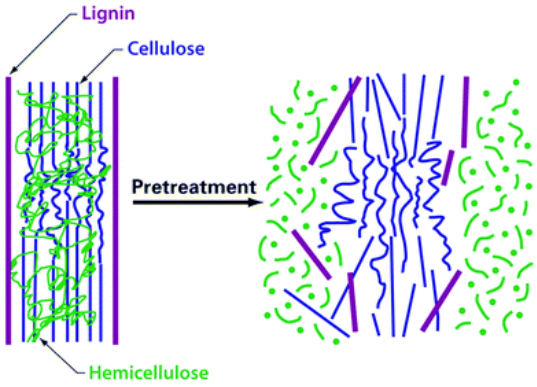


Figure 12: (Physico)chemical deconstruction of the lignocellulose structure[67]

As a consequence arising from the constant demand of novel pulping procedures towards an energy-efficient strategy for lignocellulose deconstruction and processing, ionic liquids received tremendous interest. Ionic liquids are able dissolve not only the main constituents of lignocellulose but can also directly dissolve lignocellulosic biomass. Furthermore ionic liquids represent a suitable class of solvents for the physicochemical disrupture of lignocellulose and enables not only the access to fermentable carbohydrate, but also the isolation of active ingredients located in the biopolymers.[67]

Table 5 provides an overview of ionic liquids used for the dissolution of wood as example or lignocellulosic biomass. Wood partially dissolves with [C_4mim]Cl/DMSO, [C_4mim]Cl and 1-allyl-3-methylimidazolium chloride[71, 72] but recent studies showed that [C_2mim]OAc might be more efficient for the complete dissolution of soft- and hardwood due to the higher basicity of the acetate anion.[73] According to Zavrel *et al.* [amim]Cl was more effective for the dissolution of wood chips than [C_2mim]OAc[74] whereas the research group of Welton reported that the dissolution of wood chips was best performed using [C_4mim]OAc.[22] The group of Ohno found a commercially available aqueous phosphonium ionic liquid solution (a 40% solution of tetrabutylphosphonium hydroxide ([P_{4444}]OH)) to be suitable for the dissolution of wood.[75] Generally it can be claimed that ionic liquids that dissolve cellulose can also dissolve wood.[74, 76]

Table 5: Selection of ionic liquids used for the dissolution of lignocellulose

Entry	Ionic liquid	Wood	Dissolution [%]	Conditions	Reference
1	[C_4mim]Cl	spruce	partially	110 °C	Fort, 2007[71]
2	[C_2mim]OAc	pine, oak	complete	90 °C	Sun, 2009[73]
3	[amim]Cl	beech	partially	90 °C	Zavrel, 2009[74]
4	[amim]Cl	spruce, eucalyptus	complete	75 °C	Zoia, Leskinen, 2011[77, 78]
5	aq. [P_{4444}]OH	pine, cedar, poplar	complete	r.t.	Abe, 2014[79]

Two main processes for the deconstruction and therefore dissolution of lignocellulosic biomass using ionic liquids are recently investigated: The first one is based on the solubilisation of the whole

biomass.[80, 81] An ionic liquid capable of dissolving cellulose such as [C_2mim]OAc is chosen. *Via* the addition of an anti-solvent the cellulose-rich pulp precipitates and biofuel can be produced after enzymatic depolymerisation and subsequent fermentation. The filtrate consisting of dissolved lignin, some hemicellulose and organic extractives represents a valuable source for sugars and organic compounds. To date no direct use of this solutions is reported.[67]

The other approach, the "Ionosolv Process" deals with chemical disruption without complete dissolution.[82] (Figure 13) Lignin and parts of hemicellulose are dissolved in an ionic liquid whereas cellulose and the residual hemicellulose remains undissolved. The undissolved cellulose is separated *via* filtration and can be turned into biofuel. Beneficial is the complete removal of lignin as filtrate, which also contains some hemicellulose and organic compounds that can easily be removed by extraction and aliphatic chemicals can be obtained. [83] Lignin can be precipitated by the addition of water and can be converted into aromatic chemicals. For a profitable process the ionic liquid has to be recovered but does not need to be fully dried, an important energy saving aspect. Since only a small number of papers deals with the Ionosolv Process optimization will be required.

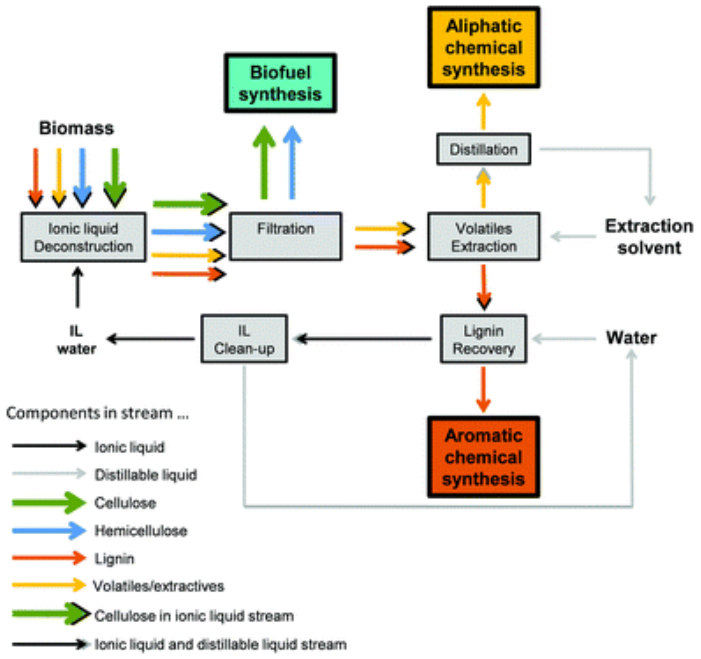


Figure 13: Flow scheme of the Ionosolv Process[67]

For both processes a quantitative removal of ionic liquid from cellulose is important, since unwanted deactivation of saccharification enzymes might occur[84] or fermentation can be inhibited.[85]

However the use of ionic liquids for lignocellulose deconstruction might become possible when the advantages of ionic liquids overcome the drawbacks, especially the price of ionic liquids has to be justified. Progress towards cheaper ionic liquids has already been made. The use of protic imidazolium ionic liquids represents a cheap alternative as they result from a simple acid-base reaction. The protic ionic liquid 1-butylimidazolium hydrogen sulphate was successfully applied for the fractionation of *Miscanthus giganteus* into a cellulose rich pulp and a lignin rich precipitate.[86] Furthermore ionic liquid recycling and therefore thermal stability plays an important role, since lignocellulose deconstruction using ionic liquids involves elevated temperatures, up to 100-190 °C.[87] Although ionic liquids are generally considered to be thermally stable at certain temperatures decomposition occurs. For

instance [C_2mim]Cl or [C_4mim]Cl were shown to be long-term stable at 120 °C but decomposition at 140 °C was observed.[88] The recovery of the ionic liquid [amim]Cl was successfully performed by Wang *et al.* after dissolution of 5 wt% pine without any loss of efficiency.[89] Another group reported a degree in saccharification yields.[81] The water content of (recovered) ionic liquids might result in e.g. lower solubility of cellulose and the cost of drying have to be considered as well. Drying is a highly energy intensive step and can be the bottleneck in such processes.

Alternatively, the application of aqueous ionic liquid solutions seems promising, as the combination of water and ionic liquid can dramatically reduce costs and avoid problems associated with the expensive drying process for anhydrous ionic liquids. Several authors have already reported lignocellulose fractionation in aqueous ionic liquid solutions such as the deconstruction of *Miscanthus giganteus*, pine (*Pinus sylvestris*) and willow (*Salix viminalis*) with aqueous solutions of 1-butyl-3-methylimidazolium methyl sulfate and 1-butyl-3-methylimidazolium hydrogen sulfate.[83]

Outstanding progress in this regard has been also made by the group of Ohno, who reported not only the dissolution of cellulose in aqueous phosphonium ionic liquids,[79] but also the dissolution of woody biomass at 60 °C[90] and the dissolution of wet wood at room temperature[75], which will be discussed in chapter 4.4.2 in detail.

2.3 Isolation of active ingredients using ionic liquids

While the use of ionic liquids for biomass dissolution for the purpose of (ligno-)cellulose processing or biofuel production has attracted tremendous interest, less attention has been paid to active ingredient isolation using ionic liquids. (Figure 14) Only a small part of published articles in the area of ionic liquids for biomass processing deals with the use of ionic liquids for the isolation of biological interesting biomolecules.

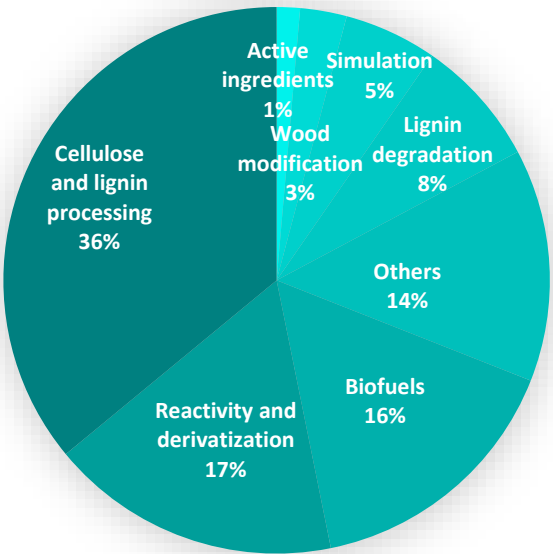


Figure 14: Literature survey on ionic liquids and biomass February 2015, performed on SciFinder™

The use of ionic liquids for the dissolution or pretreatment of biomass has demonstrated several advantages compared to conventional solvents that can also be beneficial for the isolation of active ingredients from plant material. A few years after the discovery that ionic liquids can dissolve biomass the first isolation of an active ingredient from crude biomass was reported by Lapkin *et al*. in 2006.[91]

Pioneering work was reported for the extraction of the anti-malaria drug artemisinin from plant material by alternative solvents including ionic liquids.[91-93] The extraction of opium alkaloids from plant matter using aqueous ionic liquid solutions was also described by Li *et al*. in 2005.[94] Figure 15 represents a schematic time line from the first dissolution of biomass to the isolation of active ingredients.

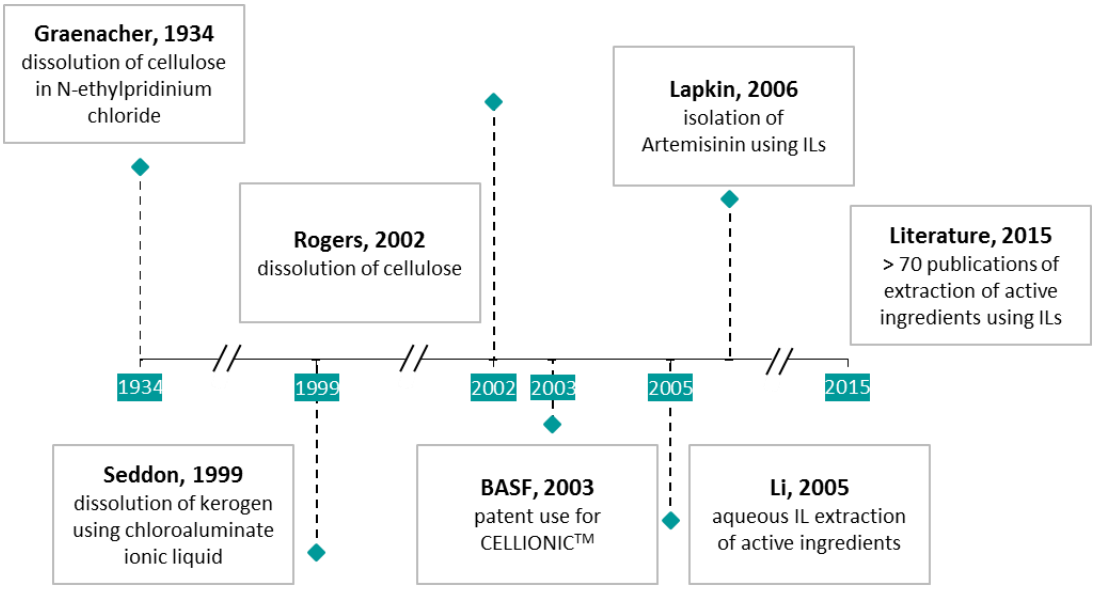


Figure 15: Schematic timeline of the evolution from biomass dissolution to isolation of active ingredients from biomass

Additionally to their unique solvent properties and potential environmental benefits compared to organic solvents, the ability of ionic liquids to swell or dissolve biomass can lead to a better access to the valuable ingredient embedded in biopolymer matrices (Figure 16).[18] Apart from that the waste use of volatile organic solvent at reflux conditions for long hours is therefore avoided. Due to the outstanding dissolution properties of ionic liquids their extraction processes can be performed at ambient temperature in short extraction times.

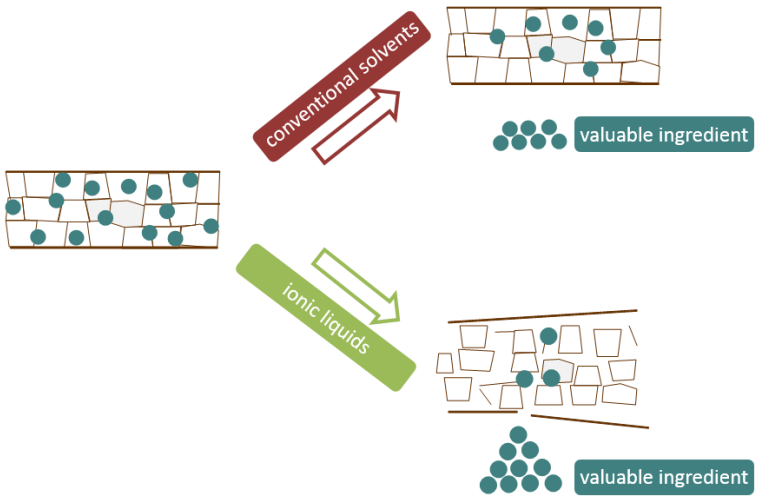


Figure 16: Ionic liquid strategy for the leaching of valuable ingredients from plant matter

To date, most examples for active ingredient leaching from biomass are performed on analytical scale, and HPLC analysis has been established as method of choice for the quantification of the valuable ingredient in the presence of ionic liquids. Only a small part of all papers deal with the actual isolation of the active ionic liquids, thus indicating that the separation of ionic liquid, active ingredient and biomass, as well as the recovery and possible recycling of the ionic liquid is the real challenge (Figure 17, left.).

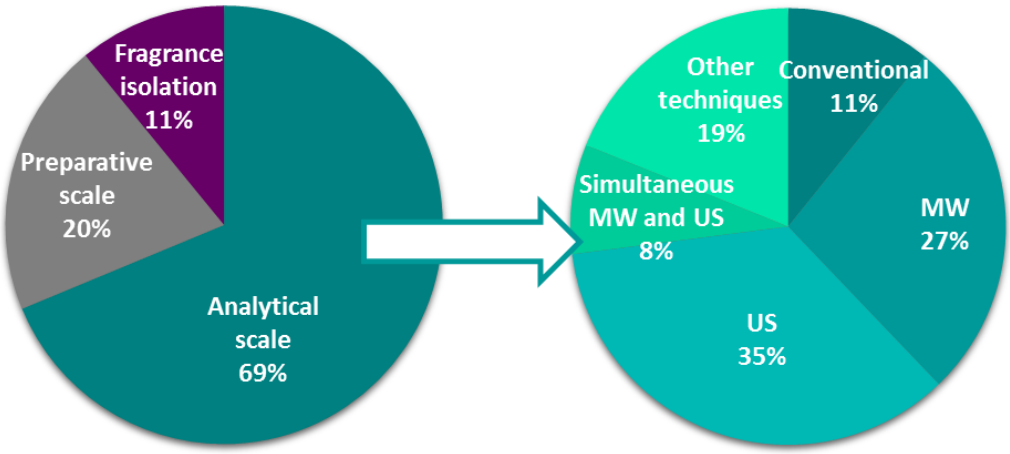


Figure 17: Left: overview of active ingredient isolation from biomass using ionic liquids; right: analytical scale in details (MW = microwave, US = ultrasound)

Although ionic liquids can improve extraction yield and efficiency compared to volatile organic compounds, biomass dissolution often requires long reaction times at elevated temperature that are always associated with high energy consumption. Consequently, different extraction technologies apart from conventional solvent extraction have evolved. (Figure 17, right). Considering the ionic nature of ionic liquids that makes them susceptible to interaction with electromagnetic fields, it is obvious that the application of microwave energy might be a highly useful tool not only for synthesis in or of ionic liquids, but also for the dissolution of biomass and for the extraction of valuable ingredients from plant materials.[95] Many examples successfully demonstrate the advantageous application of microwave irradiation (MW) for the extraction of active ingredients resulting in shorter reaction times and higher efficiency. Apart from the use of microwave energy, ultrasound (US) can be applied for the extraction of active ingredients, since US can break plant tissue allowing the solvent to penetrate through plant tissue.[96-100]

Figure 18 gives an overview of the extracted compounds using ionic liquid technologies. As it can be seen a variety of different natural products could be extracted thus underlining the diversity of ionic liquids. In most cases alkaloids were extracted using ionic liquid-based techniques. A significant part of papers also deals with the extraction of terpenoids that represent the largest group of active ingredients in nature. Apart from them flavonoids, different aromatic compounds, lipids, lignans or other class of potential pharmaceuticals were successfully extracted using either pure ionic liquids or their solutions as discussed in the following chapters in detail.

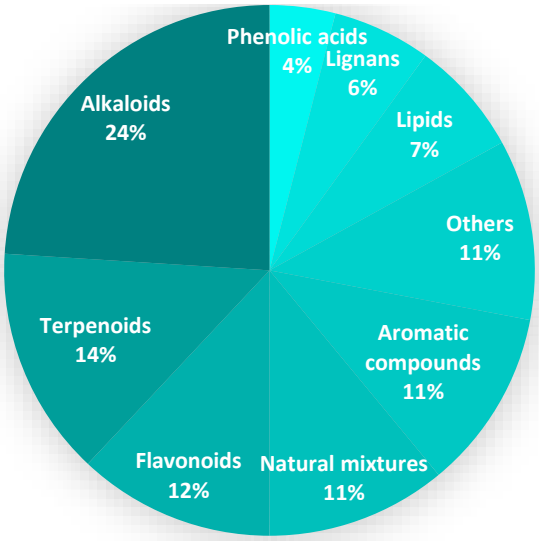


Figure 18: Distribution of extracted natural compounds from plant materials using ionic liquids based techniques[101]

2.3.1 Extraction with pure ionic liquids

The extraction of active ingredients from plant matter can be done either with pure ionic liquids or their mixtures with a co-solvent. Due to large number of potential extractant, the evaluation of ionic liquids for active ingredients isolation requires efficient screening procedures. In the past years, HPLC-based strategies for the extraction and quantification of several active pharmaceutically ingredients with ionic liquids have been reported. Typically, a sample of ground or powdered biomass is completely or partially dissolved in the ionic liquid and stirred for a certain time at elevated temperature. After dilution with a co-solvent such as MeOH, EtOH or H_2O biopolymers precipitate and can be separated. The remaining solution containing both ionic liquid and the active ingredient is further analyzed to quantify the active ingredient. Table 6 shows an overview of the experiments performed using pure ionic liquids without additional co-solvents.

Table 6: Extraction of valuable ingredients using pure ionic liquids

Entry	Active ingredient	Biomass	Ionic liquid[a]	Conditions[b]	Reference
1	artemisinin	*Artemsia annua*	[DMEA]oct, [BMOEA]N(Tf)$_2$	conv., 30 min	Lapkin 2006[91]
2	different alkaloids	various biomass of plant or fungal origin	[BMOEA]Cl	conv., r.t., 4h, slr. 3:20	Walker 2007[102]
3	anthraquinone	*Rheum officinale*	[C$_n$mim]Y n = C$_1$-C$_{10}$. Y = BF_4^-, PF_6^-, OAc^-, $CF_3SO_3^-$ or N(Tf)$_2$	conv.	Pei 2008[103]
4	paeonol	*Cynanchum paniculatum*	[C$_4$mim]Cl	MW, 70 °C, 1 min slr. 7.3:1	Jin 2011/12[104, 105]
5	shikonin, dimethylacrylshikonin	*Arnebia euchroma*	[C$_6$mim]BF_4	US, 20 °C, 5 min	Xiao 2011[106]
6	glabridin	*Glycyrrhiza glabra L.*	[C$_6$mim]N(Tf)$_2$	US, 30 °C, 40 min, pH=7	Li 2012[107]

[a] Only the best performing ionic liquid is listed, aqueous solution = aq.; [b] solid:liquid-ratio = slr. [g/mL]; MW = microwave irraditation; US = ultrasound

In a benchmark contribution in 2006 the British company Bioniqs Ltd. extracted the important antimalarial drug artemisisin from *Artemsia annua* and compared several alternative isolation techniques including ionic liquids, supercritical carbon dioxide, VOCs and fluorinated solvents for the extraction. Although artemisinin is reasonably soluble in organic solvents, its extraction with nonpolar solvents suffers from the co-extraction of essential oils, chlorophylls and waxes that it must be separated afterwards. It was shown that extraction with the ionic liquids *N*,*N*-dimethylethanolammonium octanoate and bis(2-methoxyethyl)ammonium bis(trifluoromethane)sulfonimide (Figure 19) gave higher extraction efficiencies compared to the conventional solvents ethanol and hexane and thus proved that ionic liquids are a promising class for the extraction.[91]

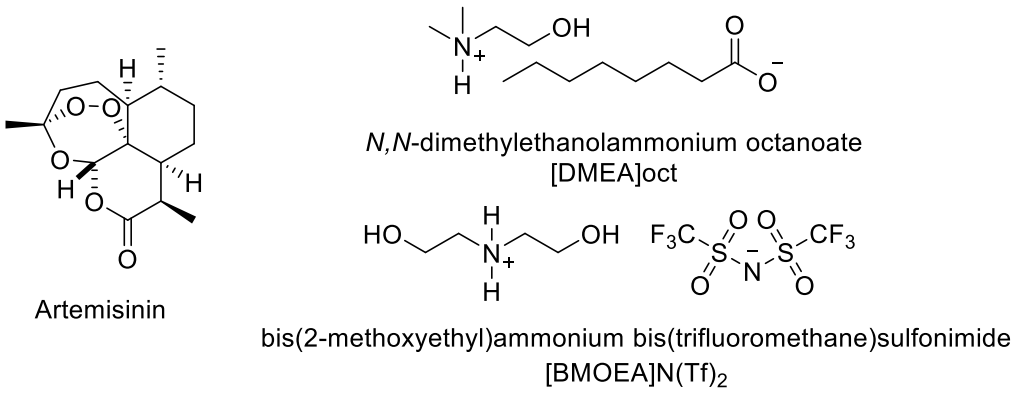


Figure 19: Ionic liquids used for the extraction of artemisinin[91]

2.3.2 Extraction of active ingredients using aqueous-ionic liquid systems

The concept of aqueous-ionic liquid systems for biomass dissolution has been recently explored, and promising work on the cellulose pretreatment with aqueous solutions of ionic liquids has been reported by Welton *et al*.[83] While ionic liquids often proved to be superior to conventional solvents in terms of extraction yield and purity, their usage is still associated with a major drawback: Ionic liquids are still relatively expensive – at least compared to conventional organic solvents – which may restrict their large-scale application as a bulk solvent. Consequently, aqueous solutions of ionic liquids represent a cheaper and interesting alternative. As a compromise between economic and environmental criteria the use of aqueous-ionic liquid systems as reaction media for synthesis and catalysis has recently attracted interest.[108] (Figure 20)

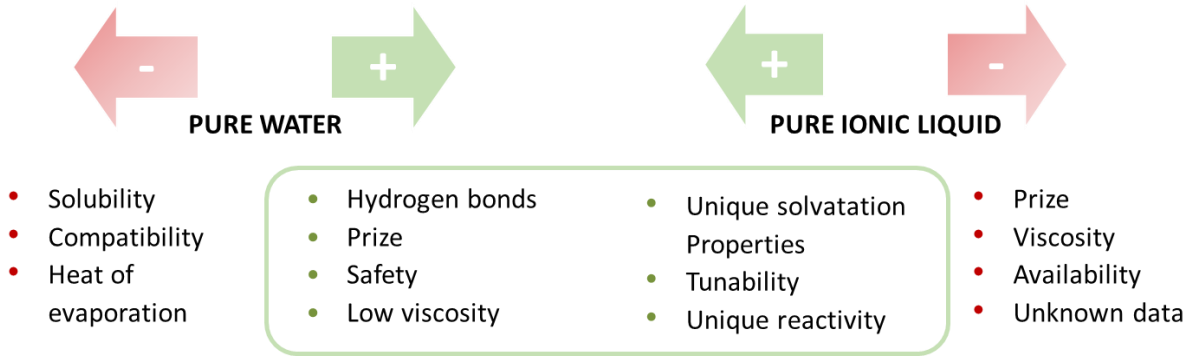


Figure 20: Compromise of properties of ionic liquid aqueous solutions[109]

The use of aqueous-ionic liquid solutions or micellar systems is particularly prominent in combination with microwave-assisted extraction. Apart from the reduction of ionic liquid required for extraction, the application of aqueous-ionic liquid solution can reduce problems associated with the high viscosity of some ionic liquids that can hinder their application as bulk solvent in microwave-assisted extraction processes. Consequently, a surprisingly large number of leaching procedures of active ingredient from plant materials with aqueous ionic liquids exist that are summarized in Table 7 for microwave irradiation based extraction. Generally relatively high concentrations of ionic liquid solution were used (0.5 M – 1.2 M) probably due to solubility limitation of the extracted compounds in aqueous solution when short chain alkyl-methylimidazolium salts, *i.e.* [$C_n$mim]Cl with n<6 or related ionic liquids were used. The application of long-chain derivatives could dramatically reduce the concentration of ionic liquids in water due to micelle formation. Investigations on the behavior of ionic liquids in the presence of water showed that certain ionic liquids can form aggregates in aqueous solution.[110-113] This is the case for long-chain 1-alkyl-3-methylimidazolium chloride salts [$C_n$mim]Cl with n = 8 - 18, where the apolar side chain stays in contact with other cations and micelles are formed.

Table 7: Aqueous ionic liquid solution for the extraction of active ingredients using microwave irradiation

Entry	Active ingredient	Biomass	Ionic liquid [a]	Extraction parameters [b]	Reference
1	*trans*-resveratrol	*Rhizma Polygoni Cuspidati*	aq. $[C_4mim]Cl$	MW, 60 °C, 10 min., slr. 1:20	Du 2007[114]
2	polyphenolic alkaloids	*Nelumbo nucifera* Gaertn.	aq. $[C_4mim]BF_4$ and $[C_6mim]BF_4$	MW 0.5 M, 90 s, slr. 1:10	Lu 2008[115]
3	polyphenols, *e.g.*	*Psidium Guajava* leaves, *Smilax china* tubers	aq. $[C_4mim]Br$	MW; 60-70 °C, 10 min, slr. 1:20	Du 2009[116]
4	rutin	*S. chinensis*, *Flos Sophorae*	aq. $[C_4mim]Br$ and $[C_4mim]OTs$	MW, 70 °C, 12 min, slr. 1:25 for *S. chinensis* and 60 °C, 8 min, slr. 1:35 for *F. Sophorae*	Zeng 2010[117]
5	3 alkaloids: N-nornuciferine, O-nornuciferine, and nuciferine	lotus leaf *Nelumbo nucifera* Gaertn.	aq. $[C_6mim]Br$	MW; 1.0 M, 280W, 2 min, slr. 1:30	Ma 2010[118]
6	dehydrocavidine	*corydalis saxicola*	aq. $[C_6mim]Br$	MW; 70 °C, 10 min, slr. 20:1	Du 2010[119]
7	myricetin, quercetin	*myrica rubra* leaves	aq. $[C_4mim]HSO_4$	Hydrolysis via MW, 2.0 mol/L [bmin][HSO_4]slr. 30:1 70 °C, 10 min.	Du 2011[120]
8	camptothecin and 10-hydroxycamptothecin	*Camptotheca acuminata*	aq. $[C_6mim]Br$	MW; 0.8-1.2 M, 8-12 min, slr. 1:8-1:12	Wang 2011[121]
9	podophyllotoxin	Chinese herbal medicine	aq. $[C_4mim]BF_4$, $[C_{10}mim]BF_4$ (*D. versipellis/S. hexandrum*); $[amim]BF_4$ (*D. sinensis*)	MW; For *D. versipellis/S. hexandrum*: c = 0.8 g/mL, 60 °C, 10 min, slr. 1:100; For *D. sinensis*: c = 0.6 g/mL, 50 °C, 15 min, slr. 1:100	Yuan 2011[122]
10	glaucine	*Glaucium flavum*	aq. $[C_4mim]$acesulfamate	conv., 1 M, 80 °C, 1 h, slr. 1:40	Bogdanov 2012[123]
11	five anthraquinones	Polygonum cuspidatum	aq. 0.6 M $[C_8mim]Br$	MW, 31 °C, 8 min	Yang 2014[124]
12	Praeruptorin A	Radix peucedani	aq. guanidine ILs	MW	Ding 2014[125]

[a] Only the best performing ionic liquid is listed, aqueous solution = aq.; [b] solid:liquid-ratio = slr. [g/mL]; MW = microwave irraditation

Table 8 shows the use of aqueous ionic liquid solutions in combination with ultrasound application or the combination of ultrasound and microwave irradiation. A considerable number of papers presents the extraction of active ingredients using hydrophilic imidazolium ionic liquids. An elegant way was presented Bi *et al*. (Entry 7) for the extraction of cryptotanshinone, tanshinone I and tanshinone II A from *Salvia Miltiorrhiza* Bunge. After extraction using aqueous solution of $[C_8mim]Cl$ HPF_6 was added to form $[C_8mim]PF_6$ and the active ingredients remained in the ionic liquid phase. A second small layer consisting of proteins was formed above the ionic liquid layer.[126]

Table 8: Aqueous ionic liquid solution for the extraction of active ingredients using ultrasound irradiation (in combination with microwave irradiation)

Entry	Active ingredient	Biomass	Ionic liquid [a]	Extraction parameters [b]	Reference
1	piperin	white pepper	aq. $[C_4mim]BF_4$	US; 2 M, 30 min, slr. 1:15	Cao 2009[127]
2	tanshinones	*Salvia miltiorrhiza bunge*	aq. $[C_{14}mim]Br$	US; 50 mM, r.t., 30min	Wu 2009[128]
3	fangchinoline and tetrandrine	*Stephaniae tetrandrae*	aq. solutions of $[C_4mim]BF_4$	US; 150 W, pH = 9.8	Zhang 2009[129]
4	aesculin and aesculetin	Cortex fraxini	aq. $[C_4mim]Br$	US; 0.5-1M, 250 W, 30-50 min, slr 2:3,	Yang 2011[130]
5	three terpenoid indole alkaloids vindoline, catharanthine and vinblastine	*Catharanthus roseus*	aq. [amim]Br	US; 0.25-0.75 M, 250 W, 30 min, slr. 1:10,	Yang 2011[131]
6	biphenyl cyclooctene lignans	*Schisandra chinensis* Baill	aq. $[C_{12}mim]Br$	US, 0.8 M, 150-250 W, 30min, slr. 1:12	Ma 2011[132]
7	cryptotanshinone,tanshinone I and tanshinone II A	*Salvia Miltiorrhiza* Bunge	aq. $[C_8mim]PF_6$/ *in situ* ion exchange to $[C_8mim]PF_6$	US; 0.5M, 105 W, 80 min, slr. 1:40, 20µL/mL HPF_6	Bi 2011[126]
8	phenolic compounds	*Laminaria japonica* Aresch	aq. $[C_4mim]BF_4$	US; 0.5 M, 200 W, 60 min, pH = 1.25	Han 2011[133]
9	carnosic acid, rosmarinic acid	*Rosmarinus officinalis*	aq. $[C_4mim]BF_4$	US; 1 M, 150-250 W, 30 min, slr. 1:20	Zu 2012[134]
10	different phenons	cynanchum bungei Decne	aq. $[C_6mim]BF_4$	US; 175 W, 25 °C, 50 min, slr. 1:35	Sun 2013[135]
11	anthraquinone	rhubarb	aq. soltutions $[C_4mim]Br$	US/MWAE; 2 M, 500 W MW, 2 min, slr.1:15	Lu 2011[136]
12	phenolic compounds	*Arctium lappa L.*,burdock leaves	aq. soltutions $[C_4mim]Br$	US/MWAE; 1.5 M, 50 W US, 400 W MW, 30 s, slr. 1:20,	Lou 2012[137]
13	tannins	*Galla chinensis*	aq. $[C_4mim]Br$	US/MWAE; 2.5 M, 400 W MW, 1 min, slr. 1:15	Lu 2012[138]
14	alkaloids	*Uncaria rhynchophylla*	aq. $[C_4mim]BF_4$	US, 0.5 M, 8 min slr. 1:100	Liu 2013[139]
15	ginsenosides	ginseng root	aq. $[C_3mim]Br$	US, 0.3 M, 20 min slr 1:10	Lin 2013[140]
16	quercetin	*Suaeda glauca*	aq. $[C_2mim]Br$	US, 0.5 M, 60 °C slr. 1:15	Wang 2014[141]
17	flavone, saponin	*Herba cynomorii*	hydrophilic ILs	US	Luo 2014[142]
18	flavoinides	*Citrus aurantium*	unkown	US	Cao 2015[143]
19	tanshinones	*Salvia miltiorrhiza*	aq. $[C_nmim]BF_4$, n=4,6,8	US, 50 °C, 5 min	Wang 2015[144]

[a] Only the best performing ionic liquid is listed, aqueous solution = aq.; [b] solid:liquid-ratio = slr. [g/mL]; MW = microwave irraditation; US = ultrasound

2.3.3 Other extraction strategies

Apart from conventional solvent extraction, or microwave assisted heating and ultrasound treatment for biomass dissolution with ionic liquid, some other techniques have been reported for the leaching of valuable ingredients from biomass as described below in detail (Table 9).

Table 9: Other strategies for ionic liquid-assisted extraction of active ingredients

Entry	Active ingredient	Biomass	Ionic liquid/Technique	Reference
1	opium alkaloids	*Pericarpium papaveris*	$[C_4mim]Cl$/ATPS	Li 2005[94]
2	hyperin, isoquercitrin	*Apocynum venetum*	$[C_4mim]BF_4$/ATPS	Lin 2012[145]
3	rutin and quercitrin	*Flos sophorae Immaturus, Crateagus pinnatifida Bunge, Hypericum japonicum Thunb* and *Folium Mori*	$[C_4mim]Cl$/IL-PLE	Wu 2012[146]
4	anthraquinone derivatives	Radix et Rhizoma Rhei	$[C_6mim]PF_6$/DLLME	Zhang 2010[147]
5	liquiritin, glycyrrhizic acid	Licorice	imidazolium based IL/SPE	Tian 2009[148]
	alkaloids	*Rhizoma coptidis* and *Cortex phellodendri*	*in situ* formation of hydrophobic ILs[#]	Cao 2013[149]
6	different tanshinones	*Salvia miltorrhiza* Bunge	imprinted functionalized IL-modified silica	Tian 2011[150]
7	different tanshinones	*Salvia miltorrhiza* Bunge	IL-modified porous polymer	Tian 2011[151]
8	tannins	*Galla chinensis*	$[C_4mim]Br$/adsorption	Lu 2013[152]
9	oxymatrine and matrine	*Sophora flavescens* Ait	silica-confined ILs/adsorption	Bi 2012[153]

[#] Paper written in Chinese.

Ionic liquid-based aqueous two-phase systems (ATPS)

Aqueous two-phase systems (ATPS) are typically generated by mixing aqueous solutions of two structurally different polymers or by mixing one polymer with certain salts at high concentration.[154] ATPS are often considered as environmentally friendly separation systems where large amounts of organic solvents for purification, extraction and enrichment are avoided and provide an interesting strategy for active ingredient isolation from plant materials.

Hydrophilic ionic liquids can form aqueous two-phase systems when contacting with concentrated solutions of water-structuring salts.[155] In 2005 Li *et al.* used a ($[C_4mim]Cl$)/K_2HPO_4 system for the extraction of opium alkaloids from *Pericarpium papaveris*, a traditional Chinese medicine. After extraction of codeine and papaverine with aqueous $[C_4mim]Cl$ solution, K_2HPO_4 was added as it led to effective phase separation and to the appropriate that allowed determination of the opium alkaloids. With higher temperatures the concentration of $[C_4mim]Cl$ in the top phase decreased without reducing the extraction efficiencies, thus allowing a wide temperature range for the extraction of codeine and papaverine. The extraction yields were compared with those from liquid-liquid-extraction (LLE) showing similar extraction yields and shorter extraction times without use of nonvolatile solvents.[94]

ATPS were also applied from Lin *et al.* in 2012 for the microwave-assisted extraction of the blood pressure lowering and anti-inflammatory hyperin and isoquercitrin from *Apocynum venetum*.[156, 157] The system $[C_4mim][BF_4]/NaH_2PO_4$ was chosen and compared to conventional ATPS-technology. Initially, the extraction efficiency of aqueous solutions of $[C_4mim]BF_4$ and $[C_8mim]BF_4$ was investigated, indicating a strong influence on the concentration of the aqueous solution as well as of the temperature. Comparison with water, methanol and ethanol-water mixtures showed that aqueous-ionic liquids solutions gave higher extraction yields. After microwave-assisted extraction of the herb with aqueous ionic liquid solution for 10 min at 30 °C, the extract was centrifuged and the supernatant liquid separated. The preconcentration of hyperin and isoquercitrin from the extract in ATPS was investigated using $[C_4mim]BF_4$ and NaH_2PO_4. While adding more ionic liquid increased the extraction yield, it also decreased the preconcentration of the active ingredients in the upper phase. The optimal concentration was 0.2 g ionic liquid ml^{-1} aqueous solution with preconcentration efficiencies of 66.4% for hyperin in 65.7% for isoquercitrin.[145]

Ionic liquid-based pressurized liquid extraction (IL-PLE)

The active components rutin and quercetin were extracted from *Flos sophorae Immaturus, Crateagus pinnatifida* Bunge*, Hypericum japonicum* Thunb and *Folium Mori* using ionic liquid-based pressurized liquid extraction. Dried sample, diatomaceous earth as supporting material and 1-alkyl-3-methylimidazolium-based ionic liquids aqueous solution were heated to 120 °C and pressurized at 1500 psi for 5 min. After dilution of the obtain extract with water the solution was filtered and an aliquot was analyzed *via* HPLC equipped with chemiluminescence detection. Compared with conventional solvent extraction or with ultrasound-assisted strategies, this technology using $[C_4mim]Cl$ aqueous solutions achieved the highest extraction efficiency in the shortest time and could also outperform conventional solvents such water and methanol.[146]

Solid-phase extraction (SPE) and dispersive liquid-liquid extraction (DLLE)

Tian *et al*. used solid-phase extraction (SPE) for the extraction of liquiritin and glycyrrhizic acid from licorice with an ionic liquid-based silica. After extracting licorice with methanol, the extracts were loaded onto the SPE cartridge consisting of the ionic liquid-absorbed silica particles, washed and eluted for HPLC analysis. Comparison with conventional C18 sorbent showed that the ionic liquid-modified material exhibited higher selectivity for the two active ingredients.

In a related paper ionic liquid-modified silica materials were developed for the extraction of different tanshinones from the traditional medicinal herb *Salvia miltorrhiza* Bunge.[150] Molecular imprinted ionic liquid modified silica was prepared with 9,10-phenantrenequinone as imprinting template and used for the separation of cryptotanshinone, tanshinone I and tanshinone IIA in the herb extract with high selectivity of the three compounds.

Later on, this group also used ionic liquid modified porous polymers for the extraction of tanshinones from the *Salvia miltorrhiza* Bunge.[151] Different side-chain functionalized imidazolium-based ionic liquids were used for the preparation of ionic liquid modified polymers that were again imprinted with 9,10-phenantrenequinone as template. The obtained polymers were then applied as sorbents for tanshinones from a methanol herb extract. It was shown that ionic liquid-modified molecular

imprinted polymers provide good absorbents for the tanshinones, and the sorbent with carboxylic acid functionalities in the side chain showed the highest selectivity for the target compounds.

Dispersive liquid-liquid microextraction for active ingredient isolation is based on a ternary solvent system which consists of disperser solvent, extraction solvent and aqueous sample and was applied by Rezaee *et al*.[158]. The herb *Radix et Rhizoma Rhei* contains the anthraquinone derivatives emodin, chrysophanol, rhein, aloe-emodin, physcion, and their glucosides which are regarded as the major active compounds with antifungal,[159] antiviral,[160] antioxidant,[161] anticancer[162] and anti-mutagenicity[163, 164] activities. Hydrophobic ionic liquids, e.g. $[C_6mim]PF_6$ were used to replace conventional organic solvents as extraction solvent and the active ingredients were transferred from aqueous solution to the ionic liquid phase by assistance of temperature.[147]

Molecular imprinted anion-functionalized poly(ionic liquids) were also used for the separation and quantification of flavonoids myricetin, quercitrin and amentoflavone with antihypertensive, anti-inflammatory and antiviral activities from *Chamaecyparis obtuse*. The poly(ionic liquid) based sorbents were functionalized with different anions, e.g. BF_4^-, PF_6^-, $N(Tf)_2^-$, lactate or sulfonate *via* anion metathesis and applied as sorbent for multi-phase dispersive extraction (MPDE). Plant powder and molecular imprinted anion-functionalized poly(ionic liquids) were placed in an empty cartridge, rinsed and eluted to finally analyze the content of flavonoids in *C. obtusa* . In a similar manner, Bi *et al*. could also apply hybrid poly(ionic liquid)-bonded silica in combination with an ionic liquid solution for multi-phase dispersive extraction (MPDE) for extraction, separation and quantification of the flavonoids myricetine and amentoflavone from *Chamaecyparis obtuse*.

Silica-confined ionic liquids

In 2012, Bi *et al.* extracted *Sophora flavescens* Ait. using silica-confined ionic liquids (SiILs) for the isolation of the active ingredients oxymatrine and matrine.[153] The evalution of the SiILs was performed *via* measurement of the adsorbed active ingredient: SiIL was placed in a flask, standard solutions of the active ingredients added, the mixture was shaken for 30 min and the supernatant collected and filtered. The sample was analyzed *via* HPLC and the amount of unadsorbed active ingredients was detected. With increasing alkyl chain length the extraction yield decreased, and best results were obtained with a protic derivative. Different anions were tested with the best cation and Cl^- still gave the highest extraction yield compared to BF_4^-, PF_6^- and $N(Tf)_2^-$ which might be related to its good water miscibility. Furthermore conventional adsorbents such as C18 and $SilprNH_2$ were applied, but gave lower yield than the Cl^- SiIL.

The extraction of the roots was performed using 50 mL of water for 4 h. The extract was mixed with the SiIL to absorb the active ingredients. Finally, the separation of oxymatrine was achieved washing the cartridges with 1 mL water for the removal of interferences. The active ingredient was then eluted using either methanol, or acetonitrile or ethanol. The SiIL was regenerated after washing with triethylamine/methanol and drying at 60 °C and could be reused for 4 cycles with only a slight loss in performance.

2.3.4 Isolation, scale-up and recycling strategies

While many ionic liquids can readily dissolve biomass and are thus able to efficiently extract active ingredients, the previous chapter focused on the extraction of different active ingredients using ionic liquids on an analytical scale. The scaled isolation of the valuable ingredient remains challenging and fewer examples exist in literature. The scale-up and isolation of active ingredients faces the problem of separating the ionic liquid from the bioactive component, but also the challenge of recovery and recycling of the ionic liquid that might be mandatory for a future application on industrial scale. Different strategies for the separation of the active ingredient and recovery of the ionic liquid have been developed that typically rely on extraction or precipitation of the active ingredient with co-solvents. The ionic liquid is typically recovered as solution in water or in water/ethanol mixtures and has to be isolated *via* evaporation of the volatile co-solvents. (Figure 21)

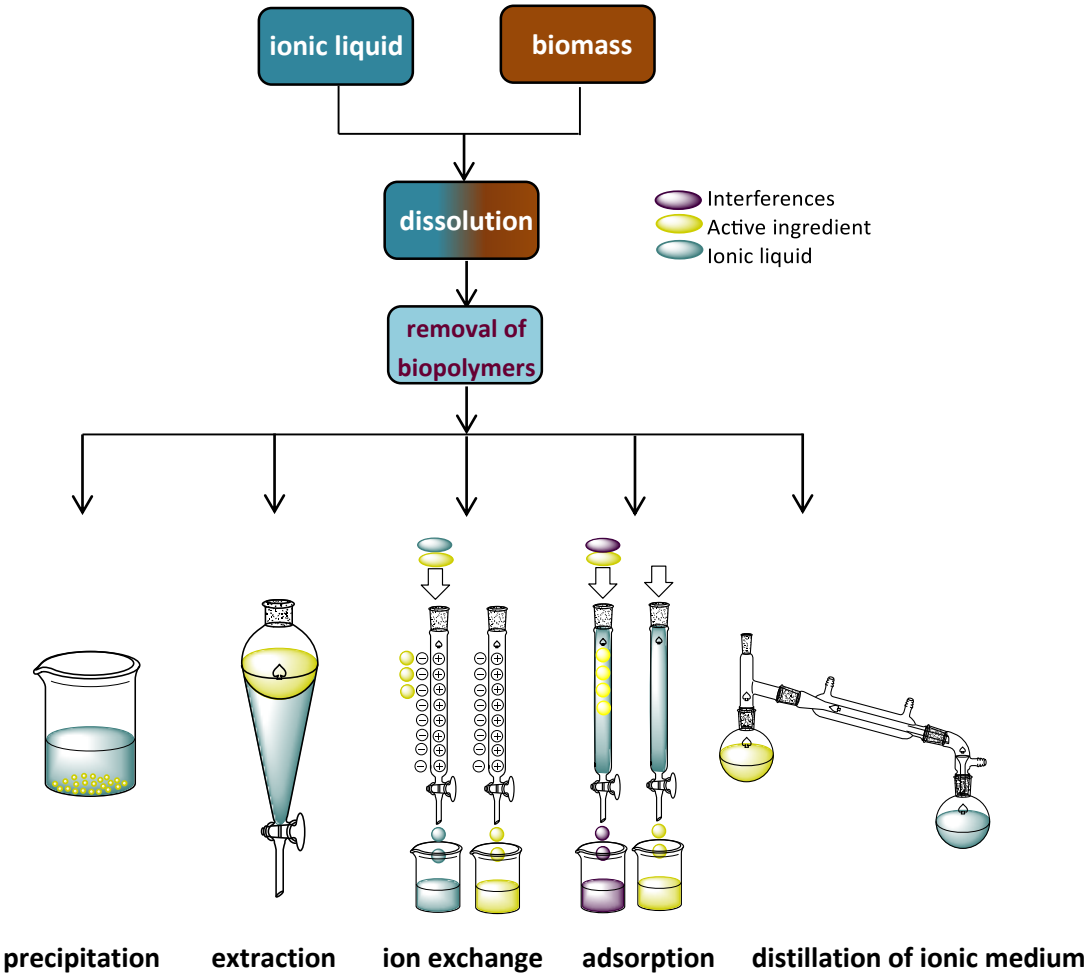


Figure 21: Different isolation strategies for active ingredients after biomass dissolution in ionic liquids

Table 10: Isolation of active ingredients using ionic liquid strategies

Entry	Active Ingredient	Biomass	Ionic liquid[a]	Extraction conditions	Isolation strategy	Reference
1	artemisinin	*Artemisia annua*	[chol]OAc	conv.; r.t. 30 min	precipitation	Bioniqs Ltd. 2008[93]
2	betulin	birch bark	$[C_2mim]OAc$	MW; 15 min, 100 °C	precipitation	Ressmann 2012[165]
3	artemisinin	*Artemisia annua*	aq. $[C_2mim]X$, X=Br, Cl, I	US; 20-60°, 5-60 min1slr. 1:5-1:70	extraction	Zhao 2009[166]
4	shikimic acid derivatives	*Illicium verum,* star anise	Brønsted-acidic ILs; $[HSO_3C_4mim]HSO_4$	conv.; 80 °C, 24 h, MW; 30 min, 100 °C	extraction	Ressmann 2011[167]
5	various lactones	*Ligusticum chuanxiong* Hort.	protic ILs, DMCEAP, DMHEEAP	MW; 300 W, 160 °C, 10 min	extraction	Yansheng 2011[168]
6	caffeine	*Gurana seeds*	aq. $[C_4mim]Cl$	conv.; 2.34 M, 70 °C, 30 min	extraction	Claudio 2013[169]
7	piperine	*Black pepper*	aq. $[C_{12}betaine]Cl$, $[C_nmim]Cl$, n= 10,12,14	conv.; r.t., 3 h,	extraction	Ressmann 2013[170]
8	(*S*)-glaucine	*Glaucium flavum* Cr.	aq. $[C_4mim]Ace$	conv. 80 °C, 30 min	enrichement/ extraction	Bogdanov 2015[171]
9	shikimic acid	Ginkgo biloba leaves	$[C_4mim]Cl$	conv.; 100 – 150 °C, 1h	anion exchange resin	Usuki 2011[172]
10	shikimic acid	*Illicium verum,* star anise	$[C_2mim]OAc$	MW; 15 min, 100 °C	anion exchange resin	Zirbs 2013[173]
11	tannins	*Acacia Catechu, Terminalia Chebula*	DIMCARB	conv.; r.t. 16 h	distillation of ionic liquid	Chowdhury 2010[26]

[a] Only the best performing ionic liquid is listed. $[C_4mim]Ace$ = 1-butyl-3-methyl imdidazolium acesulfamate

2.3.4.1 Crystallization/precipitation of the active ingredient

Already in 2008, Bioniqs Ltd. developed an isolation strategy for artemisinin from *Artemisia annua*. The conventional isolation is performed using hexane at reflux conditions, which suffers from co-extraction of impurities. In the novel ionic liquid-based strategy, biomass was extracted with choline acetate in approx. 10 wt% solution, allowing an extraction efficiency of 71% artemisinin. The active ingredient can be precipitated by the addition of water, allowing to isolate artemisinin with an overall yield of 69%. Furthermore, the ionic liquid can be recycled by the removal of water the solvent. In comparison to the conventional extraction with hexane, the ionic liquid process represents a safer, higher yielding process with a fully biodegradable ionic liquid.[93]

Betulin is a naturally occurring triterpene alcohol with a lupane skeleton found in birch bark, but also in roots or leaves of some ash trees.[174, 175] Like many members of the lupane family, betulin exhibits versatile pharmaceutical activity, including antitumor, anti-HIV, antiviral, antibacterial, anti-inflammatory, and antimalarial properties.[176, 177] In the current industrial isolation processes, betulin is extracted with high boiling hydrocarbon solvents, chlorinated solvents or with water azeotropes of

alcohols.[178-181] This is not only a rather time-consuming process with a limited yield of 10-20% but suffers from co-extraction of many impurities thus requiring several tedious purification steps to obtain betulin in pharmaceutical purity.

Comparison of organic solvents and ionic liquids for the extraction of the pharmaceutically active steroid betulin from birch bark showed significantly improved extraction yield for a range of ionic liquids. A simple and scalable isolation procedure allowed isolating betulin in excellent purities of up to 98% and recovery of the ionic liquid. Based on the high purity and isolation yield, this strategy provides a single-step, higher yielding and efficient strategy for the isolation of betulin. (Figure 22)[165]

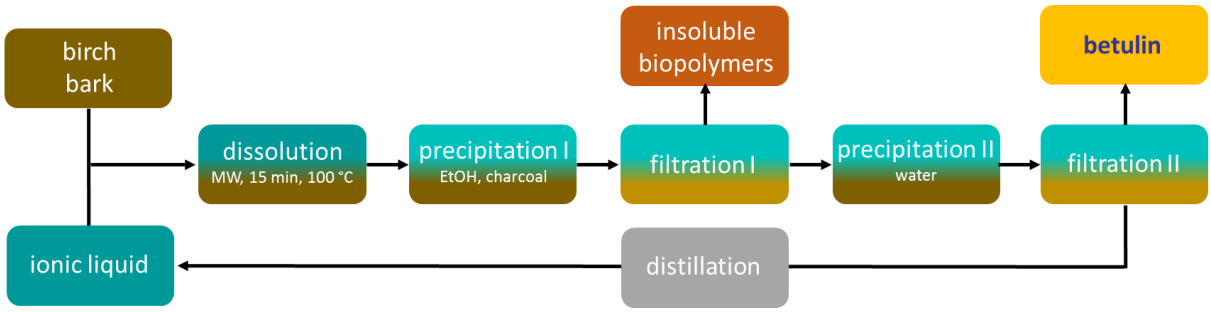


Figure 22: Isolation procedure for the pharmaceutically active ingredient betulin

2.3.4.2 Extraction of the active ingredient with a co-solvent

In 2009 Zhao *et al.* invented a patent which describes the isolation of artemisinin from *Artemisia annua* using aqueous [C_2mim]X (X=Br, Cl, I) solutions and ultrasonic irradiation. The extract was further treated with organic solvents to obtain artemisinin and purified *via* column chromatography, recrystallization while the ionic liquid was recovered.[166]

In the current manufacturing process, the synthesis of Tamiflu™ involves the formation of shikimic acid ethyl ester followed by a ketal intermediate that is further transferred into the final drug.[182] The initial step in the industrial process is typically done using stoichiometric amounts of toxic and corrosive thionyl chloride for the generation of anhydrous hydrochloric acid as catalyst. The toxicity of thionyl chloride as well as the formation of greenhouse gases after hydrolisation do not only raise serious safety and environmental concerns, but requires a more involved manufacturing process. An ionic liquid-based strategy was developed for the reactive dissolution of star anise seeds using different Brønsted-acidic ionic liquids as solvent and reaction media towards the isolation of important pharmaceutical intermediates. (Figure 23)[167] Based on Brønsted-acidic ionic liquids, this procedure provides a single-step, higher yielding and environmentally benign strategy towards the synthesis of the anti-influenza drug Tamiflu™.

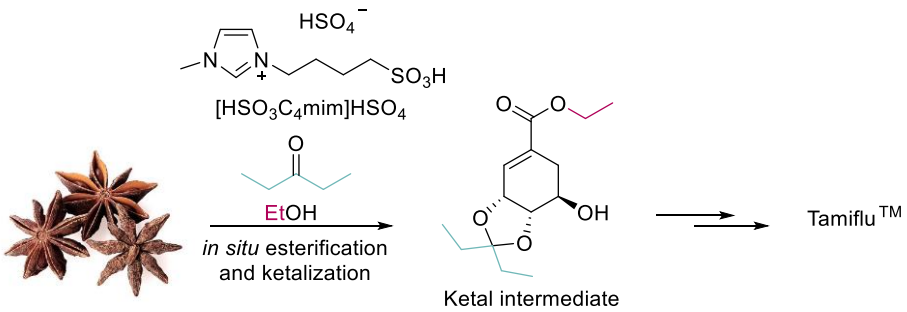
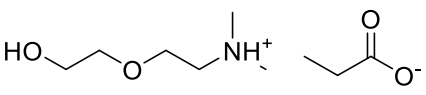
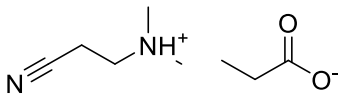


Figure 23: In situ extraction and derivatization of shikimic acid from star anise powder

Lactones, such as the pharmaceutically active senkyunolide I, senkyunolide H and *Z*-ligustilide have been extracted from *Ligusticum chuanxiong* Hort. using two protic ILs, *N*,*N*-dimethyl-*N*-(2-hydroxyethoxyethyl)ammonium propionate (DMHEEAP) and *N*,*N*-dimethyl(cyanoethyl)ammonium propionate (DMCEAP) by Yansheng and coworkers. (Figure 24) After microwave irradiation of the crude plant material with the ionic liquids for 1-5 min, the sample was diluted with methanol and the methanol phase filtered and analyzed *via* HPLC. The recovery of DMHEEAP was performed according to following procedure: The reaction mixture was diluted by methanol and filtered. Methanol was evaporated and the recovered ionic liquid reused. After the third circle the extraction yield decreased dramatically due to higher viscosity caused by co-extraction of contaminants and coproducts.[168] An additional back-extraction of the ionic liquid with *n*-hexane could improve the concentration of senkyunolide I and senkyunolide H hardly decreased, but the concentration of *Z*-ligustilide still decreased by 39.7%.[168]



N,*N*-dimethyl-N-(2-hydroxyethoxyethyl)ammonium propionate (DMHEEAP)



N,*N*-dimethyl(cyanoethyl)ammonium propionate (DMCEAP)

Figure 24: Ionic Liquids used for the extraction of Ligusticum chuanxiong Hort

In 2013, Claudio *et al.* reported an enhanced and selective extraction of caffeine from guaraná seeds using aqueous solutions of ionic liquids based on imidazolium or pyridinium cations combined with the chloride, acetate and tosylate anions. An elegant response surface methodology allowed identifying optimal conditions for the extraction process such as the ionic liquid concentration, the contact time, the solid-liquid ratio and the temperature. Outstanding extraction yields (up to 9 wt% of caffeine per guaraná dry weight) were obtained at a moderate temperature and in a short-time, thus showing that aqueous solutions of ionic liquids are superior alternatives for the solid liquid extraction of caffeine from biomass. The recyclability and reusability of the ionic liquids was realized *via* back-extraction conventional solvents; however, the toxic organic solvent chloroform had to be used for a complete removal of caffeine of the ionic liquid aqueous solution. Furthermore they observed an significant change of the structure of biomass treated with ionic liquid solution compared to pure water, as it can be seen in Figure 25.[169]

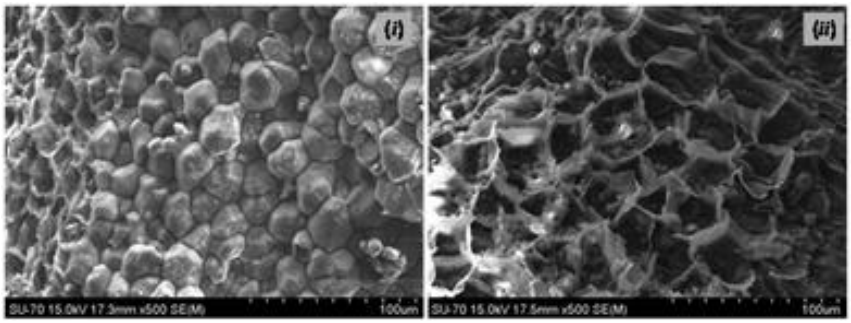


Figure 25: SEM pictures of guaraná samples after extraction with (i) water and (ii) an aqueous solution of [C4mim]Cl

One part of this thesis deal with the use of ionic liquid-aqueous micellar solutions as isolation media for the pharmaceutically active ingredient piperine from black pepper.[170]

Several surface-active ionic liquids including a biodegradable betaine-derivative (Figure 26) were used for the extraction of piperine, and a strong correlation between extraction yield and the critical micelle concentration of the respective ionic liquid was found.

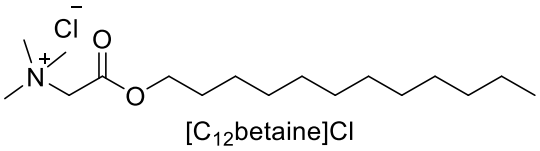


Figure 26: Betaine-based biodegradable and surface-active ionic liquid

A scaled strategy for the isolation of piperine based on back-extraction of aqueous solutions with a small volume of environmentally benign *n*-butyl acetate was developed. This strategy allowed recovery and recycling of the aqueous ionic liquid micellar solution for five runs without any loss in extraction efficiency.

The importance of the aqueous ionic liquid solution compared to pure water was also visible from electron microscopy that was performed on the recovered biomass after extraction. Although micellar solution of 1-alkyl-3-methylimidazolium based ionic liquids [C$_n$mim]Cl cannot completely dissolve biomass as it would be the case with neat ionic liquids, some changes in biomass morphology can be observed in electron microscopy that are not present when pure water was used as extraction media. A similar effect was observed by Coutinho *et al*. reported an increase in the ratio of broken cells to intact cells of guaraná seed in the presence of ionic liquid/aqueous mixtures, although the biomass was not completely dissolved, as shown above.[169]

Another elegant approach was presented by Bogdanov *et al.* in 2015.[171] Aqueous solutions of the ionic liquid [C$_4$mim]Ace were used for the isolation of glaucine from *Glaucium flavum* since this ionic liquid had previously shownfull extraction efficiency for glaucine in analytical scale in short extraction times.[183] (Figure 27)

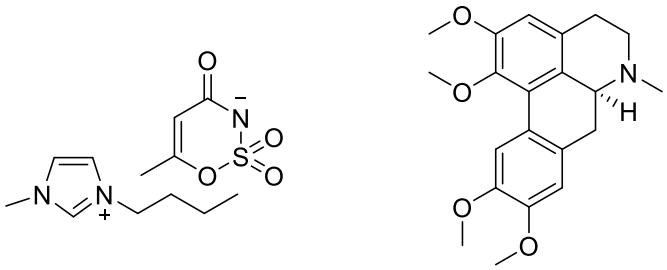


Figure 27: Used ionic liquid for the extraction of glaucine by Bogdanov et al.[183]

Instead of performing a back-extraction of the active ingredient from the aqueous ionic liquid solution with a co-solvent, the ionic liquid solution was directly reused for a next extraction step up to 10 cycles. Therefore not only the costs of the ionic liquid are reduced but also the extraction efficiency is improved by accumulation of the active ingredient in the aqueous ionic liquid solution. The observed a linear increase of glaucine in the ionic liquid solution for 10 cycles allowed extraction efficiencies increased about ~35% compared to methanol, which is currently used for conventional isolation process of glaucine. Unfortunately for each cycle approx. 8% of the ionic liquid was lost due to

absorption on the matrix micro pores on the biomass. The back-extraction of glaucine from the enriched aqueous ionic liquid solution using different organic solvents was also investigated. Only chlorinated solvents such as dichloromethane and chloroform resulted in quantitative back-extraction, whereas non-chlorinated solvents e.g. ethyl acetate, diethyl ether, toluene only showed a moderate extraction performance. Eventually chloroform was chosen since dichloromethane formed a stable emulsion, although an environmentally benign alternative is needed. Figure 28 shows the isolation and ionic liquid recovery strategy. After enrichment of the active ingredient, back-extraction and a filtration step is performed. Glaucine is precipitated as hydrobromide salt in high purities. The ionic liquid was quantitatively recovered after a work-up and reused without any loss of extraction efficiency.

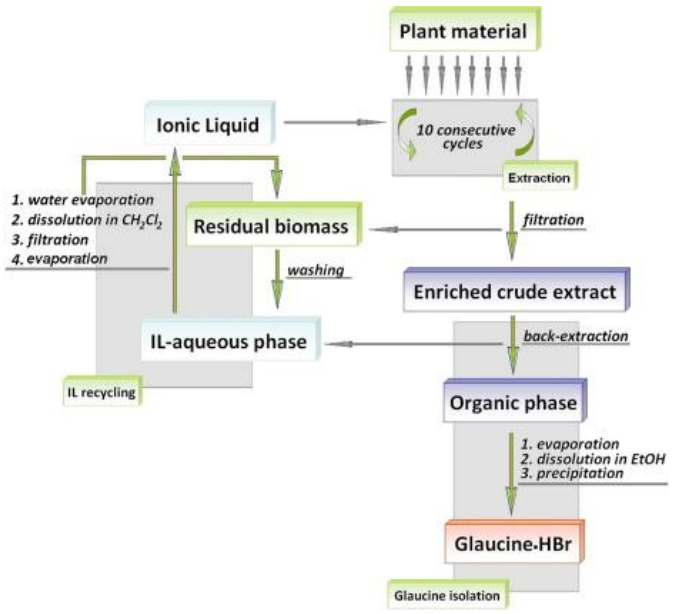


Figure 28: Isolation strategy for glaucine published by Bogdanov et al.

2.3.4.3 Separation of active ingredient and ionic liquid *via* exchange resins/macroporous resins

The importance of active ingredient isolation for the pharmaceutical industry is particularly true for the case of shikimic acid, the major starting material for the production of the neuraminidase inhibitor Tamiflu™ (Oseltamivir phosphate) which is well known for the treatment and prevention of influenza.[184, 185] The production of Tamiflu™ is still dependent on the isolation of shikimic acid from Chinese star anise seeds, and the low isolation yield of 3-7% was held responsible for the world-wide shortage in Tamiflu™ in 2005.[186-188]

The Tamiflu™ precursor shikimic acid was also extracted from *Ginkgo biloba* leaves with [C_4mim]Cl. Their HPLC analysis indicated that at 150 °C the ionic liquid yielded 2.5 times as much shikimic acid than methanol at 80 °C and that the ionic liquid performed better than H_2O and DMF. An ion exchange resin was used for the isolation of shikimic acid. An extract containing 7.1 mg of shikimic acid was charged to the resin column, washed with water to obtain the recovered ionic liquid (99%). Washing with 25% acetic acid released shikimic acid that could be isolated in 87% yield.[172]

In 2013, Zirbs *et al.* reported that the dissolution of star anise seeds from *Illicum verum* in imidazolium-based ionic liquids can lead to better access to the valuable ingredient embedded in the biopolymer, and thus to a higher extraction yield. Different imidazolium-based ionic liquids were investigated, and the extraction yield of shikimic acid was correlated with their hydrogen-bonding properties *via*

polarizable molecular dynamics simulations, indicating that the hydrogen bonding of the anion to shikimic acid is responsible for a good extraction performance. A scale-up strategy for the isolation of shikimic acid with the ionic liquid 1-ethyl-3-methylimidazolium acetate ([C_2mim]OAc) was developed based on ion-exchange resins, thus allowing to isolate shikimic acid in up to 10 wt% yield with complete recovery of the ionic liquid.[173]

Lu *et al*. presented an interesting strategy for extraction of *Galla chinensis* which contains the hydrolysable tannins gallotannins using [C_4mim]Br solutions and macroporous resins for the removal of the ionic liquids.[152] After extraction of crude biomass with microwave- and ultrasound assisted extraction with aqueous [C_4mim]Br solutions, macroporous resin adsorption technology was further employed to purify the tannins and remove the ionic liquid from crude extract. Initial adsorption and desorption experiments identified XDA-6 resin was identified as best adsorbent, as it had higher separation efficiency than other tested resins. With XDA-6 resin adsorption isotherms at different temperatures were obtained for tannins, showing a saturation plateau at tannins concentration of 24 mg mL^{-1} and 28 °C as ideal temperature. More parameters were adjusted to optimal conditions and optimum conditions were as follows: the ratio of column height to diameter bed was 1:8, flow rate 1 BV/h (bed volume per hour), 85% ethanol was used as eluent while the elution volume was 2 BV. Under the optimized conditions, the adsorption and desorption rate of tannins in XDA-6 were 94.81 and 91.63%, respectively. Furthermore, the result of ultra-performance liquid chromatography analysis showed that [C_4mim]Br could be removed from extract.

2.3.4.4 Distillation of the ionic medium

The group of MacFarlane presented an elegant strategy for the extraction of biomass using the distillable ionic liquid, *N*,*N*-dimethylammonium *N*'*N*'-dimethylcarbamate (DIMCARB) to extract hydrolysable tannin materials from plant sources such as catechu (*Acacia Catechu*) and myrobolan (*Terminalia Chebula*).[26] Tannins are generally defined as water soluble organic substances present in plant extracts that effect the transformation of animal hide into leather. Typically, vegetable tannins are phenol-rich compounds that show antitumor, anticarcinogenic, antimicrobial and antiviral effects.[189] In the leather industry tannins replace „chrome tanning" and can therefore avoid the handling with Cr(VI), which is considered being highly toxic, mutagenic and carcinogenic. Conventional extraction methods for tannins require harsh conditions and a high solvent/solid ratio resulting in poor extraction yields. DIMCARB is a distillable, protic ionic liquid and is formed by combining CO_2 and dimethylamine in an approximately 1:2 ratio. Figure 29 represents the dynamic equilibria in the DIMCARB system showing a two step proton transfer for the formation of the dimethyl ammonium ion and the dimethylcarbamate ion. In contrast to conventional ionic liquids, the formation of DIMCARB is reversible, and distillation at 45 °C reforms CO_2 and dimethylamine.

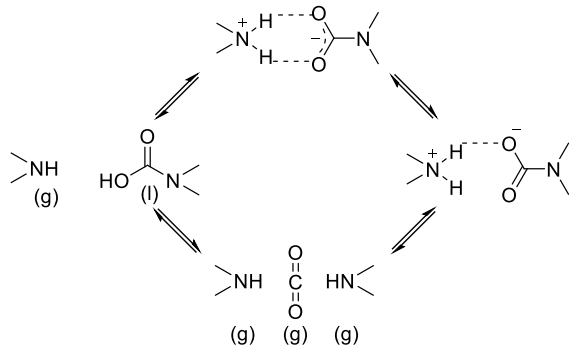


Figure 29: Dynamic equilibria in the DIMCARB system.

A mixture of 5.0 g of either myrobalan nut or catecheu was treated with 25.0 g of DIMCARB ionic liquid and stirred at room temperature for varying times. (Figure 30) After filtration of undissolved plant material and evaporation of DIMCARB, water was added, and the aqueous solution was filtered to remove the so-called “condensed tannins”. The filtrate consisting of so-called “water-soluble tannins” could be directly used for the leather tanning process or evaporated to yield the pure hydrolysable tannins such as ellagic acid. In contrast to conventional solvents ellagic acid was obtained in higher yields and the products are more stable against bacterial moulds as evidenced by microbial analysis. Furthermore only a third of water was necessary compared to the conventional process.[26]

Figure 30: Isolation procedure of tannins using DIMCARB

2.3.5 Fragrance isolation

Apart from active ingredients plant matter may also consist a variety of fragrances and essential oils. Valuable essential oils are typically a complex mixture of individual fragrance components obtained from plant material and are widely used in various domains of human activities including perfumery, cosmetics, nutrition, and pharmaceuticals.[190] According to the United Nation’s COMTRADE database, global imports of essential oils stood at US$ 2bn in 2005.

Essential oils are traditionally obtained by steam distillation, solvent extraction, or cold pressed from crude fragrance materials. Solvent extraction is always associated with the risk of handling large quantities of combustible and often toxic solvents, and the products suffer from contamination. Steam distillation is not only a highly energy consuming process, but can induce thermal degradation, hydrolysis, and water solubilization, thus often requiring expensive waste water redistillation for fragrance recovery.[191]

The versatile features of ionic liquids do not only allow the dissolution of plant materials for efficient fragrance release, but enable the direct and mild distillation directly from the ionic liquid media without excessive steam production, solvent contamination or losses from water solubilization. Figure 31 represents the general isolation scheme for fragrances and essential oils.

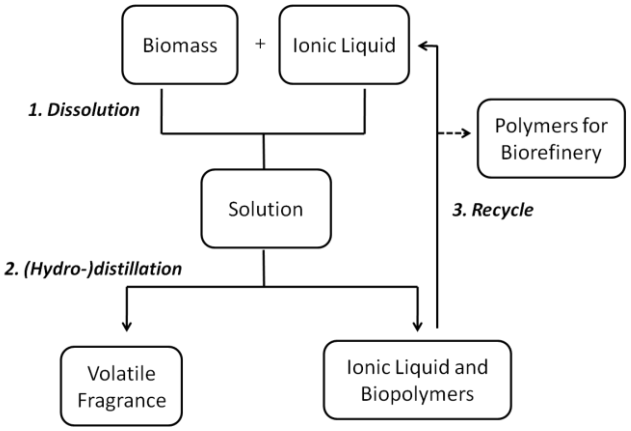


Figure 31: General procedure for the isolation of fragrances and essential oils

Table 11 summarizes fragrances and essential oil extracted or isolated using ionic liquid-based strategies using either pure ionic liquids or aqueous solutions thereof.

Table 11: Isolation of fragrances and essential oils using ionic liquids

Entry	Fragrance	Biomass	Ionic Liquid	Reference
1	pine needle oil	pine needles	hydrophilic methylimidazolium -based	Zhu 2008[192]
2	essential oils	diospyros kaki	hydrophilic methylimidazolium -based	Zhu 2008[193]
3	essential oils	*Illicium verum* Hook. f. and *Cuminum cyminum* L.	$[C_4mim]PF_6$	Zhai 2009[194]
4	orange essential oil	orange peels	$[C_2mim]OAc$	Bica 2011[195]
5	biphenyl cyclooctene lignans	*Schisandra chinensis* Baill	aq. $[C_{12}mim]Br$	Ma 2011[196]
6	carnosic acid, rosmarinic acid and essential oil	*Rosmarinus officinalis*	aq. $[C_8mimBr]$	Liu 2011[197]
7	essential oil	*Dryopteris fragrans*	$[C_2mim]OAc$	Jiao 2012[198]
8	essential oil	*Fructus forsythiae*	$[C_2mim]OAc$	Jiao 2013[199]
9#	fragrances	tea leaf	$[C_4mim]Br/BF_4$, $[C_8mim]BF_4$	Liu 2013[200]
10#	essential oil	ginger	$[C_2mim]OAc$	Hu 2014[201]
11#	flavor	*Passiflora edulis*	$[C_nmim]PF_6$, n = 4,6	Xia 2015[202]

Paper written in Chinese.

In 2008 Zhu *et al.* disclosed a method for extracting essential oil from leaves of *Diospyros kaki* and of pine needles using imidazolium-based hydrophilic ionic liquids, e.g. [C_4mim]OAc, [C_4mim]Cl and [amim]Cl under microwave conditions. For the isolation and ionic liquid recovery they either applied conventional steam distillation or ion exchange resins.[192,193]

Zhai *et al.* extracted the bioactive essential oils from *Illicium verum* Hook. f. and *Cuminum cyminum* L. that are widely used for their flavors and pharmaceutical characters with the ionic liquid [C_4mim]PF_6 as microwave absorption media.[194] The distillate was concentrated continuously by a cooler outside the microwave oven, dried and analyzed them vie GC-MS. The ionic liquid-assisted microwave-extraction process was compared to hydro distillation (HD). The authors did not observe any obvious difference between main constituents in essential oils obtained by ionic liquid-assisted extraction or hydrodistillation, however, the extraction time was considerably shortened to 15 minutes only using microwave-assisted extraction with ionic liquids, whereas 180 min were required for complete extraction with conventional hydrodistillation.

Bica *et al.* presented the dissolution of orange peel in various ionic liquids and compared direct distillation and solvent extraction for the isolation of orange essential oil.[195] Only partial dissolution was observed for [C_4mim]Cl and [amim]Cl after 24 h, whereas complete dissolution of orange peels was obtained using [C_2mim]OAc after 3 h. The dissolved biomass was immediately subjected to vacuum resulting in a two-layer distillate consisting of limonene and water from the orange peel. Limonene was obtained in approx. 5 wt% after phase separation in excellent purity, and no traces of the ionic liquid or its degradation products were found. As an alternative to direct distillation liquid-liquid extraction was applied using ethyl acetate but the isolated limonene suffered from low purity and yield. For recycling of the ionic liquid [C_2mim]OAc water was added and filtration of the coagulated biopolymers and evaporation of water allowed a simple recovery of the ionic liquid in spectroscopically pure form but dark in color. For further purification, the recovered ionic liquid was refluxed over charcoal and could be isolated in excellent purity and 90-95%.

Ma *et al.* extracted essential oils as well as cyclooctene lignans which show antioxidant, antimicrobial, antitumoral activites[203] from *Schisandra chinensis* Baill fruits using aqueous solutions of [$C_n$mim] ionic liquids under microwave irradiation.[196] Starting with a [C_4mim]$^+$ backbone different anions were investigated. Since the bromide anion showed the highest extraction efficiency, the alkyl chain of [$C_n$mim] was varied (n = 2,4,6,8,10,12). With increasing chain length, the extraction yield increased and [C_{12}mim]Br was therefore chosen for further investigations. Compared to conventional extraction strategies which required 180 min for complete extraction, the microwave-assisted strategy suing ionic liquids shortened the reaction time to 20 min. However, the authors found that a longer extraction time lead to a decrease of lignans indicating that carbonization or isomerization of lignans took place.

Liu *et al*. simultaneously extracted carnosic acid, rosmarinic acid and essential oil from *Rosmarinus officinalis* with aqueous solutions of ionic liquid using a combined microwave dissolution-hydrodistillation approach.[197] The essential oils containing in rosemary species are not only used in foodstuffs, perfumes or cosmetic product , but exhibit biological activities such as antioxidant, antimicrobial, anti-inflammatory, antitumor and chemopreventive activities.[204] Starting with the [C_4mim]$^+$ cation, the anions Br^- and NO_3^- showed the highest extraction yield for carnosic acid, whereas BF_4^- and Br- were the most efficient anions for rosmarinic acid. For further studies a 1 M [C_8mim]Br

aqueous solution was chosen and the optimization of parameters lead to 15 min irradiation time at 700 W and a solid-liquid ratio of 1:12. The present method showed slightly higher extraction yield carnosic acid and rosmarinic acid than microwave-assisted extraction with ethanol and significantly higher extraction yields than conventional hydrodistillation and could reduce the reaction time to 20.

Dryopteris fragrans was extracted by Jiao *et al.* using MW irradiation and different 1-alkyl-3-methylimidazolium-based ionic liquids, since the essential oil has antioxidant potential in terms of the free radical scavenging and lipid peroxidation inhibitory activities.[198] After pretreating the plant material using 300 W irradiation power, the dark slurry was mixed with 100 ml water and hydrodistillation was applied to isolate the essential oil. Recovery of the ionic liquid was successfully achieved by filtration of the ionic-liquid aqueous solution and successive azeotropic distillation of $EtOH/H_2O$. Different ionic liquid were tested and the extraction of essential oil decreased in the order $[C_2mim]OAc$ > [amim]Cl > $[C_4mim]Cl$ > $[C_4mim]Br$ > pure water. The recovered $[C_2mim]OAc$ could be reused for five cycles; however a loss of performance from 0.9 % essential oil after the first run to 0.5% after the 5^{th} cycle was observed.

3 Task

In the first part of this thesis a set of ionic liquids with different should be synthesized for the dissolution of biomass. Different ammonium or imidazolium based ionic liquids with either a hydrophilic or a hydrophobic anion should be obtained *via* alkylation, acid base reaction or metathesis and further characterized. Furthermore different properties of the ionic liquid should be varied, for example *via* the prolongation of the alkyl chain length of the cation.

In the next part the focus will be set on different ionic liquid-based technologies for the extraction and isolation of several valuable ingredients from different types of biomass. Micellar extraction should be applied for the isolation of piperine from black pepper. The same strategy should be used for the extraction of eugenol from cloves to allow the direct conversion of the extracted eugenol towards the valuable isoeugenol. Betulin should be extracted using a biphasic mixture consisting of an aqueous ionic liquid solution and an organic solvent. After separation, betulin should be directly oxidized to obtain the value-added derivatives betulinic acid, followed by an esterification to bevirimat. Eventually, the combination of ionic liquids for extraction and use in biocatalytic reactions should be explored by the *in situ* extraction of limonene and conversion towards a valuable chiral polymer building block.

In the last part of this thesis the extraction of DNA from different food matrices should be investigated. Biomass should be dissolved in pure ionic liquids or their mixtures with aqueous buffer systems to develop fast extraction strategies for DNA with importance for food quality control.

4 Results and discussion

4.1 Synthesis of ionic liquids

For the dissolution of biomass a diverse set of more than 50 ionic liquids containing various anions and cations was synthesized. A majority of the ionic liquids consist of an imidazolium cation. The general methods applied for the synthesis of imidazolium based ionic liquids are shown in Figure 32: In a first step alkylation of the nucleophile, e.g. *N*-methylimidazole with different alkyl halides was performed to obtain a halide-based ionic liquid. The anion could then be further exchanged either *via* metathesis using a metal salt of the corresponding acid or *via* an acid basic reaction using the stronger acid.

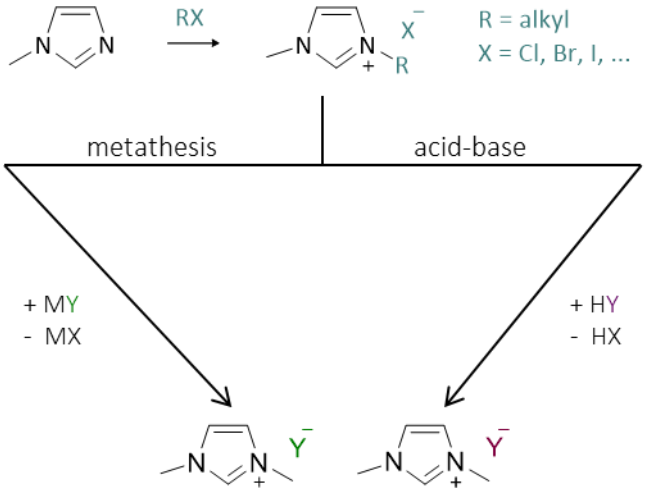


Figure 32: Synthesis pathway of imidazolium ionic liquids applied in this thesis

While the general synthesis of the ionic liquids in discussed in this chapter, the choice of functionalized ionic liquids for a specific active ingredient isolation will be discussed separately for each separation problem.

4.1.1 Synthesis of ionic liquids *via* alkylation

Starting with the synthesis of imidazolium based ionic liquids using alkylation reaction, ionic liquids with manifold properties were obtained. Not only a variety of "classical" biomass dissolving ionic liquids could be obtained, but also long alkyl chain derivatives with surface activity were successfully synthesized. (Chapter 4.1.5) Apart from the classical imidazolium based ionic liquids, phosphonium and ammonium based cations were used, typically in connection with halides as anion.

Initially, a small set of imidazolium ionic liquids that are already known to dissolve biomass[33] was synthesized *via* alkylation of 1-methylimidazole with the corresponding alkyl halide, including [C_4mim]Br (**1**), [C_4mim]Cl (**2**) and [C_2OHmim]Cl (**3**). For the synthesis of [C_2mim]Me_2PO_4 (**4**) 1-ethylimidazole was alkylated with Me_3PO_4. (Figure 33) All ionic liquids were obtained in excellent yields.

While [C_4mim]Br (**1**) and [C_4mim]Cl (**2**) could be purified *via* crystallization from a mixture of acetonitrile (ACN) and ethyl acetate (EtOAc), the other two ionic liquids were obtained in high purities after washing with organic solvents. Those ionic liquids were used not only for the extraction of betulin from birch bark but also for the extraction of DNA from different food matrices. (Chapter 4.4 and 4.6)

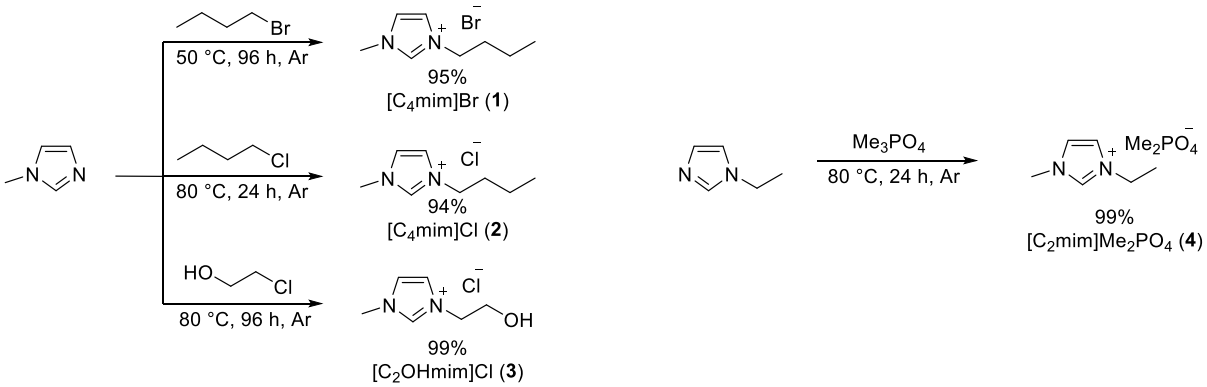


Figure 33: Imidazolium ionic liquids synthesized via alkylation

4.1.2 Synthesis of ionic liquids *via* anion exchange

For a broader variation of anions, a small set of ionic liquids was synthesized from the corresponding halide derivatives *via* anion exchange. For this purpose [C_2mim]Cl (**5**) or [C_4mim]Cl (**2**) served as precursor. The imidazolium chlorides were further reacted with the corresponding metal salt or acid as already shown in the general scheme Figure 32. Reactions were typically performed in water to obtain the hydrophobic bistriflimide ionic liquid as a second layer. The remaining by-product LiCl was separated *via* liquid-liquid extraction of dichloromethane containing the ionic liquid with water, and chloride removal was checked with a silver nitrate test. In case of dicyanamide and thiocyanat-based ionic liquids the by-product salt, *i.e.* silver chloride or sodium chloride was precipitated in acetone and separated *via* filtration over celite. Hexafluorophosphoric acid was used for the synthesis of [C_4mim]PF_6 (**10**). Except for the latter, ionic liquids were obtained in very good yields. (Figure 34)

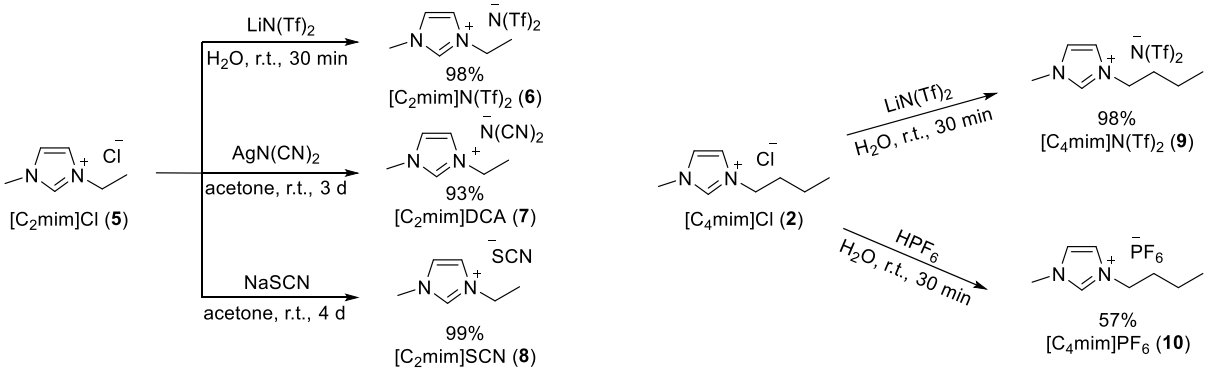


Figure 34: Imidazolium ionic liquids synthesized via anion exchange

The synthesized ionic liquids were used for the extraction of DNA from different food matrices. (Chapter 4.6)

4.1.3 Synthesis of basic ionic liquids

A combination of the two synthetic strategies presented so far enabled the creation of a set of basic ionic liquids that differ from the previous ionic liquids by the presence of an additional unalkylated nitrogen unit. In a first step *N*-heterocyclic bases were reacted with alkyl halides to obtain the chloride salt. In a second step the chloride anion was further exchanged to the bistriflimide anion *via* metathesis as described above.

Basic precursors 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 1,4-diazabicyclo[2.2.2]octane (DABCO), pyridine and 4-dimethylaminopyridine (DMAP) were reacted with butylchloride to yield their corresponding chloride ionic liquids in moderate to good yields. The anion exchange gave the hydrophobic bistriflimide ionic liquids in excellent yields. (Figure 35)

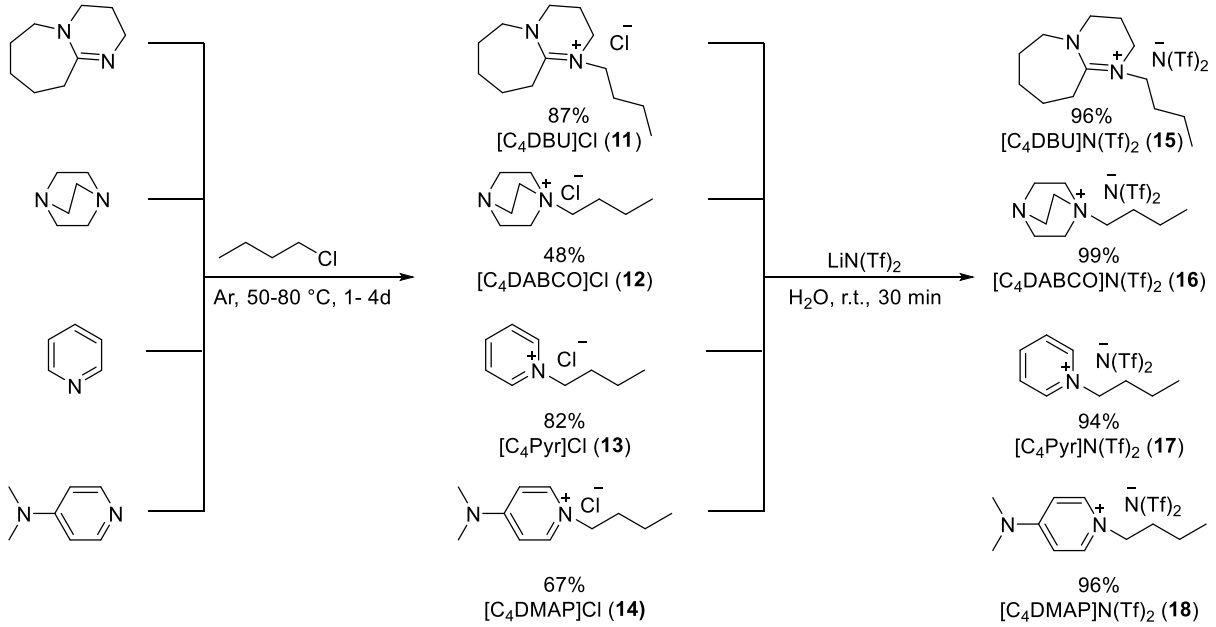


Figure 35: Alkylation and ion exchange of DBU, DMAP, pyridinium and DABCO ionic liquids

Additionally, a small set of ionic liquids bearing an imidazolium core with an additional pyridine moiety in their cations was synthesized. Two different synthetic pathways were chosen: For the synthesis of [C_1PyrImd]Cl (**20**) 2-(chloromethyl)pyridine hydrochloride was neutralized using sodium hydrogen carbonate. After isolation *via* simple extraction and evaporation the obtained unstable product **19**, which was directly converted with 1-methylimidazole in methanol to the chloride based ionic liquid [C_1PyrImd]Cl. Anion exchange yielded the bistriflimide ionic liquid **21** in excellent yield. (Figure 36)

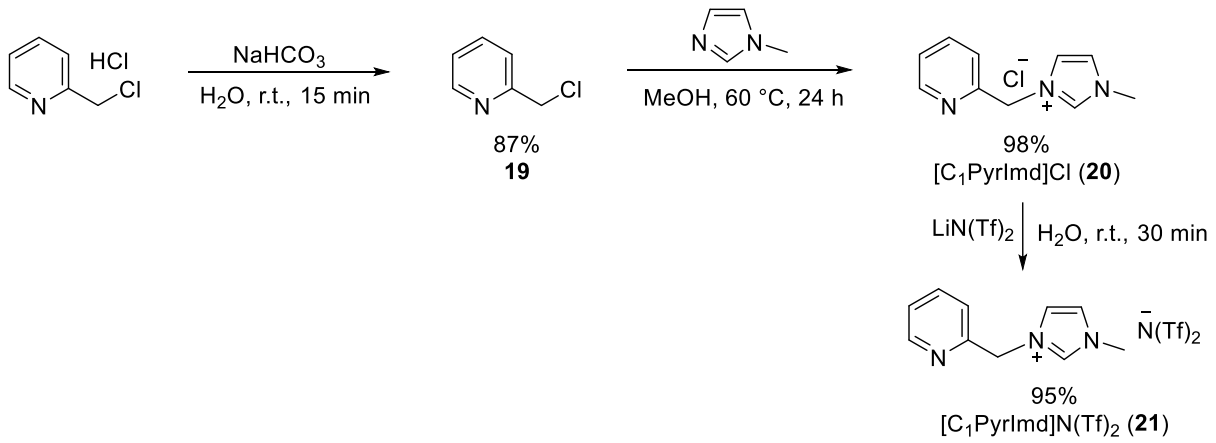


*Figure 36: Synthesis of [C_1PyrImd]Cl **20** and [C_1PyrImd]N(Tf)$_2$ **21***

The second approach was chosen for the synthesis of [C_4PyrImd]Cl (**20**). With this method the intermediate can be alkylated with different alkyl halides and to provide access to different alkyl chain length or halide anions. While the yield of the precursor **22** was moderate, alkylation could be

performed solely on the imidazole unit, and ionic liquid **23** was obtained in good yields after crystallization. (Figure 37)

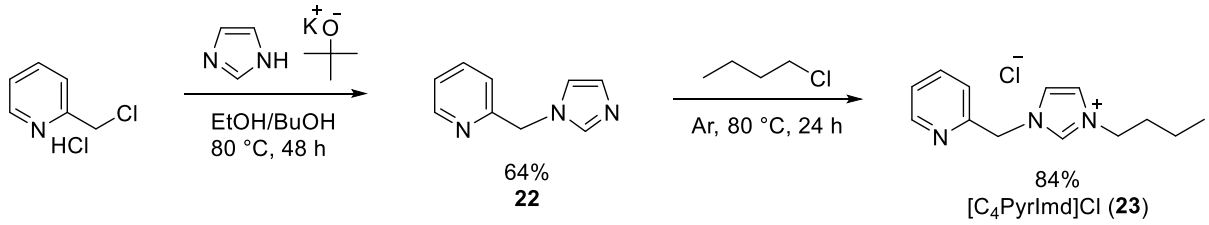


*Figure 37: Synthesis of [C_4PyrImd]Cl **23***

Basic ionic liquids were used for the esterification of betulinic acid to bevirimat. (Chapter 4.4.5.1.2)

4.1.4 Synthesis of ionic liquids *via* acid-base reaction

Another strategy for the synthesis of ionic liquids relies on acid-base reactions, e.g. neutralization or protonation. One very interesting class of ionic liquids is represented by choline derivatives since they are generally classified as environmentally benign and biodegradable ionic liquids with low toxicities.[205,206] For the synthesis of choline derivatives commercially available choline hydrogencarbonate solution (**24**), whose exact concentration was determined prior to its use *via* titration, served as precursor that was reacted with the corresponding acid to yield choline ionic liquids in short time and quantitative yield. (Figure 38)

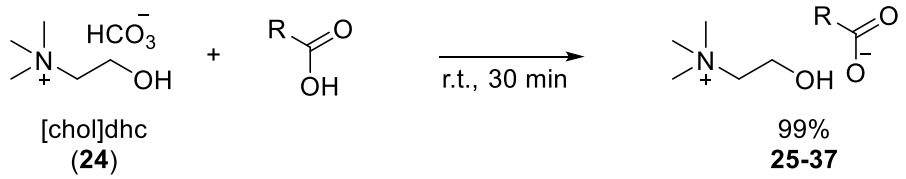


Figure 38: General synthesis of choline derivatives

By applying this simple reaction a set of 13 choline derivatives was synthesized: Apart from carboxylate choline ionic with different chain length, choline phophates and phosphinates were also obtained. (Figure 39)

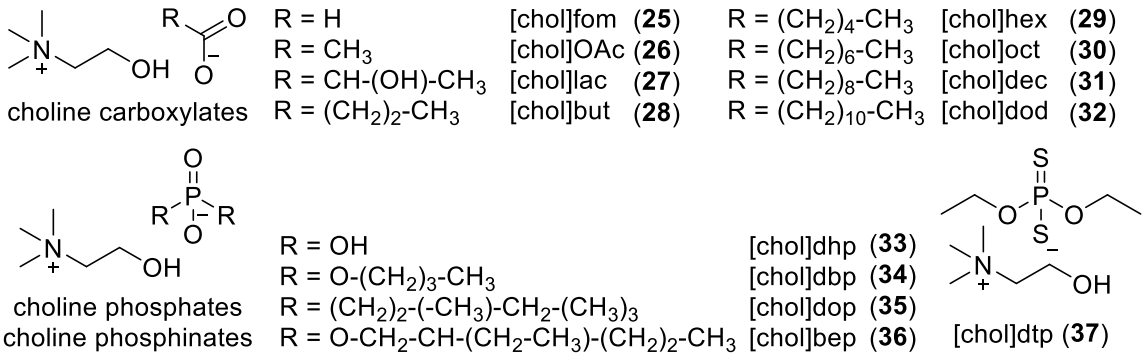
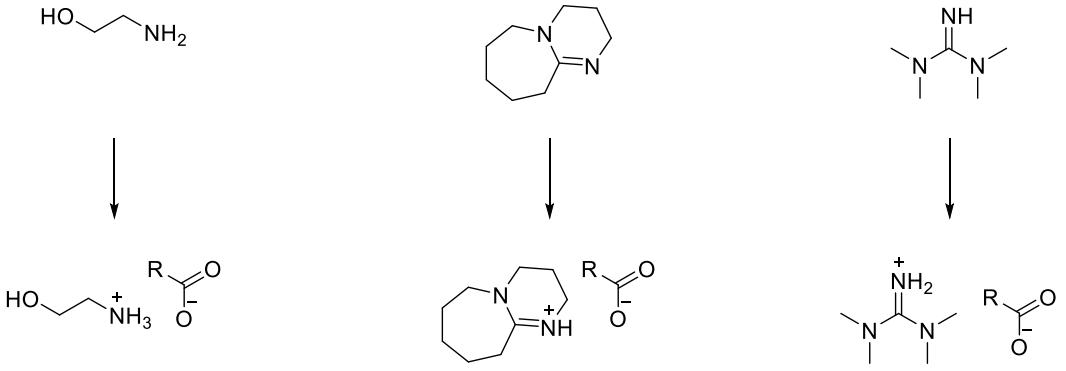


Figure 39: Set of choline derivatives synthesized

In contrast to the ionic liquids previously shown, protic ionic liquids are synthesized *via* a proton transfer from an acid to a basic moiety to obtain the ionic character. In the ideal case the proton transfer is complete, but it is more likely that neutral acid and base species are present as well.[207] Beneficial is their cheap synthesis due to its simple procedure.

Protic ionic liquids were synthesized in a comparable manner than the permanently ionized choline based derivatives. Different acids were used for the introduction of the anion *via* protonation of a nitrogen precursor. Again all ionic liquids were obtained in quantitative yield. (Figure 40)



R = H [N_{0002OH}]fom (**38**)
R = CH_3 [N_{0002OH}]OAc (**39**)
R = CH-(OH)-CH_3 [N_{0002OH}]lac (**40**)
R = $(CH_2)_4$-CH_3 [N_{0002OH}]hex (**41**)

R = H [DBU]fom (**42**)
R = $(CH_2)_4$-CH_3 [DBU]hex (**43**)

R = H [guan]fom (**44**)
R = CH_3 [guan]OAc (**45**)
R = $(CH_2)_2$-CH_3 [guan]but (**46**)
R = $(CH_2)_4$-CH_3 [guan]hex (**47**)
R = $(CH_2)_6$-CH_3 [guan]oct (**48**)
R = $(CH_2)_8$-CH_3 [guan]dec (**49**)

Figure 40: Set of ionic liquids synthesized via acid-base reaction

4.1.5 Surface-active ionic liquids

Apart from the previously described ionic liquids that were mostly based on short chain alkylated systems *i.e.* ethyl or butylimidazolium derivatives, long chain imidazolium derivatives with alkyl chain lengths > 8 carbon atoms are of particular interest for extractions. Investigations on the behavior of ionic liquids in the presence of water showed that certain ionic liquids can form aggregates in aqueous solution.[110-113] Since they consist of a hydrophilic head and a hydrophobic tail, they are able to form micelles upon a certain concentration in water (critical micelle concentration, CMC).

These surface-active ionic liquids can be used as aqueous solutions, as the price of ionic liquids still represents an issue. The use of aqueous solutions of ionic liquids represents a good compromise between costs, solubility properties and safety. Therefore not only enhanced solubility of the active ingredients in the micellar solution as well as enhanced solubility of the biomass in the ionic liquid solution compared to pure water are expected.

A set of imidazolium based surface-active ionic liquids was prepared *via* alkylation of 1-methylimidzaole with long chain alkyl halides in excellent yields. High temperature and long reaction time was necessary for the synthesis of these ionic liquids. Since the boiling point of the alkylchlorides is increasing with increasing chain length, higher temperatures were necessary for longer alkyl chains. Furthermore the increasing viscosity of the solution with increasing reaction time made the stirring difficult. While [C_8mim]Cl (**50**) and [C_{10}mim]Cl (**51**) were obtained as highly viscous liquids, ionic liquids containing an alkyl side chain of 12, 14 or 16 carbon atoms were crystallized from THF to yield colorless crystals in high purity. (Figure 41)

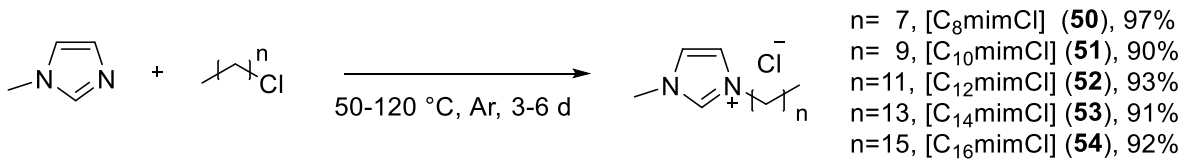


Figure 41: Synthesis of surface-active imidazolium based ionic liquids

An interesting correlation between the CMC and the chain length of the ionic liquid was found in literature. (Figure 42) With increasing chain length the CMC decreases on a logarithmic scale.[208-210]

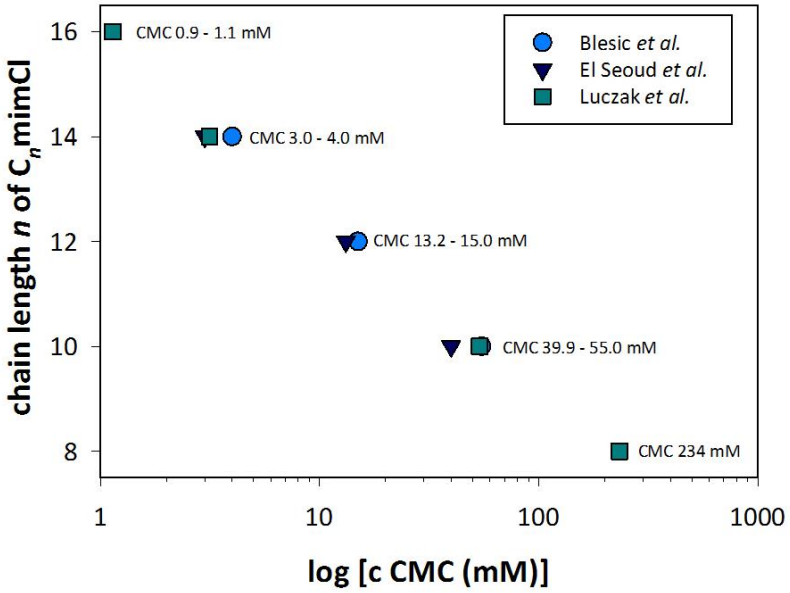


Figure 42: Relation of the chain length and the CMC

In order to avoid the aqua toxicity of long chain ionic liquids, environmentally benign biodegradable ionic liquids were synthesized. In general biodegradable ionic liquids are readily synthesized *via* introducing an ester side chain to an ionic liquid allowing cleavage at a specific position. Their biodegradation is improved compared to commonly used dialkylimidazolium ionic liquids, whereas the amide analogues are poorly degradable.[211-213]

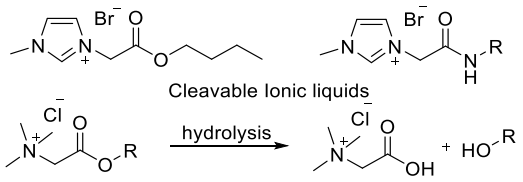


Figure 43: Cleavable ionic liquids in literature and hydrolysis of the biodegradable ionic liquid used in further experiments

Apart from alkyl imidazolium surface-active ionic liquids, biodegradable ionic liquids based on a betaine cation and consisting of an ester group were synthesized. The two step synthesis started with an esterification of dodecanol and chloroacetyl chloride which gave the intermediate **55** in 95% yield. The quaternisation of trimethylamine in THF with the precursor **55** yielded the betaine ionic liquid [C_{12}betaine]Cl (**56**) in 88% yield. Using 1-methylimidazole led to the biodegradable imidazolium derivative [C_{12}COmim]Cl (**57**) in 81% yield. (Figure 44)

The synthesis of the betaine based ionic liquid required careful adjustment of solvent and reaction conditions, and several solutions of trimethylamine were tested. If the alkylation was performed in water, an undesired hydrolysis of the ester bond occurred. Transesterification towards the ethyl ester derivative was observed when applying trimethylamine in ethanol. Therefore it was decided to perform the alkylation with a solution of trimethylamine in THF, allowing to isolate the product in excellent yields after crystallization.

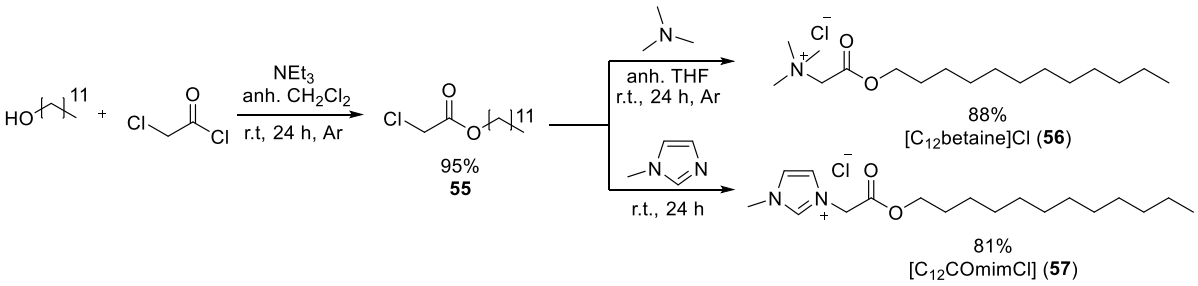


Figure 44: Synthesis of cleavable ionic liquids

The stability of the two cleavable ionic liquids in aqueous media was further investigated. Therefore solutions of the ionic liquids in D_2O and D_2O/DCl were stirred at room temperature or 100 °C for 24 h and analyzed *via* NMR. In case of the imidazolium based ionic liquid **57** a good stability was observed in D_2O and D_2O/DCl at r.t. and in D_2O at 100 °C. If the D_2O/DCl solution was heated to 100 °C ester cleavage depending on the concentration of the added DCl was observed. If 0.1 eq. of DCl in D_2O is used, 3% degradation is observed after 1 h, 20% after 4 h and full degradation after 24 h. (Figure 45) If the amount of DCl is increased to 1 eq., full degradation was observed after 2 h only.

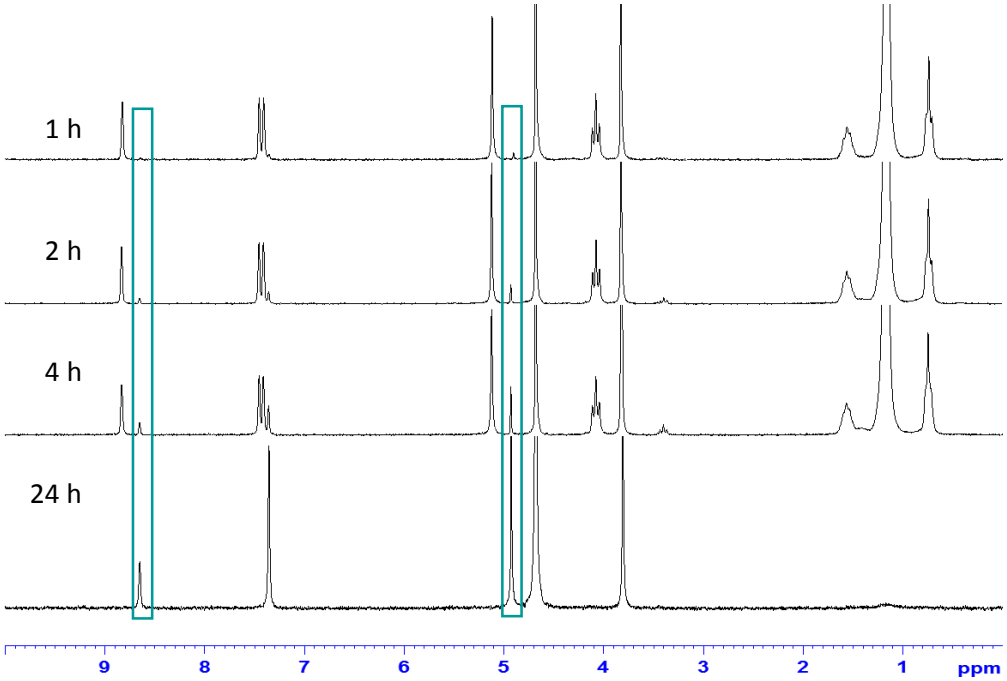


*Figure 45: Cleavage of the ester group of [C_{12}COmim]Cl (**57**) using 0.1 eq. DCl at 100 °C*

A similar trend was observed when performing the experiment with [C_{12}mim]betaine (**56**). Only with the addition of 1 eq. DCl at 100 °C cleavage of the ester was observed. However the cleavage proceeded slower compared to the imidazolium derivative. While in case of the imidazolium derivative

78% of the ester were degraded after 1 h, only 3% degradation was observed in case of the trimethylammonium ionic liquid. After 8 h 60% degradation was observed and the ester was fully cleaved after 24 h. Basic hydrolysis was not investigated so far.

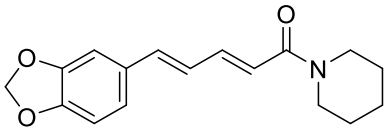
The long chain ionic liquids [$C_n$mim]Cl (n = 10, 12, 14) and the biodegradable betaine based ionic liquid[211-213] were successfully applied as aqueous solution for the extraction of piperine and eugenol (chapter 4.2 and 4.3).

4.2 Isolation of piperine (58) from black pepper

In this part of the thesis, several surface-active ionic liquids including a biodegradable betaine derivative should be used for the extraction of piperine (**58**) from black pepper. After optimization of extraction conditions a scale-up strategy should be developed, allowing to isolate the active ingredient and recycle the ionic liquid aqueous solution.

4.2.1 Piperine (58)

Piperine ((2*E*,4*E*)-, 5-(1,3-benzodioxol-5-yl)-1-(1-piperidinyl)-, 2,4-pentadien-1-one, **58**, Figure 46), a common alkaloid that is responsible for the pungent taste of pepper, is naturally occurring in *Piper nigrum* (6-9%), in *Piper longum* fruits (4%) and in *Piper retrofractum* (4-5 %).[214] Apart from its nutraceutical uses, it has attracted attention for its inhibitory influence on enzymatic drug biotransforming reactions in the liver. The pharmacological properties of piperine (**58**) are widespread, e.g. antifungal,[215] anti-inflammatory,[216] and it serves as a positive $GABA_A$ receptor.[217, 218]



piperine (**58**)

*Figure 46: Structure of piperine (**58**)*

As most plant-derived active ingredients, piperine (**58**) is typically isolated from cultivated plant material using excessive refluxing with volatile organic solvents such as ethanol, toluene or chlorinated hydrocarbons.[219, 220] This extraction process is not only time consuming and requires waste amounts of often toxic solvents, but suffers from co-extraction of undesired by-products such as gums, polysaccharides or essential oils. Additional purification steps are thus required making the isolation and purification of a natural product a large obstacle in any cost-efficient and sustainable drug manufacturing process. Therefore an alternative isolation strategy for piperine (**58**) is needed.

4.2.2 Choice of ionic liquids

For an efficient extraction of the bioactive molecule piperine (**58**) surface-active imidazolium ionic liquids [$C_n$mim]Cl (n = 10, 12, 14) and the biodegradable betaine based ionic liquid[211-213] shown in Figure 47 should be applied as aqueous solution for the extraction of piperine (**58**) from black pepper. The influence of the alkyl chain length of the cation and the concentration of the ionic liquid in water should be investigated. Furthermore the influence of the anion of [C_{12}mim]X (X = Br^-, OTf^-, $N(CN)_2^-$ and OAc^- should be evaluated.

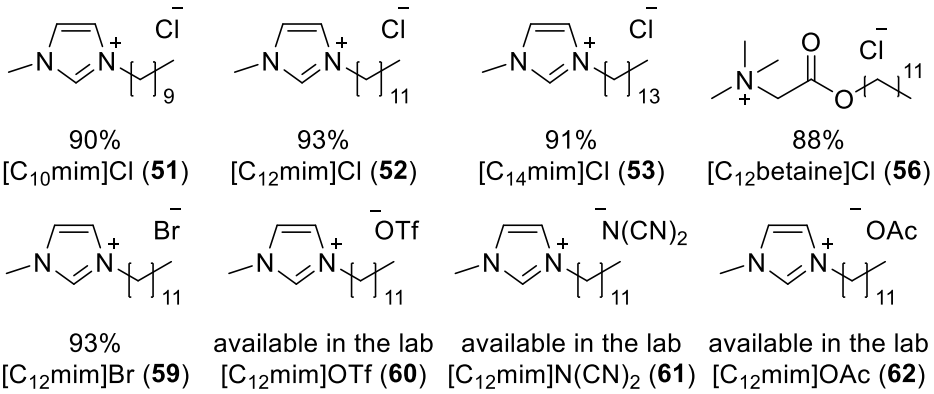


*Figure 47: Surface-active ionic liquids used for the extraction of piperine (**58**)*

The strategy for the extraction of piperine (**58**) from pepper relies on biomass pretreatment, *i.e.* the swelling or dissolution of the biomass in the aqueous solutions of the surface-active ionic liquids. Beneficial for the extraction of the active ingredient is the formation of micelles of the surface-active ionic liquids. As a result of the enhanced solubility of the biomass due to the powerful dissolution properties of ionic liquids and the increased solubility of the active ingredient in the ionic liquid water mixture compared to pure water can be expected. (Figure 48)

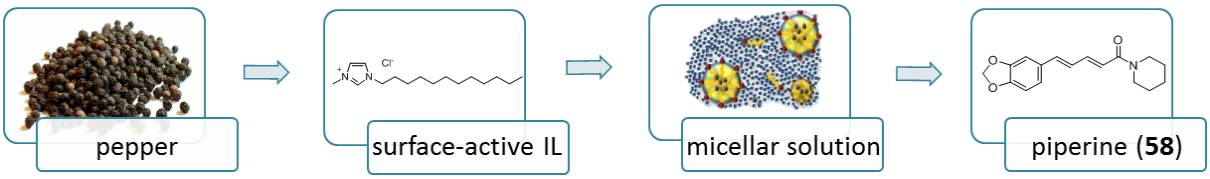


*Figure 48: Concept for the isolation of piperine (**58**)*

4.2.3 Extraction of active ingredients using aqueous (micellar) ionic liquid solutions

As previously discussed (chapter 2.3.2), a number of publications deals with the extraction of active ingredients from plant matter using aqueous ionic liquid solution. Cao *et al.* investigated ionic liquid ultrasound assisted extraction for the isolation of piperine (**58**) from white pepper.[127] Different aqueous solutions of short-chain 1-alkyl-3-methylimidazolium bromide [C$_n$mim]Br with *n* = 3 and *n* =4 were used in rather high concentration > 5 M. . A strong influence on the anion was observed, as the extraction efficiency decreased in the order BF_4^- > Br^- > $H_2PO_4^-$ > PF_6^- which was explained by a reduced hydrophilicity and water miscibility.[127] Although [C$_4$mim]Br performed significantly better than [C$_3$mim]Br, longer chain lengths of the alkyl imidazolium backbone were not investigated. Recently Coutinho *et al.* published an elegant strategy for the isolation of caffeine from guaraná seeds using aqueous ionic liquid solutions.[169]

However, only few papers explore the extraction of active ingredients with surface-active ionic liquids. Yao *et al.* extracted different tanshinones, namely tanshinone I, tanshinone IIA and cryptotanshinone from *Salvia miltiorrhiza* bunge. Based on bromide anions, the chain length of 1-alkyl-3-methylimdazolium cation was varied, as tanshinones are lipophilic compounds. While [C$_8$mim]Br failed to extract the active ingredients, the extraction yield increased dramatically for tanshinone I and tanshinone IIA when the chain length was increasing from C_{10} to C_{16}.[128] Microwave assisted micellar extraction was also successfully applied by Lin *et al.* in 2012 using an aqueous two-phase system consisting of [C$_n$mim]BF_4/NaH_2PO_4 for the active ingredients hyperin and isoquercitrin from *Apocynum*

venetum; however, better results were obtained with the butyl chain length rather than with the surface-active 1-methyl-3-octylimidazolium tetrafluoroborate.[145]

As these literature examples indicated that micellar extraction can be beneficial for the isolation of active ingredients, this technique was further investigated for the isolation of piperine (**58**).

4.2.4 Optimization of conditions

After developing a reliable HPLC strategy that allowed quantification of piperine (**58**) in the presence of the aqueous ionic liquid systems for a quick identification of the optimum conditions for the extraction process, the screening was started using a 50 mM [C_{12}mim]Cl (**52**) solution. With this concentration micelles are present (CMC = 13-15 mM) and the ionic liquid concentration was kept economically low. The work flow for the extraction of piperine (**58**) from pepper is represented in Figure 49. Ground pepper and the aqueous ionic liquid solution was stirred for a defined time at room temperature. A sample of the supernatant was taken, diluted, filtered over a syringe filter and analyzed *via* reversed phase HPLC.

*Figure 49: Work flow for the isolation of piperine (**58**) from black pepper*

In a first step biomass extraction time and biomass loading was investigated: A 5 wt% solution of pepper in a 50 mM [C_{12}mim]Cl (**52**) solution was stirred at room temperature for a defined time. With increasing extraction time, extraction efficiency also increased (Table 12, entries 1-3). As a compromise between time demand and extraction yield, an extraction time of 3 hours was chosen, since already 3.74 wt% of piperine (**58**) per black pepper dry weight were extracted. When the ratio between biomass and ionic liquid solution was further investigated, only a slight difference between 1 wt% and 5 wt% biomass loading was found. However, with a higher biomass loading of 10 wt% the extraction efficiency decreased significantly, which might be related to mixing problems or saturation of the micellar solution, (Table 12, entries 4-6) since black pepper usually contains up to 6-9 wt% piperine (**58**).[214] However the first results were already promising as a high yield of 4 wt% piperine (**58**) after a short extraction time at room temperature was obtained.

*Table 12: Influence of extraction conditions on the extraction yield of piperine (**58**) from black pepper using a 50 mM solution of [C_{12}mim]Cl (**52**) in water.*

Entry[a]	Conditions	Piperine (58) (wt%)[b,c]
1	1 h, 5 wt%	3.42 ± 0.38
2	3 h, 5 wt%	3.74 ± 0.19
3	24 h, 5 wt%	4.64 ± 0.30
4	3 h, 1 wt%	3.86 ± 0.27
5	3 h, 5 wt%	3.74 ± 0.19
6	3 h, 10 wt%	2.93 ± 0.11

[a] Performed with 50.0 ± 0.1 mg ground black pepper in 950 µL of ionic liquid (**52**) solution at 25 °C; [b] Yield was determined *via* HPLC using phenol as internal standard; [c] Average of three independent experiments (mean ± STD, *n* = 3).

Therefore it was decided to perform further investigations using a 5 wt% solution of pepper in ionic liquid solution and a stirring time of 3 hours at room temperature.

4.2.5 Influence of the aqueous ionic liquid solutions on the extraction of piperine (58)

Based on the optimum conditions, the focus was set on the influence of different concentrations of [C_{12}mim]Cl (**52**) in water. An interesting correlation of the CMC of the respective ionic liquid with the extraction yield of piperine (**58**) was found: At low concentrations of ≤ 10 mM, only small amounts of piperine (**58**) < 0.20 wt% could be extracted, which is only slightly higher than the extraction yield that was obtained with pure water at similar conditions. However, as soon as the concentration of [C_{12}mim]Cl (**52**) reached the CMC (15 mM) a significant increase in the extraction efficiency was observed.[208-210] At higher concentrations the extraction yields leveled off, which might be caused by the saturation of the solution. (Figure 50)

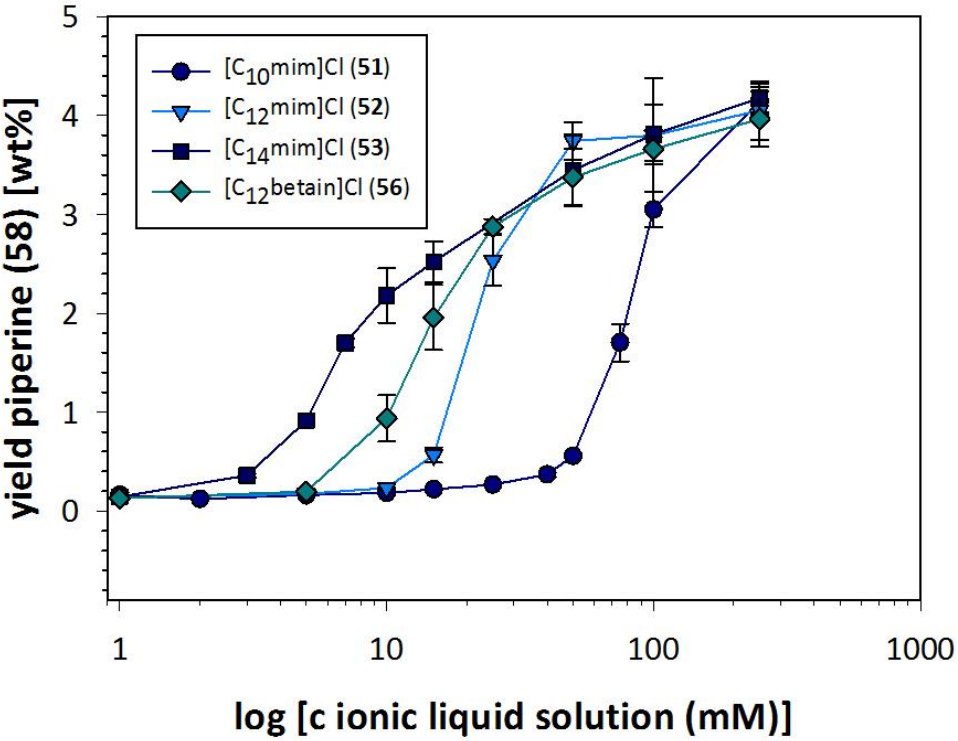


*Figure 50: Concentration dependency of ionic liquids in water on the extraction of piperine (**58**)*

A similar pattern was observed when expanding the range of ionic liquids. For solutions of [C_{10}mim]Cl (**51**), [C_{14}mim]Cl (**53**) as well as the biodegradable [C_{12}betaine]Cl (**56**) the extraction efficiency dramatically increased at concentrations higher than CMC of the respective ionic liquid. For all ionic liquids, the extraction yield eventually leveled off at concentrations higher than 100 mM, and up to ~4 wt% of piperine (**58**) could be extracted.

The increase in the extraction efficiency can be correlated with the chain length and in the order of [C_{14}mim]Cl (**53**) > [C_{12}mim]Cl (**52**) ≈ [C_{12}betaine]Cl (**56**) > [C_{10}mim]Cl (**51**) was found. This again is in accordance with literature data, as a linear relationship between the logarithm of CMC and the number of carbon atoms *n* is found for 1-alkyl-3-methylimidazolium-based ionic liquids [$C_n$mim]Cl. (Figure 42) As the CMC is decreasing with increasing chain length, the formation of micelles at low concentrations might be responsible for the higher extraction yields for the ionic liquids containing a longer alkyl chain.

In comparison to the large effect of the chain length in the cation, the anion in the surface-active ionic liquids [C_{12}mim]X had less influence on the extraction efficiency. (Table 13) With the exception of the triflate anion (X = OTf (**60**)) that gave only moderate yields of 2.28 wt%, all other anions including halides (X = Cl^- (**52**) or Br^- (**59**)), dicyanamide (X = $N(CN)^-$ (**61**)) or acetate (X = OAc^- (**62**)) were suitable for the extraction and gave comparable yields between 3.20 and 3.74 wt%. (Table 13) This is not surprising as the CMC of those compounds are below 50 mM.[ii] In case of using a 50 mM [C_{10}mim]Cl (**51**) solution only 0.56 ± 0.02 wt% piperine (**58**) was extracted, as the CMC is between 39 and 55 mM. The amount of micelles formed in the [C_{10}mim]Cl (**51**) solution is lower and therefore the solution cannot take up the same amount of piperine (**58**) compared to a [C_{12}mim]Cl (**52**) solution.

Table 13: Influence of different anions on the extraction yield of piperine from black pepper using a 50 mM solution of [C_{12}mim]X in water.

Entry[a]	Ionic liquid solution	Piperine (58) [wt%][b,c]
1	50 mM [C_{12}mim]OTf (**60**)	2.28 ± 0.19
2	50 mM [C_{12}mim]Cl (**52**)	3.74 ± 0.19
3	50 mM [C_{12}mim]Br (**59**)	3.08 ± 0.13
4	50 mM [C_{12}mim]$N(CN)_2$ (**61**)	3.28 ± 0.33
5	50 mM [C_{12}mim]OAc (**62**)	3.35 ± 0.45

[a] Performed with 50.0 ± 0.1 mg ground black pepper in 950 µL of ionic liquid solution at 25 °C; [b] yield was determined *via* HPLC using phenol as internal standard; [c] average of three independent experiments (mean ± STD, *n* = 3).

4.2.6 Comparison to conventional solvents

When comparing the extraction performance of micellar solutions of 1-alkyl-3-methylimidazolium-based ionic liquids and the biodegradable ionic liquid **56**, it was found that these solutions can compete with different conventional organic solvents reported in literature. The volatile and toxic solvents such as chloroform, methanol and toluene gave only slightly higher yields of 3.81 - 4.10 wt%, whereas the environmentally more benign butyl acetate (BuOAc) extracted around 3.10 wt% and gave the lowest yield among the organic solvents screened (Figure 51).

[ii] [C_{12}mim]Br 9 mM, [C_{12}mim]OTf 2 mM, unpublished results by Alice Cognigni.

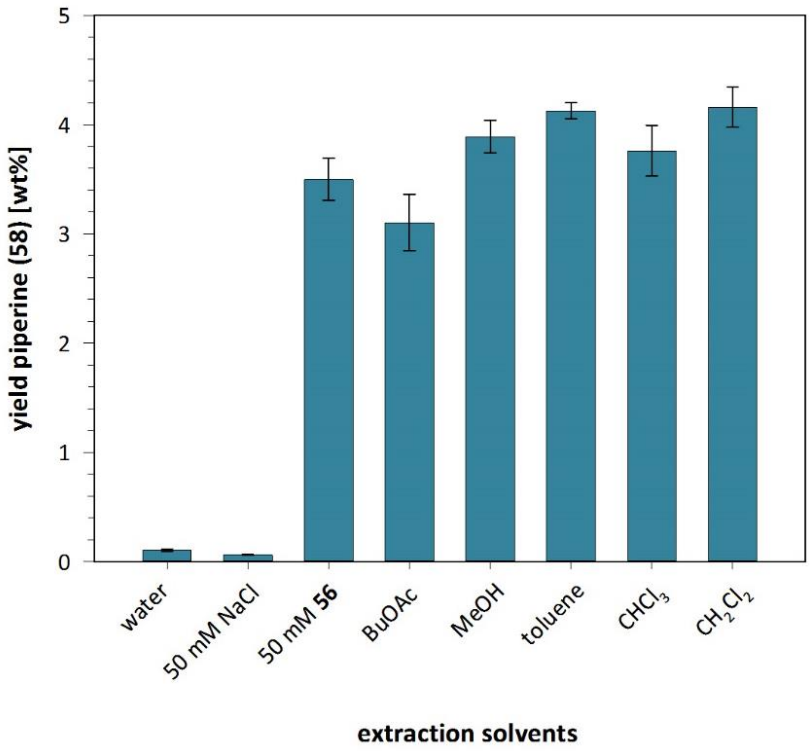


*Figure 51: Comparison of conventional solvents and a 50 mM [C_{12}betaine]Cl (**56**) solution*

The importance of the surface-activity of the applied ionic liquid solution is obvious when the extraction yield is compared with the yield of a 50 mM solution of NaCl in water: Only 0.06 wt% could be isolated here, which is even below the extraction yield with pure water that reached 0.11 wt%. The influence of aqueous ionic liquid solutions compared to pure water is also visible from electron microscopy that was performed on the recovered biomass after extraction. Although micellar solution of 1-alkyl-3-methylimidazolium based ionic liquids [$C_n$mim]Cl did not completely dissolve biomass, some changes in biomass morphology were observed that could not be observed when pure water was used as extraction media (Figure 52). A similar effect was observed by Coutinho *et al.* who investigated the extraction of caffeine from guaraná seed and reported an increase in the ratio of broken cells to intact cells in the presence of ionic liquid/aqueous mixtures, although the biomass was not completely dissolved.[169]

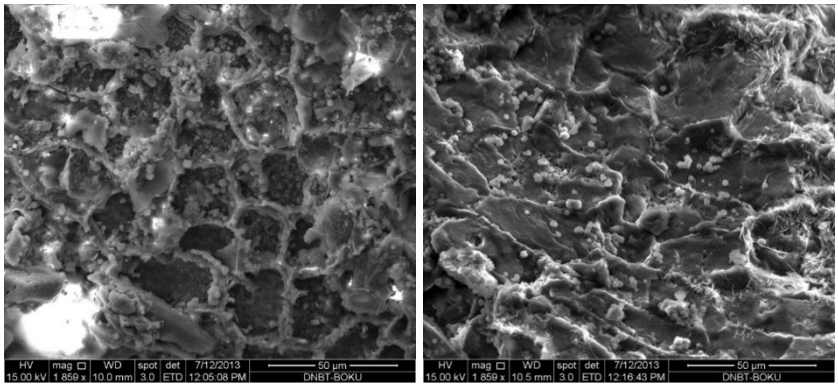


Figure 52: SEM pictures of the biomass treated with water (left) and with ionic liquid solution (right)

Consequently, it seems that complete solubilization of the biopolymer matrix is not always necessary for efficient extraction of the valuable ingredients: When comparing the extraction yields of aqueous

solution of the ionic liquids [C$_n$mim]Cl with those obtained with pure ionic liquids, it was found that the micellar systems can be a superior alternative to the neat ionic liquid (Table 14, entries 1-3). It should be noted that the extraction with pure ionic liquids had to be performed at 80 °C as [C$_{12}$mim]Cl (**52**) and [C$_{14}$mim]Cl (**53**) were solid at room temperature. Even at this temperature, viscosity problems occurred with longer alkyl chains and the extraction efficiencies decreased. Only [C$_{10}$mim]Cl (**51**), which is liquid at room temperature gave comparable yields to the aqueous micellar solution.

*Table 14: Extraction efficiencies of pure ionic liquids [C$_{10}$mim]Cl (**51**), [C$_{12}$mim]Cl (**52**) and [C$_{14}$mim]Cl (**53**)*

Entry[a]	Ionic liquid	Piperine (58) [wt%][b,c]
1	[C$_{10}$mim]Cl (**51**)	3.74 ± 0.28
2	[C$_{12}$mim]Cl (**52**)	3.57 ± 0.28
3	[C$_{14}$mim]Cl (**53**)	3.52 ± 0.28

[a] Performed with 50.0 ± 0.1 mg ground black pepper in 950.0 ± 5.0 mg of pure ionic liquid at 80 °C; [b] yield was determined *via* HPLC using phenol as internal standard; [c] average of three independent experiments (mean ± STD, n = 3).

4.2.7 Scaled procedure and reusability of the extraction media

Apart from the optimization of the extraction process, an isolation process including the recovery of the ionic liquid solution was developed. (Figure 53)

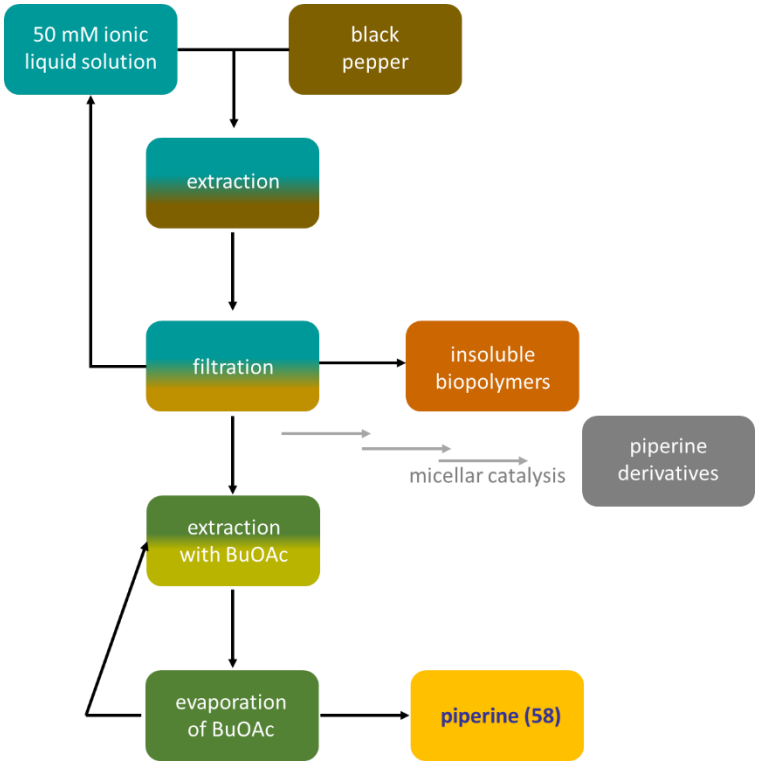


*Figure 53: Isolation flow scheme of piperine (**58**)*

Using the optimum conditions established (5 wt% solution of pepper in ionic liquid solution, 3 hours extraction time at room temperature) a 50 mM solution of ionic liquid of the biodegradable ionic liquid [C$_{12}$betaine]Cl (**56**) was chosen as extraction medium. The concentration of 50 mM represents a

compromise between price and extraction efficiency. A simple process was developed allowing not only the isolation of piperine (**58**), but also the recovery of the micellar ionic liquid solution. After extraction of 1.00 g ground black pepper with 19 mL of a 50 mM solution of [C_{12}betaine]Cl (**56**) in water for 3 h at room temperature, the remaining biomass was removed *via* filtration. Once the biomass was separated, the micellar solution was extracted with a small amount of environmentally benign BuOAc. It should be mentioned that other solvents tested (heptane, methyl-THF) showed either an unsatisfying extraction behavior or ionic liquid was co-extracted. After extraction with only 5 ml BuOAc piperine (**58**) was nearly quantitatively separated leaving less than 0.2 wt% piperine (**58**) in the ionic liquid micellar solution. This allowed not only a quick and clean separation of piperine (**58**) in excellent purity, but also the recovery of the aqueous solution containing the ionic liquid that could be directly used for four additional runs without any loss in performance (Figure 54).

Alternatively, the micellar solution containing piperine (**58**) could be directly subjected to any consecutive synthetic modification towards piperine-derivatives, which will be the object of our future studies.

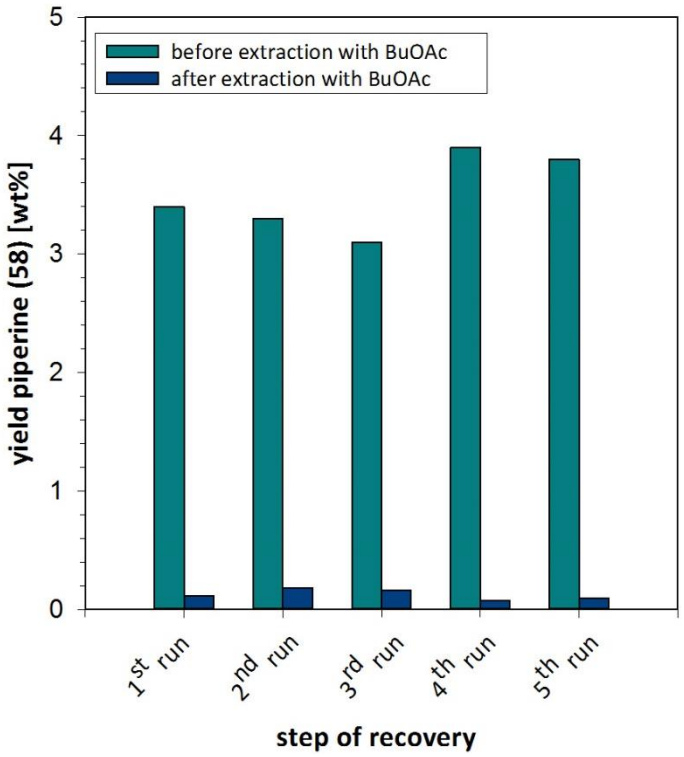


Figure 54: Recovery of the ionic liquid solution in the isolation process of piperine

*Purity of the extracted piperine (**58**)*

In order to assess the purity of extracted piperine (**58**) HPLCs and NMRs studies were performed. HPLC chromatograms of isolated piperine (**58**) showed no co-extracted impurities as can be seen in Figure 55. However, according to NMR, impurities were detected especially in the aliphatic area as it can be seen in Figure 56. However, if required piperine (**58**) could be further purified using either crystallization or column chromatography.

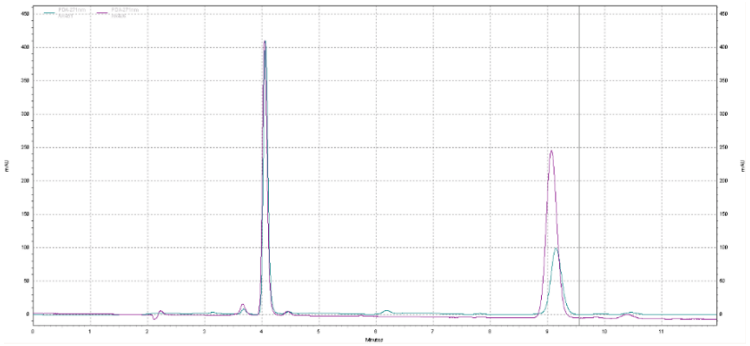


*Figure 55: RP-HPLC chromatograms of extracted piperine (**58**) (green) and reference piperine (**58**) (violett)*

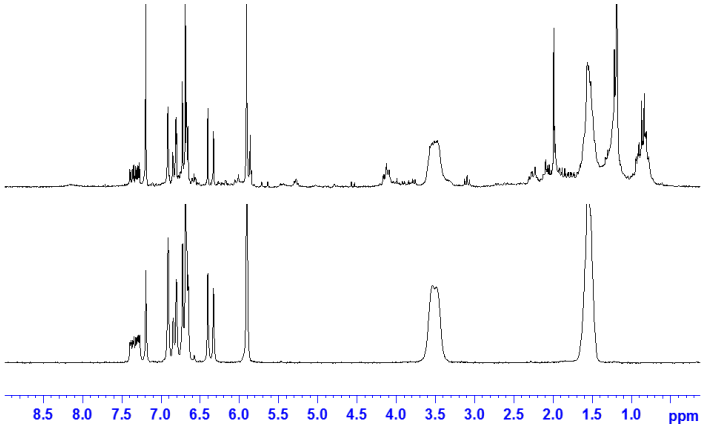


*Figure 56: ^{1}H-NMRs of extracted piperine (**58**) (top) and refernce piperine (**58**) (bottom)*

4.3 Towards the synthesis of isoeugenol (64)

The aim of this project was the combination of the previously shown micellar extraction[170] and *in situ* catalysis in the system, providing a one-pot strategy for the valorization of the extractant toward a valuable-added product. Long chain imidazolium ionic liquids have been previously reported as suitable medium for organic reactions, *i.e*. Diels-Alder reaction,[108] a nucleophilic substitution reaction or Heck reaction.[3] In the first two examples, the micellar media was responsible for a tremendous increase of the reaction rate compared to pure water. An interesting correlation between the concentration of the ionic liquid in water and the reaction rate was observed and the maximum reaction rate occurred above the CMC of the ionic liquids. In case of the Heck reaction the concentration of the ionic liquid was responsible for the formation of different palladium species. Best reactions yields were obtained with rather low concentration of the ionic liquid.

As a consequence of these aspects, this part of the thesis focuses on the *in situ* extraction and valorization of eugenol (**63**) from cloves. Eugenol (4-allyl-2-methoxyphenol, **63**) should be extracted from cloves in a first step and directly be converted to isoeugenol (2-methoxy-4-(1-propenyl)phenol, **64**). (Figure 57)

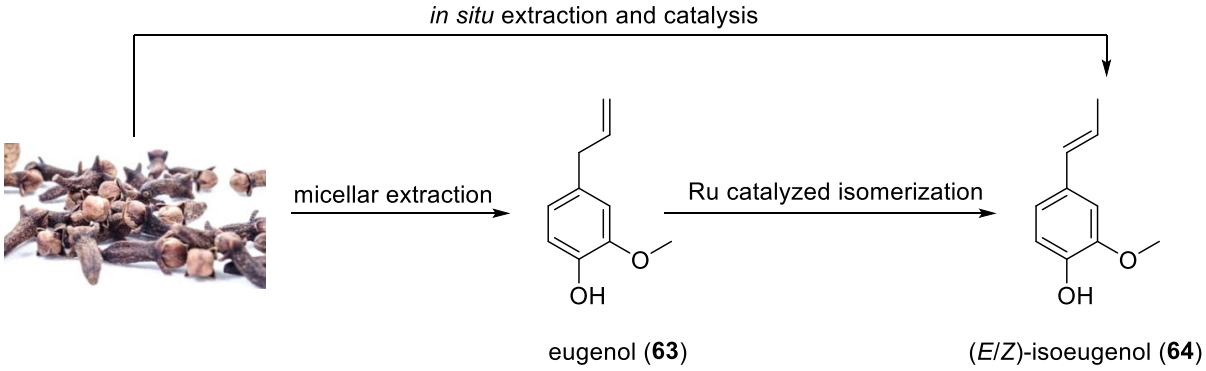


Figure 57: Micellar catalysis followed by Ru catalysis

4.3.1 Eugenol (63) and its impact for vanillin production

The fragrances eugenol (**63**) and isoeugenol (**64**) are widely used in industry for e.g. perfumeries but also for the production of vanillin. In a conventional synthesis isoeugenol (**64**) used to be synthesized from eugenol (**63**) using stoichiometric amounts of KOH in the presence of mostly higher alcohols at higher temperatures, followed by an ozonolysis to obtain vanillin.[221] (Figure 58)

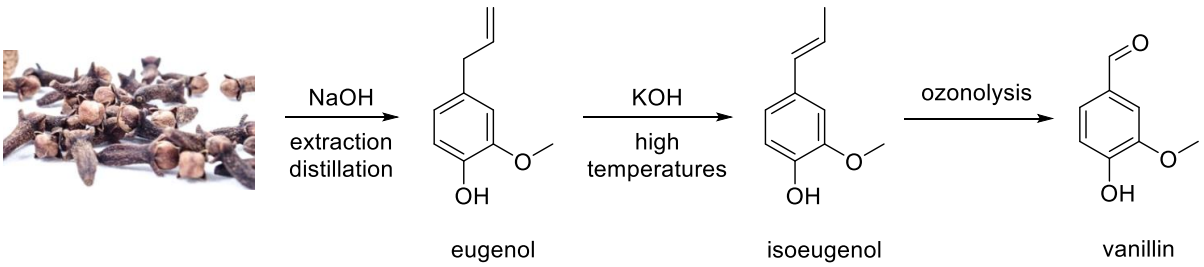


*Figure 58: Eugenol (**63**), isoeugenol (**64**) and vanillin*

[3] Unpublished results in the group.

Eugenol (**63**), that received its name from the Latin word for clove *Eugenia aromaticum* or *Eugenia caryophyllate*, is naturally occurring in the essential oil of clove in up to 72-90%.[222] It is responsible for the specific taste of cloves. Eugenol (**63**) has already been extracted using supercritical CO_2,[223] pressurized hot water[224] or water-organic solvents mixtures.[225] Conventionally it is isolated from clove or cinnamon leaf *via* extraction with sodium hydroxide solution. Nonphenolic materials are removed by steam distillation and eugenol (**63**) can be obtained by distillation after acidification.

E-isoeugenol (*E*)-(**64**) is used in manufacture of perfumeries, stabilizers and antioxidants for plastic and rubber industries. Furthermore application can be found in antiseptic and analgesic medicines.[226]

Vanillin has been isolated and identified as the chief constituent of vanilla bean. The demand of vanillin exceeds its natural occurrence about a factor of 10.[227] It used to be synthesized from eugenol (**63**) *via* isomerization and ozonlysis or as a by-product in the sulfite process.[228] Nowadays vanillin is mainly produced from the petrochemical raw material guaiacol in a 2 step process.[228] Guaiacol is converted to vanillylmandelic acid with glyocylic acid, which is oxidized to 4-hydroxy-3-methoxyphenylglyoxylic acid. After decarboxylation vanillin is obtained. (Figure 59)

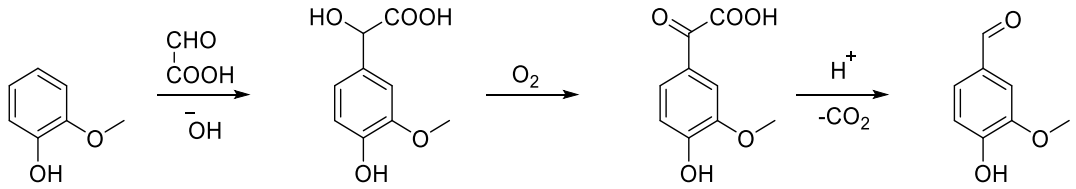
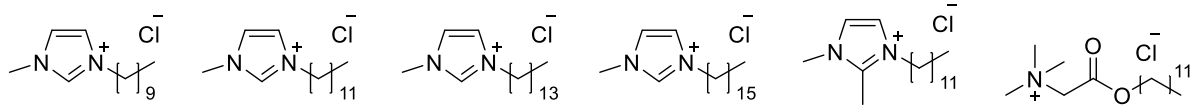


Figure 59: Industrial synthesis of vanillin

To aim for a natural product derived alternative synthesis of isoeugenol (**64**), eugenol (**63**) should first be extracted from cloves followed by isomerization to isoeugenol (**64**) using micellar ionic liquid systems.

4.3.2 Choice of ionic liquids

The choice of ionic liquids was based on the previous experience of isolation of piperine (**58**) using micellar solutions. (Chapter4.2) Different alkyl-methyl-imdazolium derivatives with alkyl chain length from 10 to 16 were chosen, since they showed good extraction efficiency for piperine (**58**) from black pepper. The biodegradable betaine ionic liquid **56** was also investigated. Furthermore the alkylated dimethyl-imidazolium chloride ionic liquid [$C_{12}m_2$im]Cl (**65**) was also tested, since this ionic liquid would be particular interesting for the catalysis step. (Figure 60)



90%	93%	91%	92%	available in the lab	88%
[C_{10}mim]Cl (**51**)	[C_{12}mim]Cl (**52**)	[C_{14}mim]Cl (**53**)	[C_{16}mim]Cl (**54**)	[$C_{12}m_2$im]Cl (**65**)	[C_{12}betaine]Cl (**56**)

*Figure 60: Surface-active ionic liquids used for the extraction of eugenol (**63**)*

4.3.3 Micellar extraction

After establishing a reliable HPLC strategy, a screening for identifying optimized conditions was performed: Therefore a 5 wt% solution of ground cloves in a 50 mM [C_{12}mim]Cl (**52**) solution was extracted for a defined time to obtain the ideal extraction time.

*Table 15: Time screening for the extraction of eugenol (**63**) from cloves*

Entry	Time [h]	Extraction yield 63 [wt%]
1	1	0.51 ± 0.01
2	3	0.50 ± 0.01
3	6	0.50 ± 0.01
4	24	0.45 ± 0.01

A 50 wt% solution of ground cloves in 50 mM [C_{12}mim]Cl (**52**) solution (50 mg cloves in 950 µl ionic liquid solution) was stirred for a defined time. Yields were analyzed using RP-HPLC. All extractions were performed in triplicates.

Table 15 shows that the extraction is rather independent on time. The extraction yield did not change with longer extraction time. After 24 h the extraction yield slightly decreased, which might be due to losses caused by the volatility of eugenol (**63**). In a next step the biomass loading was investigated using a fixed extraction time of 1 h.

*Table 16: Screening of biomass loading for the extraction of eugenol (**63**) from cloves*

Entry	Biomass loading[wt%]	Extraction yield 63 [wt%]
1	1	2.63 ± 0.16
2	2.5	2.45 ± 0.02
3	5	0.67 ± 0.05
4	10	0.36 ± 0.02

For a 1 wt% solution 10 mg cloves and 910 µl 50 mM [C_{12}mim]Cl (**52**) solution, for 2.5 wt% solution 25 mg cloves and 975 µl 50 mM [C_{12}mim]Cl solution, for a 5 wt% 50 mg cloves and 950 µl 50 mM [C_{12}mim]Cl (**52**) solution, and for 10 wt% 100 mg cloves and 900 µl 50 mM [C_{12}mim]Cl (**52**) solution, were stirred for 1 h at r.t. Yields were analyzed using RP-HPLC. All extractions were performed in triplicates.

As it can be seen in Table 16 the biomass loading significantly influenced the extraction yield of eugenol (**63**). The extraction yield dramatically decreased with increasing biomass loading. While a 1 wt% biomass loading yielded 2.6 wt% eugenol (**63**), the extraction yield was almost similar with 2.5 wt% when 2.5 wt% biomass loading were used. The extraction yield further dropped to 0.7 wt% and 0.4 wt% eugenol (**63**)when the biomass loading was increased to 5 wt% and 10 wt%. Therefore further investigations were performed using 2.5 wt% biomass loading and an extraction time of 1 h.

In a next step the influence of chain length of the cation of the ionic liquid and the concentration of the ionic liquid was investigated. (Figure 61)

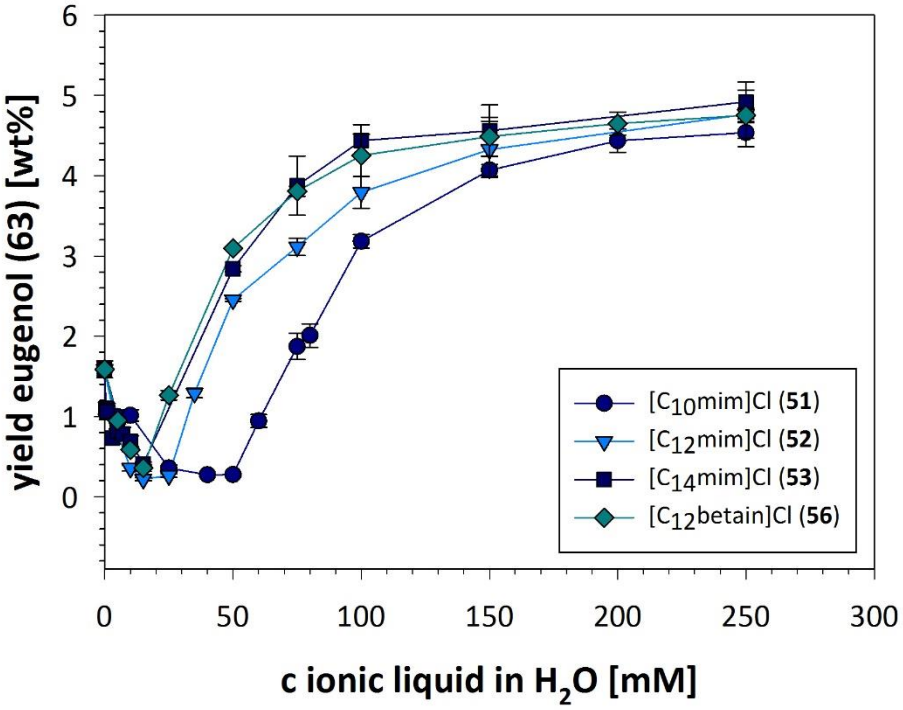


*Figure 61: Micellar extraction of eugenol (**63**) using micellar solutions*

Surprisingly, a decrease at of extraction efficiency was observed at very low concentrations, indicating that the pure water was a more suitable extraction medium than low concentrated ionic liquid solutions. Since the solubility of eugenol (**63**) in water is very low (2.5 g/l at 25 °C[4]) the addition of ionic liquid in low concentrations might saturate the aqueous system, thus reducing the uptake of eugenol (**63**). However, the extraction yield was tremendously increased when if the CMC was exceeded. The same phenomena was already observed for the micellar extraction of piperine (**58**). (Chapter 4.2.5) Therefore, shorter chain length ionic liquids with a length of 10 carbon atoms needed higher concentrations than longer chain ionic liquids for the same extraction efficiency at concentrations around the CMC. However at concentrations around 100 mM all ionic liquids gave an extraction yield of 3.0-4.5 wt%. With increasing concentration of the ionic liquid in water the extraction levelled off and a maximum extraction yield of 5.0 wt% was observed.

Furthermore, the C2-methylated [$C_{12}m_2$im]Cl (**65**) ionic liquid was also tested as extraction medium, as it might provide a different reactivity compared to [C_{12}mim]Cl in metal catalysis, as discussed later in detail. (Figure 66) However, as it can be seen in Figure 62 the extraction performance of the two C_{12}-imidazolium ionic liquids was the same. Not only the same initial decrease at low concentrations, but also the same increase at higher concentrations was observed.

4 http://gestis.itrust.de/nxt/gateway.dll/gestis_de/492573.xml?f=templates$fn=default.htm$3.0, last access 21.7.2015

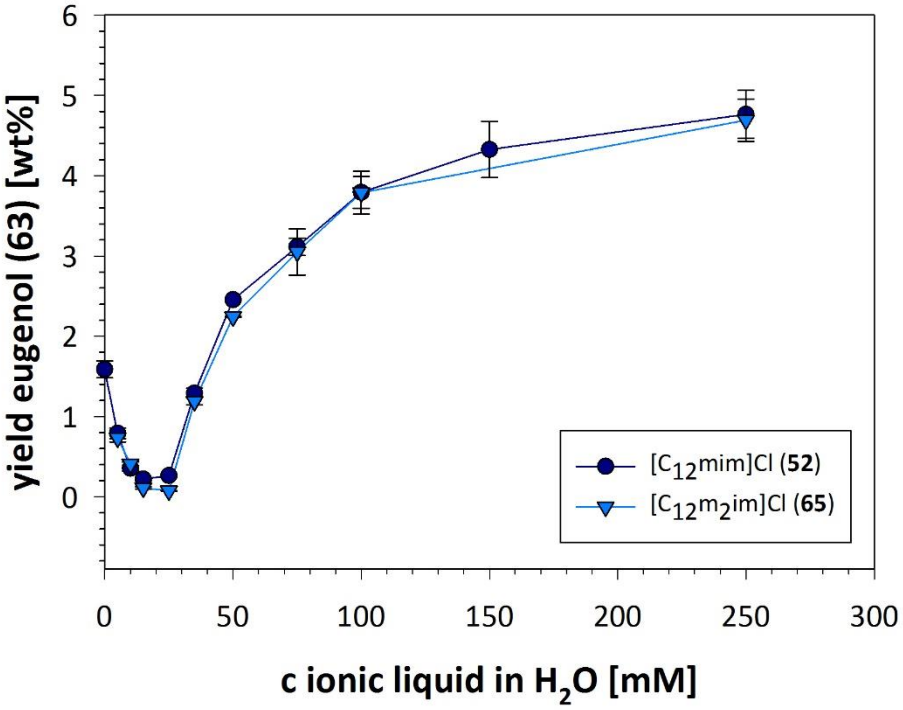


*Figure 62: Extraction efficiency of [C_{12}mim]Cl (**52**) and [$C_{12}m_2$im]Cl (**65**)*

Furthermore [C_{16}mim]Cl (**54**) was also briefly investigated. Table 17 shows a similar trend as previously seen for shorter alkyl-imidazolium chain lengths, although only a set of three concentrations was investigated.

*Table 17: Extraction of eugenol (**63**) from cloves using aqueous [C_{16}mim]Cl (**54**) solutions*

Entry	Concentration ionic liquid 54 [mM]	Extraction yield [wt%]
1	5	0.73 ± 0.05
2	15	0.47 ± 0.04
3	50	3.23 ± 0.20

In conclusion it can be stated that very low concentrated ionic liquid solutions (50 mM) already improved the extraction efficiency tremendously compared to pure water. Reliable extraction conditions have been established allowing focusing on the metal assisted isomerization of eugenol (**63**) to isoeugenol (**64**) in the next step.

4.3.4 Ruthenium catalyzed isomerization

During the past years considerable research efforts have been spent on ruthenium-catalyzed isomerization of double bonds.[229] However, the rapid and selective isomerization of eugenol (**63**) to isoeugenol (**64**) (Figure 63) *via* metal assisted catalysis remains challenging. High temperatures of approximate 200 °C are required and low yields with only moderate diastereoslectivity towards the *E*-isomer of iseugenol (**64**) are obtained. This is related to the competing deprotonation of the hydroxyl group and the methylene hydrogens of the allyl functionality.[230, 231]

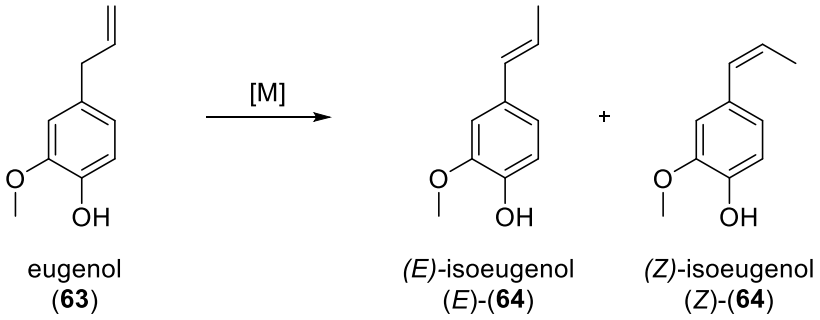


*Figure 63: Metal catalyzed isomerization of eugenol (**63**)*

After evaluation of literature, $RuCl_2(PPh_3)_3$ (**66**) was initially selected as catalyst for the isomerization of eugenol (**63**) to isoeugenol (**64**) as it is commercially available and reasonably priced. Sharma *et al*. obtained very good yields of 95% of isoeugenol (**64**) with an *E*/*Z* ratio of up to 95:1 in ethanol at 86 °C. A possible mechanism of the isomerization is represented in Figure 64. Generally two different mechanisms for the double bond isomerization cataylzed by transition metal complexes exist: π-allylic mechanism (1,3-hydrogen shift) or alkyl mechanism (1,2-hydrogen shift). While the allylic mechanism is based on β-C-H activation including a three-carbon arrangement in π-bonding to the metal, the alkyl mechanism is mostly followed by metal hydride complex formation.[232] For the $RuCl_2(PPh_3)_3$ (**66**) catalyzed isomerization of eugenol (**63**) high temperature of 100 °C activate the catalyst by cleavage of a triphenylphosphine group in a first step. (Figure 64) This is followed by the interaction of π-allylic electron of eugenol (**63**) with the transition metal to form a π-complex with the coordinative unsaturated metal complex. After hydrogen migration (oxidative addition) followed by reductive elimination *via* a hydride shift from π-allyl metal-hydride complex to α-carbon. In a next equilibrium step the double bond is formed and dissociation from the catalyst leads to the two isomers. Finally the catalyst is released and the cycle can restart.

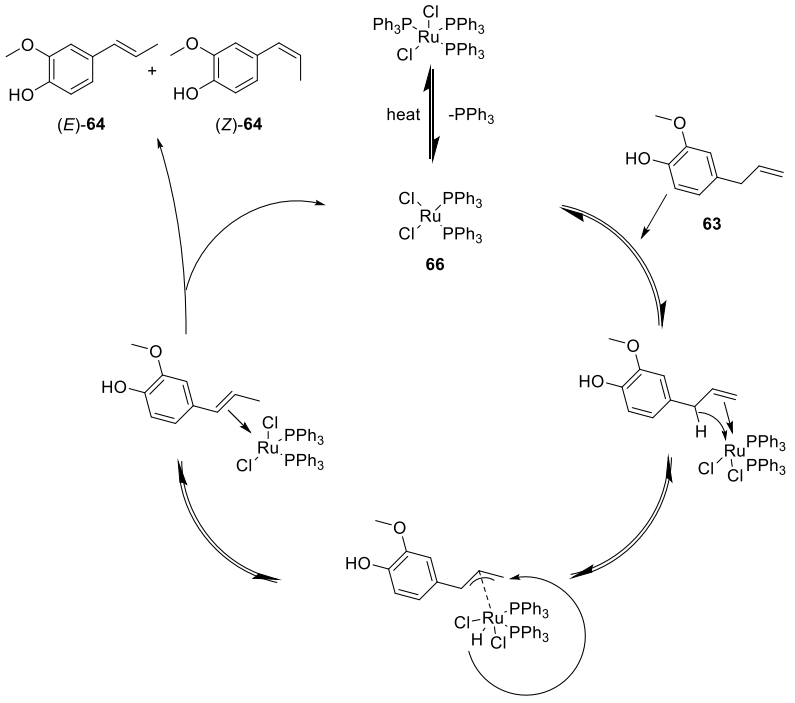


*Figure 64: Possible mechanism of isomerization of eugenol (**63**) to isoeugenol (**64**) using a ruthenium catalyst[233]*

However in protic polar solvents the mechanism for the $RuCl_2(PPh_3)_3$ (**66**) catalyzed isomerization might be different, as outlined in Figure 65: Due to the high conversion and selectivity in alcohols, it is assumed that that the catalyst is faster transformed to the active species. It is possible that the ruthenium hydride complex is formed *via* oxidative addition by the solvent. Hydride ruthenium complexes have been reported to have a high activity towards double bond isomerization.[234] However, Sharma *et al*. stated that none of the previous shown reaction mechanism was proven yet.[233]

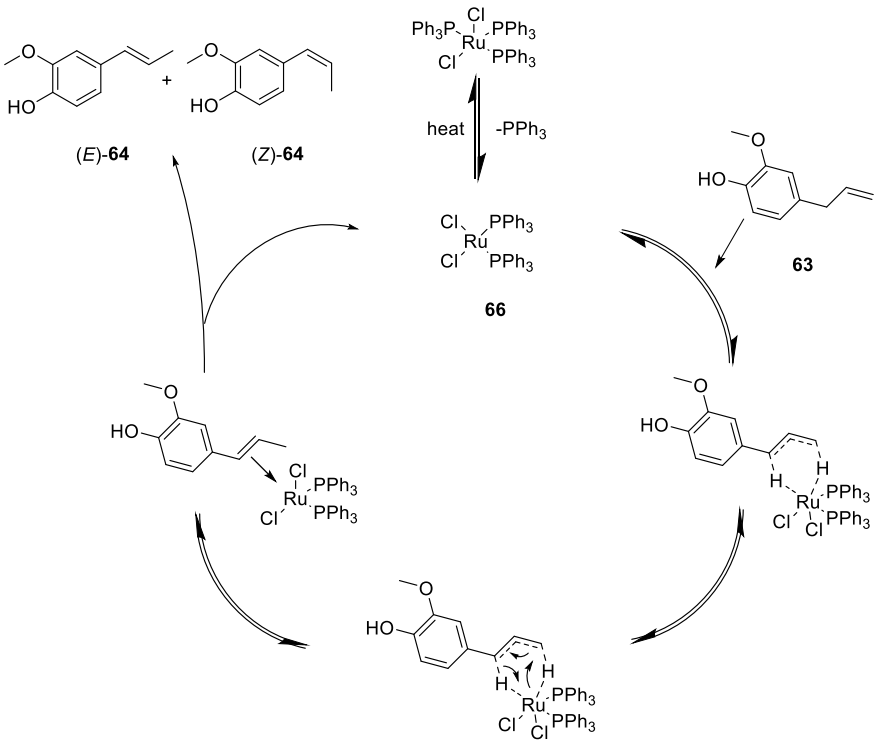


*Figure 65: Possible mechanism of isomerization of eugenol (**63**) to isoeugenol (**64**) using a ruthenium catalyst in protic solvents*[233]

1-Alkyl-3-methylimidazolium based ionic liquids such as $[C_{12}mim]Cl$ (**52**) have an acidic hydrogen in position 2 available. In the presence of a base this proton can easily be cleaved and a carbene can be formed with a transition metal. In case of 1-alkyl-2,3-dimethylimidazolium based ionic liquids e.g. $[C_{12}m_2im]Cl$ (**65**)position 2 is blocked with the bulky methyl group. Therefore carbene formation is not possible and a difference in reactivity might be expected.

*Figure 66: Reactivity of $[C_{12}mim]Cl$ (**52**) and $[C_{12}m_2im]Cl$ (**65**)*

Investigations for the $RuCl_2(PPh_3)_3$ (**66**) catalyzed isomerization were started after setting up reliable GC conditions. (Figure 67)

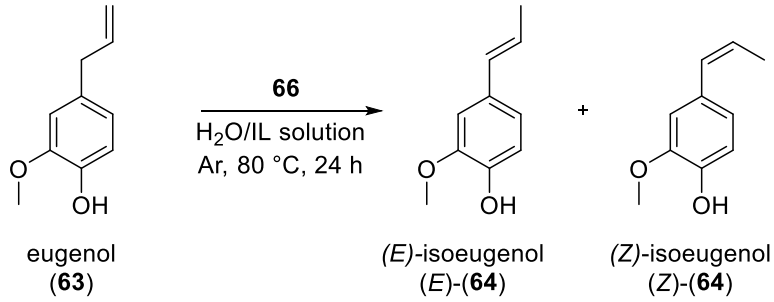


Figure 67: Isomerization catalyzed by $RuCl_2(PPh_3)_3$

In order to investigate the necessity of the degassing step, the reaction was performed in water and in degassed water. (Table 18) Entry 1 states that 10% of the product is formed using degassed water, while only 2% product are observed without degassing step.

Table 18: Isomerization using degassed and not degassed water

Entry	Water	Conversion eugenol (63) [%]	Yield isoeugenol (64) [%]
1	degassed	53.1	10.0
2	not degassed	42.9	2.4

Therefore all further experiments are carried out in degassed solution of ionic liquid in water. In a first step the concentration of ionic liquid in water was varied. (Figure 68) Full conversion with a moderate yield around 52% was observed in case of an ionic liquid concentration of 5 mM and 15 mM. If the concentration of the ionic liquid was increased to 25 mM a decrease in conversion to 82% with a yield of only 43% was obtained. If the concentration was further increased to 50 mM and 100 mM the conversion significantly dropped to 53% and 43% and poor yields around 10% isoeugenol (**64**) were obtained. Several byproducts, including dimerized products obtained *via* a competitive metathesis step were also observed in the GC spectra for all concentrations.

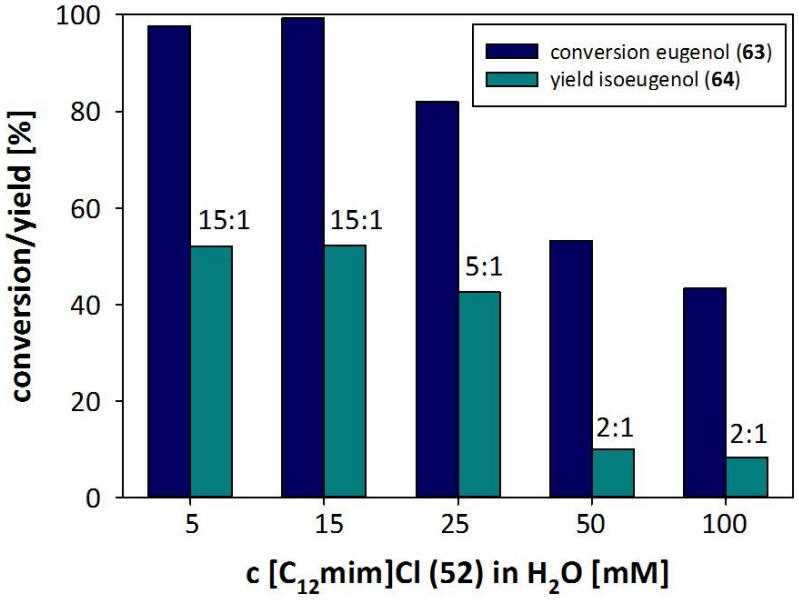


*Figure 68: Isomerization with E/Z ratio using different concentrations of [C_{12}mim]Cl (**52**) in water*

Next the influence of the concentration of eugenol (**63**) on the reaction was investigated. Table 19 shows that with increasing concentration of eugenol (**63**) the conversion also increased.

Table 19: Variation for the concentration of eugenol

Entry	Concentration eugenol (63) [mg/ml]	Conversion eugenol (63) [%]	Yield isoeugenol (64) [%]	*E/Z* ratio
1	6	53.7	-	-
2	12	53.0	10.0	2:1
3	24	99.3	65.3	13:1
4	48	100	68.5	18:1

Performed in 1 ml degassed water with a concentration of 50 mM of [C_{12}mim]Cl (**52**) and 1 mol% catalyst under argon atmosphere at 100 °C with 24 h stirring time.

The amount of catalyst was reduced from 2 mol% with a yield of 69% to 1 mol% with a yield of 67% and 0.5 mol% to 54% yield isoeugenol (**64**).

To obtain isoeugenol (**64**) in high yields a significant higher concentration of eugenol (**63**) has to be used. According to the literature procedure a ratio of **63**:solvent of approximately 1:5 is needed to obtain high yields. This would correspond to a biphasic mixture of eugenol (**63**) and water and would not be comparable to eugenol (**63**) concentrations obtained *via* micellar extraction.

Since these isomerization did not meet the expectations, the catalyst was changed. According to Lastra-Barreira *et al.* the highly in water soluble catalyst **68** (Figure 69) gave yields of >99% in favour of (*E*)-isoeugenol (*E*)-(**64**) with a diastereomeric ratio of 97:3. They also evaluated the influence of pH value, showing that a neutral pH of 7.2 yielded more than 99% of the product after 15 min stirring at 80 °C. The pH did not have any influence on the yields, whereas the *E*:*Z* ratio changed slightly. Furthermore they did not observe isomerization using acidic (pH = 4.8) or basic (pH = 12.9) conditions at 80 °C for 48 h without the presence of catalyst.[235]

Therefore the catalyst was synthesized in a two-step synthesis with comparable yields to literature values. (Figure 69)

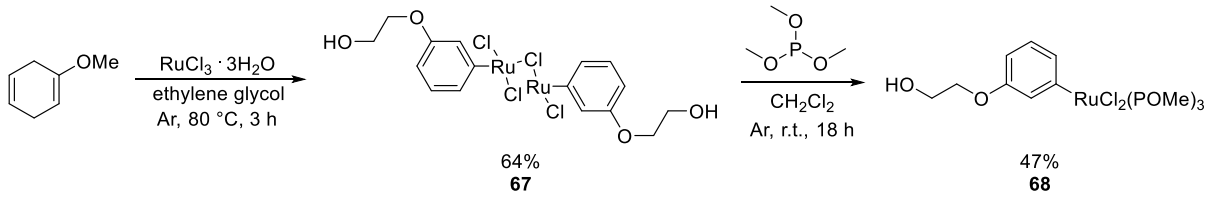


*Figure 69: Synthesis of catalyst **68***

The catalyst was briefly tested in different concentrations of ionic liquid solutions. (Table 20) Moderate to good conversion of eugenol (**63**) and selectivity for the desired product was observed. However, the diastereomeric ratio was significantly lower than described in literature.[235]

*Table 20: Isomerization using catalyst **68***

Entry	Concentration ionic liquid solution [mM]	Conversion eugenol (63) [%]	Yield isoeugenol (64) [%]	E/Z ratio
1	15	80.8	78:7	47:1
2	50	41.8	40.5	65:1
3	100	48.4	48.4	60:1

Performed using 1 ml degassed water, 17 mg eugenol (**63**), 1 mol% catalyst under argon atmosphere, 80 °C and 3 h stirring time.

Unfortunately, again concentration issues concerning eugenol (**63**) appeared. According to literature a two-phase system using eugenol (**63**) and water was used, which is not suitable for a possible combination of extraction and isomerization.[235]

4.3.5 *In situ* extraction and catalysis

After obtaining the optimal conditions for the extraction and some pre-experiments for the isomerization a short comparison between the different conditions indicated that the combination of extraction and catalysis might be challenging. (Table 21) While for the extraction a low concentrations up to 2 mg eugenol (**63**)/ml micellar solution was the maximum yield, the isomerization requires rather high concentrations, ideally 0.2 g eugenlol/ml solvent. On the other hand the concentration of the ionic liquid in water should exceed the critical micellar concentration (in case of [C_{12}mim]Cl (**52**) 45-55 mM). However concentrations higher than 15 mM were not beneficial for the Ru-catalyzed isomerization. Dilution of a more concentrated ionic liquid solution after extraction would not be a solution in this case, since the eugenol (**63**) concentration would be also decreased, which is not beneficial for the isomerization. Additionally, degassed water and Argon atmosphere is necessary for the isomerization reaction, requiring the extraction to be carried out under similar conditions.

Table 21: Comparison of optimal conditions for extraction and catalysis

Entry	Condition	Extraction	Catalysis
1	concentration of eugenol (**63**)	low	high
2	concentration of ionic liquid in water	high	low
3	water	not degassed	degassed
4	atmosphere	air	argon

Further experiments would address different chain lengths of the alkyl group in the cation of the ionic liquid. As the CMC is decreasing with increasing chain length, [C_{16}mim]Cl (**54**) showed a better extraction performance at lower ionic liquid concentrations in water. It might be possible to find a compromise of ionic liquid concentration for extraction and isomerization. However the isomerization should be tested in [C_{16}mim]Cl (**54**) for evaluation of best conditions. Still, it is doubtful that the ideal concentration of eugenol (**63**) for extraction and isomerization can be found.

The isolation of isoeugenol (**64**) *via in situ* extraction and isomerization was unfortunately not possible. Optimum conditions for extraction were contra productive for the isomerization and *vice versa*. Intensive screening of an appropriate ruthenium catalyst is necessary.

4.4 Towards the synthesis of betulinic acid (70) and bevirimat (71)

Since ionic liquids are suitable solvents for the dissolution of wood,[67] a strategy for improved synthesis of betulinic acid (**70**) and bevirimat (**71**) should be developed. Several strategies were investigated during this project towards the extraction and consecutive transformation towards the value-added products **70** and **71**.

- Extraction of betulin (**69**) from birch bark, oxidation to betulinic acid (**70**) and esterification to bevirimat (**71**)
- *In situ* extraction and oxidation of betulin (**69**) from birch bark to obtain **70** and esterification to bevirimat (**71**)
- Extraction of betulinic acid (**70**) from plane bark and esterification to bevirimat (**71**)
- *In situ* extraction and esterification of betulinic acid (**70**) from plane bark to obtain **71**

The enhanced isolation and *in situ* derivatization of betulin (**69**) from birch bark should be developed in this part of the thesis. The extracted betulin (**69**) should be directly oxidized to betulinic acid (**70**) and further reacted to bevirimat (**71**). Alternatively betulinic acid (**70**) should be extracted from plane bark and could be directly converted to bevirimat (**71**). (Figure 70).

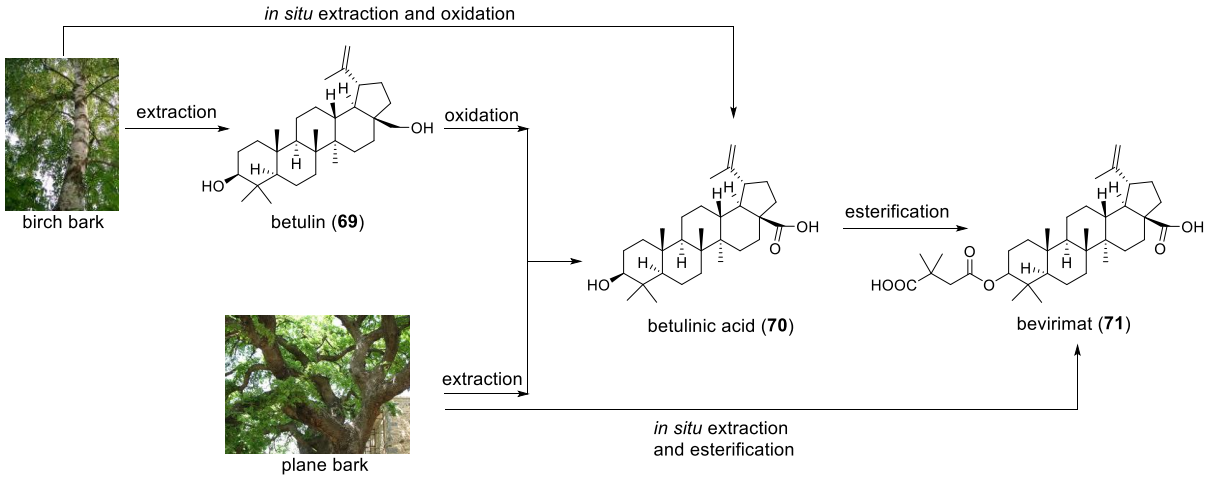


*Figure 70: Different approaches for the isolation/synthesis of betulinic acid (**70**) and bevirimat (**71**)*

4.4.1 Betulin (69)and derivatives

Betulin ((3β)-lup-20(29)-en-3,28-diol, **69**, Figure 71) is a naturally occurring triterpene alcohol present in birch bark (up to 30%).[236, 237] Betulin (**69**) is mostly accumulated in peridermal cells during spring and fill the intracellular space in summer.[238, 239] Already several hundred years ago people used betulin (**69**) and its derivatives obtained from birch bark as remedies against skin diseases. Pliny the elder, a roman naturalist first mentioned birches as "*gallica arbor*" which means "tree of the gauls", and produced a sap of the birch bark that was used as plaster. Later on during the 12th, 13th and 14th century scientists were using birch bark against wounds and renal calculi. In 1788, Lowitz described betulin (**69**) in pure form for the first time as white flakes which occur by putting the white cortex of a birch next to a fire. However, it was only in 1950 when the structure of betulin (**69**) was first determined and the lupane skeleton identified.[5] To date, there is no total synthesis reported in literature.

5 www.betulin.de, last accessed 18/4/2015.

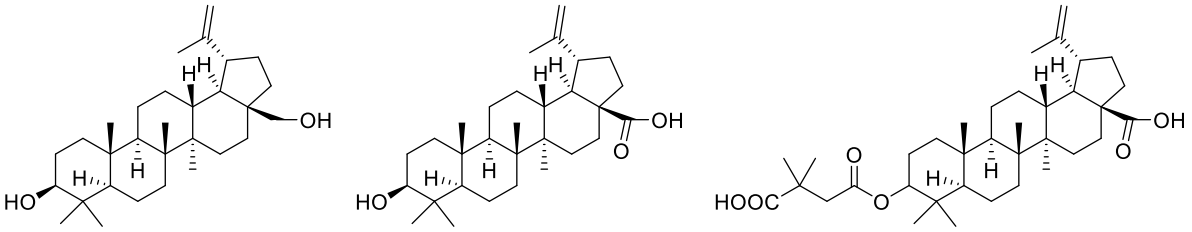


*Figure 71: Betulin **(69)**, betulinic acid **(70)** and bevirimat **(71)***

The extraction of **69** is usually performed using refluxing organic solvents, such as dichloromethane, chloroform, ethanol or methanol, since betulin (**69**) is a non-structural element of the plant cell and can be isolated from the outer bark. (Table 22)

*Table 22: Comparison of different extraction and isolation methods of **69** using conventional solvents*

Entry	Solvent	Yield 69 [wt%]	Purity	Purification	Reference
1	EtOH	26 (crude)	n.a.	chromatography	Drag[178]
2	Chloroform	17	>95%	chromatography	Pichette[179]
3	Toluene	0.3	96%	recrystallization	Sauter[180]

However, all processes require large amounts of volatile solvents and hours of refluxing to obtain moderate yields, and the obtained crude actives need to be further purified *via* expensive column chromatography or repeated crystallization.

Therefore other techniques such as microwave assisted extraction with limonene,[240] water-organic solvents mixtures,[225] supercritical CO_2 extraction,[241] ultrasound assisted extraction,[242] or microwave assisted ionic liquid extraction[165] have been reported.

Betulin (**69**) and its derivates, e.g. betulinic acid ((3β)-3-Hydroxy-lup-20(29)-en-28-oic acid, **70**), bevirimat (3-O-(3',3'-dimethylsuccinyl)betulinic acid, **71**) show antitumor, anti-HIV, antiviral, antibacterial, anti-inflammatory and antimalarial activities.[243] Betulin (**69**) and betulinic acid (**70**) show attractive antiviral activities, *e.g.* against herpes simplex type 1. In contrast, their antibacterial activities are rather poor, and they were found to be inactive against *Escherichia coli* and other bacteria. Anti-inflammatory activity has been reported for betulin (**69**) and betulinic acid (**70**), and they were effective against skin inflammation and ear edema. **69** is inactive against malaria whereas betulinic acid (**70**) shows moderate activity against *Plasmodium falciparum*.[243]

Tang *et al.* discovered that betulin (**69**) could be used as a leading compound for development of drugs for hyperlipidermia. Betulin (**69**) inhibits the pathway of SREBPs (sterol regulatory element-binding proteins) that offers a treatment against metabolic diseases, for instance type II diabetes and atherosclerosis. SREBPs are major transcription factors and responsible for the expression of genes involved in biosynthesis of cholesterol, fatty acid and triglyceride.[244]

Apart from its pharmaceutical properties, betulin (**69**) in the form of birch bark extract is commonly used as additive to shampoo, skincare, dental-care and hair-care products. Furthermore, the remedy

Betual® consisting of betulin (**69**) and birch bark extract is used as a dietary supplement, for liver protection, prevention and treatment of acute alcoholic intoxication and against hangover intensity.[237]

The derivative bevirimat (**71**) that is probably one of the most promising derivatives of betulin (**69**) was found to be active against Human Immunodeficiency Virus (HIV) infection. Bevirimat (**71**) is well tolerated and is currently in clinical trials.[245] Due to the fact that bevirimat (**71**) is well absorbed and tolerated, it passed clinical phase I and II and is at the moment in clinical phase III.[176]

4.4.2 Extraction of betulin (69) from birch bark

In this chapter the "upper" path towards the synthesis of betulinic acid (**70**) should be investigated. (Figure 72) The focus was set on the extraction of betulin (**69**) form birch bark using an ionic liquids based pretreatment step.

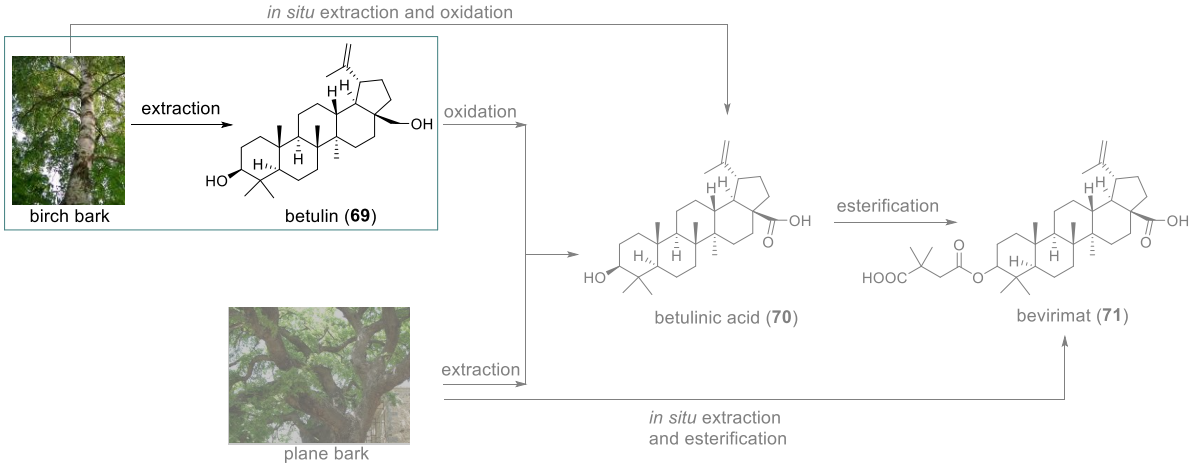


*Figure 72: Synthesis of betulinic acid (**70**) and bevirimat (**71**)*

The pretreatment of birch bark using pure ionic liquids or aqueous solutions thereof should deconstruct the lignocellulose biomass and therefore enable the access to active ingredients embedded in the biopolymer matrix. (Figure 82)

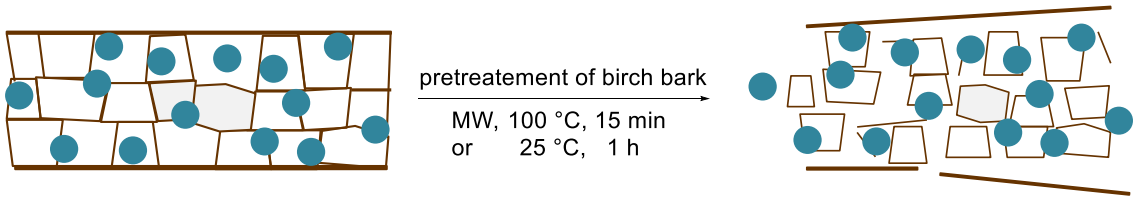


Figure 73: Pretreatment of birch bark

The pretreated bark should then be extracted using an organic solvent for the selective and enhanced isolation of the valuable ingredient. For the extraction of betulin (**69**) from birch bark the biomass should be dissolved in hydrophilic ionic liquids. After the formation of a two-phase system *via* the addition of water and an organic solvent betulin (**69**) should be enriched in the organic layer.

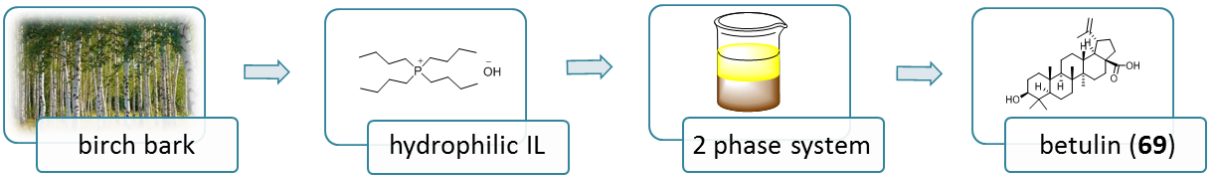


*Figure 74: Isolation approach for betulin (**69**)*

The extracted lupane derivative should then be directly converted to betulinic acid (**70**) followed by the synthesis to bevirimat (**71**).

4.4.2.1 Choice of ionic liquids

As desired approach for the isolation of betulin (**69**) a two-phase system should be applied, using an organic solvent as the upper phase and a mixture of water and ionic liquid as the lower phase. Therefore ionic liquids with a great hydrophilicity were chosen. Not only imidazolium derivatives such as [C_2mim]OAc (**72**), [C_2mim]Me_2PO_4 (**4**), [C_4mim]Cl (**2**), and [C_4mim]$MeSO_3$ (**73**), but also the cheap ammonium based ionic liquid [$HNEt_3$]HSO_4 (**74**), as well as the two phosphonium based ionic liquids [P_{4444}]Br (**75**) and [P_{4444}]OH (**76**). (Figure 75)

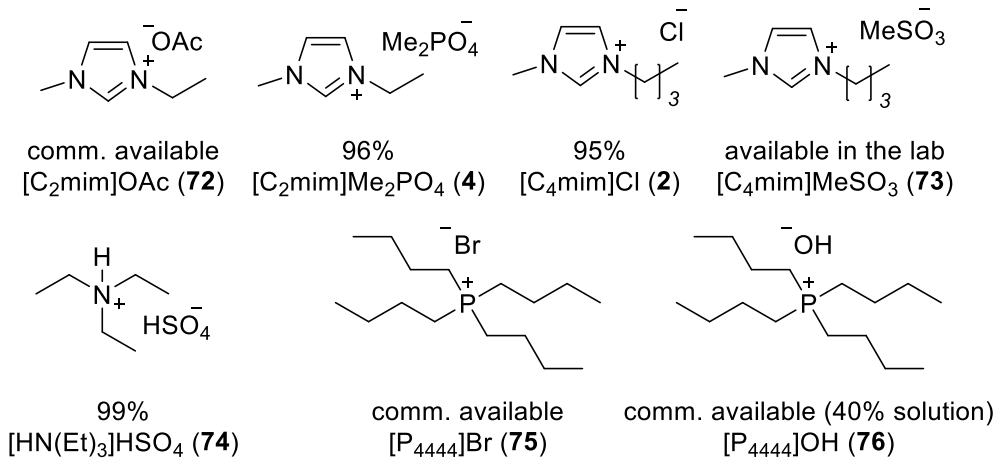


*Figure 75: Choice of hydrophilic ionic liquids for the extraction of betulin (**69**)*

Some of the imidazolium based ionic liquids have already been applied for the dissolution of lignocellulosic biomass and seemed therefore suitable for those extraction experiments: Wood was partially dissolved with a mixture of [C_4mim]Cl (**2**) and DMSO,[45] [C_2mim]OAc (**72**)[73] or in aqueous solutions of [P_{4444}]OH (**76**).

In 2012, Ohno *et al*. reported the dissolution of cellulose in aqueous [P_{4444}]OH (**76**) solutions. The authors could show that 15 wt% of cellulose were soluble in up to 50% water content in only 5 min at room temperature. If the system contained 40% of water, 20 wt% cellulose were soluble in 5 min only at room temperature.[79] The water present in the ionic liquid solution decreased the viscosity significantly. However a certain amount of hydroxide concentration is necessary, since a 10 wt% solution of [P_{4444}]OH (**76**) did not dissolve cellulose at all. They performed ^{1}H-NMR measurements of solutions of (**76**) and cellulose and observed the hydroxide anion/water peak shifted to lower magnetic field with increasing cellulose content, suggesting an interaction between hydroxide anion and the protons of the hydroxyl groups of cellulose. This observation is in accordance with literature, since a

typical mechanism of the dissolution of cellulose in ionic liquids is based on those hydrogen bond formations.[51]

Furthermore the aqueous ionic liquid [P_{4444}]OH (**76**) was applied for the dissolution of woody biomass at 60 °C[90] and the dissolution of wood at room temperature.[75] Pine, cedar and poplar wood were completely dissolved at room temperature in 6 months in a 40% solution of the ionic liquid [P_{4444}]OH (**76**) in water. The extraction degree of polysaccharides from poplar wood was up to 37% when using a 40% solution of (**76**) after 1 hour only. A longer extraction time of 24 h gave an extraction degree of 62% using a 30% solution of [P_{4444}]OH (**76**) in water at room temperature.

4.4.2.2 Extraction of betulin (69) using hydrophilic ionic liquids

In a recent paper the isolation of betulin (**69**) in high yields and purity using several hydrophilic and hydrophobic ionic liquids was reported.[165] Best isolation yields were obtained by dissolving birch bark in [C_2mim]OAc (**72**) for 15 min at 100 °C. Biopolymers were precipitated *via* the addition of ethanol and the solution was filtered. After the addition of water betulin (**69**) precipitated and was isolated after a second filtration step. The remaining filtrate was concentrated and the ionic liquid was successfully recovered. However, aiming for the consecutive *in situ* oxidation of betulin (**69**) this strategy had to be modified in order to avoid ethanol as co-solvent. Consequently, a two-phase extraction process was developed, starting initially with the dissolution of biomass in the ionic liquids or their aqueous solutions for 15 min at 100 °C under microwave irradiation. Water and BuOAc were added and MW irradiation was applied for a second time. After these stepwise dissolution and extractions steps, the betulin (**69**) content in the upper organic phase was quantified *via* HPLC.

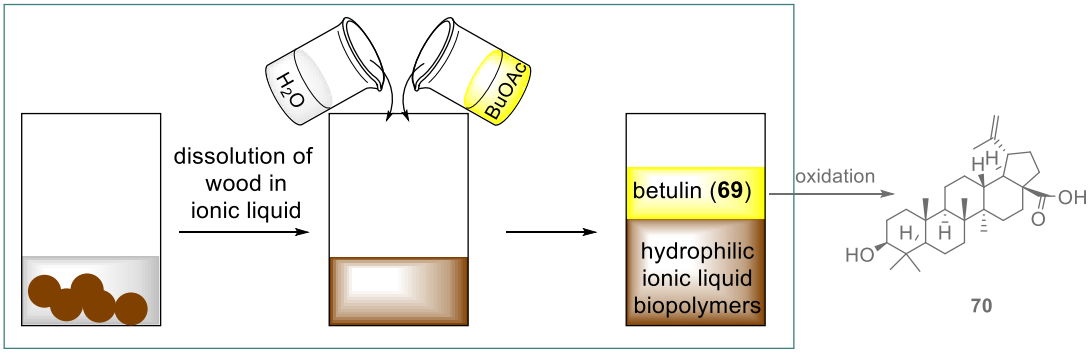


*Figure 76: Extraction of betulin (**69**) using a biphasic system*

The imidazolium based ionic liquids, e.g. [C_2mim]Me_2PO_4 (**4**) and [C_4mim]$MeSO_3$ (**73**) gave moderate yields of 19 wt% and 10 wt% betulin (**69**), whereas the application of [C_2mim]OAc and [C_4mim]Cl resulted in poor yields (< 5 wt% betulin (**69**)) for the combined dissolution and extraction into BuOAc. These low yields might be related to a phase transfer problem. It was observed that [C_2mim]OAc (**72**) and BuOAc were not miscible. The cheap ionic liquid [$HNEt_3$]HSO_4 (**74**)[27] showed poor performance and yielded only 4 wt% betulin (**69**).

However when aqueous solutions of phosphonium based ionic liquids were tested, the extraction efficiency increased tremendously. A solution of [P_{4444}]Br (**75**, 40%) gave good yields of 24 wt%, a 40% solution of [P_{4444}]OH (**76**) gave excellent yields of 29%. It was observed that the wood was dissolved in the ionic liquid. The concentration of [P_{4444}]OH (**76**) was decreased and with a 20% solution and good yields of 25 wt% were obtained. If the concentration was further decreased to 10%, the yield dropped

again and with a 5% solution the yield reached the same level as performing the pretreatment of birch bark with water (22%). A saturated NaCl solution gave the same extraction values as when performing the extraction with water. Furthermore a 40% of NaOH was also tested but only moderate yields were obtained, thereby emphasizing the unique role of this ionic liquid on the dissolution process.

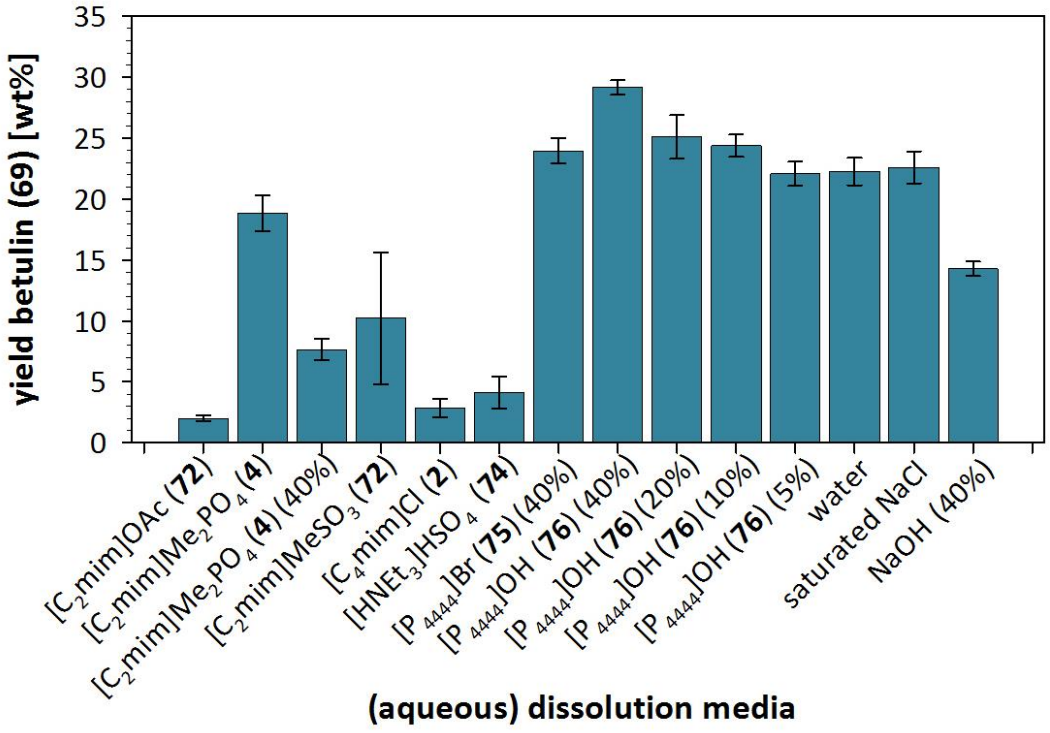


*Figure 77: Betulin (**69**) extraction using hydrophilic ionic liquids, water and BuOAc with MW irradiation*

In order to develop an energy saving process, the extraction efficiency of the ionic liquid/water/BuOAc system at room temperature was also investigated, as it was already shown that the phosphonium ionic liquid is able to dissolve wood at room temperature.[75] Bark was pretreated in water, saturated NaCl solution, a 40% solution of NaOH, [P_{4444}]OH (**76**) and [P_{4444}]Br (**75**), diluted with water and BuOAc and stirred again for 1 h. Figure 78 shows the comparison between microwave irradiation and room temperature stirring. It can be seen that the yield of betulin (**69**) dropped significantly when applying room temperature extraction in case of water, saturated NaCl solution or [P_{4444}]Br (**75**). The yield using aqueous NaOH solution was slightly increased probably due to avoidance of degradation. When using the ionic liquid [P_{4444}]OH (**76**) no significant difference between MW irradiation at 100 °C and room temperature extraction was observed. This outlines the powerful dissolution properties of the ionic liquid even at room temperature.

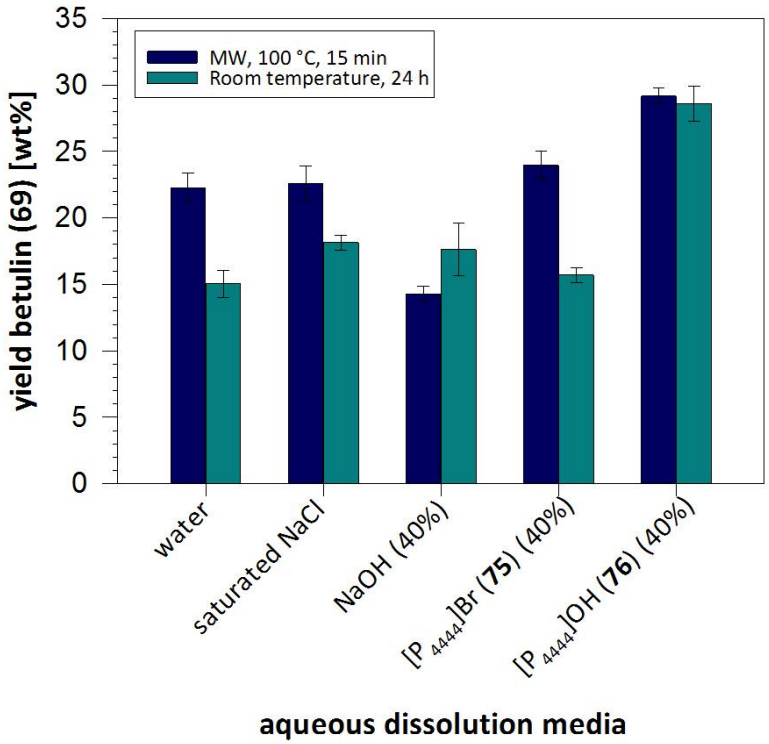


*Figure 78: Extraction of betulin (**69**) at room temperature and MW irradiation*

A time screening using aqueous $[P_{4444}]OH$ (**76**) was performed, showing a strong increase within the first hour. However, very good yields of 27 wt% were obtained after 1 hour pretreatment only and were increased to 28.6 wt% after 24 h, whereas 29.1 wt% were obtained using microwave irradiation.

*Table 23: Time screening of the extraction at r.t. using **76***

Entry	Time [h]	Yield betulin (69) [wt%]
1	0.5	20.4 (± 0.8)
2	1	26.5 (± 0.6)
3	3	27.5 (± 1.2)
4	6	27.0 (± 0.1)
5	24	28.6 (± 1.3)

Performed using 100 mg ground birch bark, 900 mg $[P_{4444}]OH$ (**76**) at defined stirring time at room temperature, addition of 2 ml water and 3 ml BuOAc and stirring of 1 h.

Furthermore the extraction time after the addition of water and BuOAc was investigated showing that the stirring time of 1 h is necessary as the extraction yield of betulin (**69**) was decreased to 12 wt% using a stirring time of 30 min. Therefore further extraction experiments are performed using microwave irradiation at 100 °C for 15 min for both the dissolution process of birch bark in ionic liquid and the two-phase extraction. Alternatively, dissolution process is performed at room temperature with stirring times of one hour each.

Betulin (**69**) was also isolated after performing the MW based process. However column chromatography was necessary for purification, since a byproduct lupeol (**77**, (3β)-lup-20(29)-en-3-ol) was co-extracted. Betulin (**69**) was obtained in 25 wt% yield, whereas lupeol (**77**) was and isolated in

3 wt% yield. The content of **77** in birch bark varies between 1-4 wt% according to Rizhikovs *et al*.[246] Crystallization from BuOAc was also possible, however the yield was significant lower.

SEM pictures

While preparing the biomass for the SEM-pictures it was already visible with the eye that pretreatment using [P_{4444}]OH (**76**) tremendously changed the biomass morphology. After filtration only small residues were remaining in the filter. As it can be seen in Figure 79 the morphology of the birch bark changed when treated with different solvents. Tremendous changes in the structure of the cell walls were obtained using the two phosphonium salts.

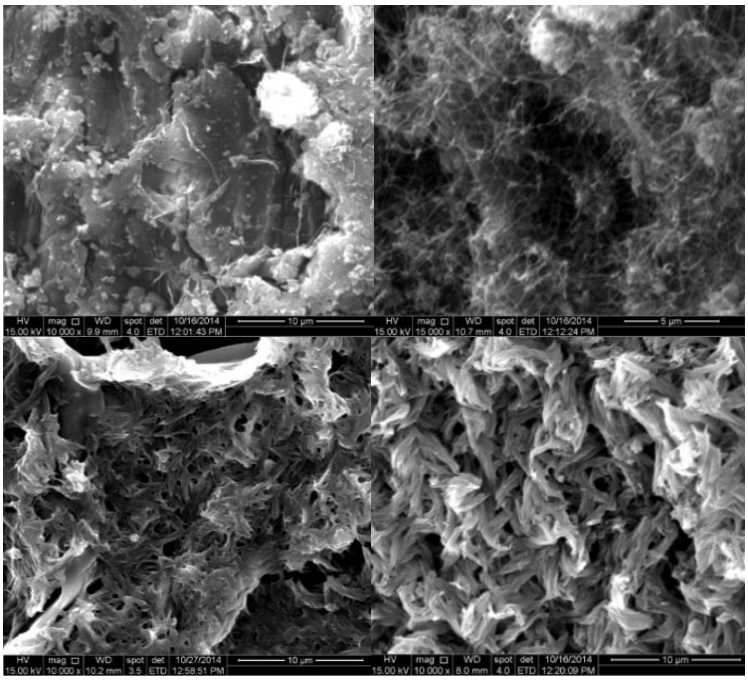


*Figure 79: SEM pictures of untreated birch bark (top, left), birch bark treated with NaOH (top, right), [P_{4444}]Br (**75,** bottom, left) and [P_{4444}]OH (**76**, bottom, right)*

Apart from the powerful biomass dissolution ability of the ionic liquid [P_{4444}]OH (**76**), the basic character of this ionic liquid represents another advantage for the extraction of betulin (**69**). After addition of water and BuOAc to the ionic liquid pretreated birch bark, he hydrophobic target molecule betulin (**69**) should be present in the organic layer, whereas biopolymers and the hydrophilic ionic liquid should be accumulated in the aqueous layer. Due to the basic character of the ionic liquid alcohols present in the lignin structure shown in Figure 80, might be deprotonated and exist in the aqueous layer as their salts. In contrast, betulin (**69**), betulinic acid (**70**) and even its carboxylate anion will be extracted in the organic phase due to its highly hydrophobic character.

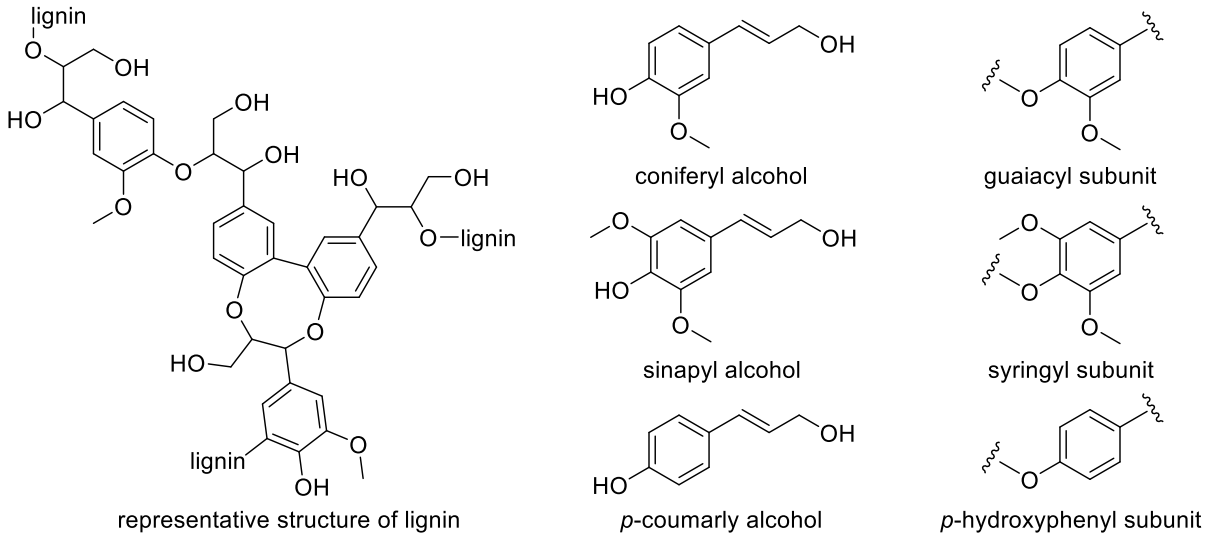


Figure 80: Structure of lignin and its subunits

4.4.3 Pretreatment of plane bark using aqueous ionic liquids

Following the "lower"path towards the synthesis of betulinic acid (**70**) and bevirimat (**71**), the biomass of choice is plane bark. Plane bark consists up to 3 wt% betulinic acid (**70**).[175]

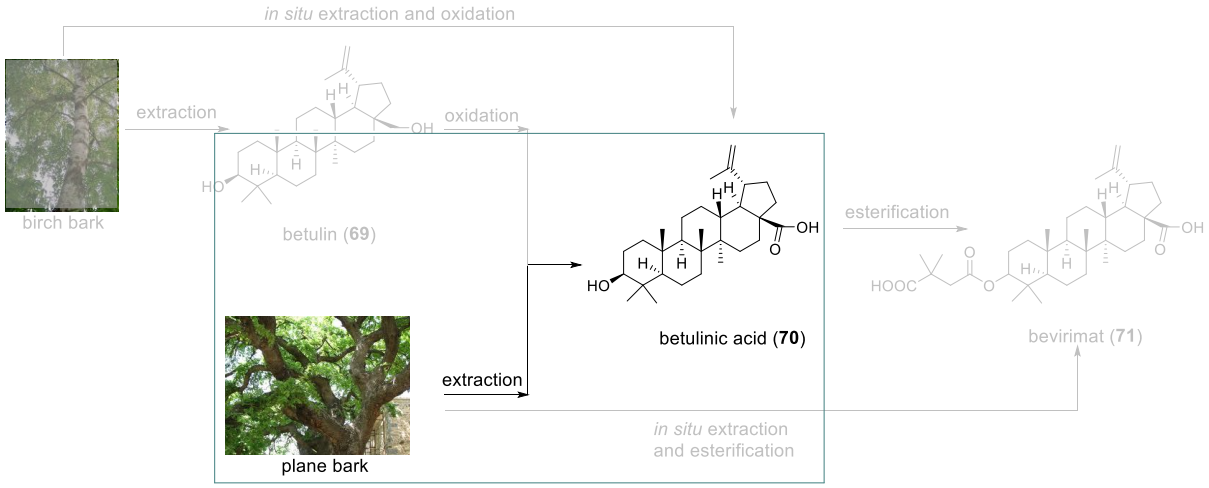


*Figure 81: Synthesis of betulinic acid (**70**) and bevirimat (**71**)*

The pretreatment of plane bark using aqueous ionic liquid solutions should deconstruct the lignocellulose biomass and therefore enable the access to active ingredients embedded in the biopolymer matrix. (Figure 82)

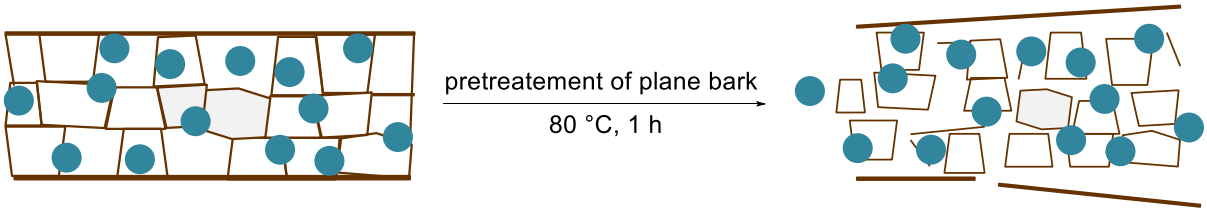
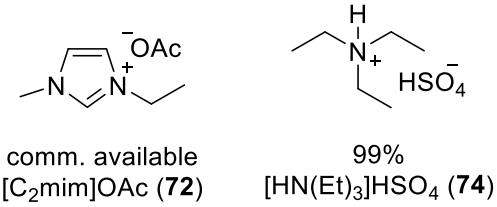


Figure 82: Pretreatment of plane bark

The pretreated bark should then be dried and be extracted using an organic solvent for the selective and enhanced isolation of betulinic acid (**70**).

4.4.3.1 Choice of ionic liquids

Based on the experience on the isolation of betulin (**69**)[165] [C_2mim]OAc (**72**) was an obvious choice for the pretreatment of plane bark. Additionally, the low-cost cheap protic ionic liquid [$HNEt_3$]SO_4 (**74**)was selected due to its reasonable price.



comm. available [C_2mim]OAc (**72**)

99% [$HN(Et)_3$]HSO_4 (**74**)

Figure 83: Choice of hydrophilic ionic liquids for the pretreatment of birch bark

4.4.3.2 Extraction of pretreated plane bark

After stirring the ground bark at 80 °C for one hour with aqueous solutions of ionic liquids to swell and partially dissolve the biomass, the solution was filtered and the bark was dried. A better access to the biomolecules located in the cell walls should therefore be guaranteed. The dried bark was then stirred with butyl acetate (BuOAc) or dimethylcarbonate (DMC) for one hour and the supernatant analyzed *via* HPLC.

Figure 84 shows the extraction yields of betulinic acid (**70**) using pretreated bark. The extraction yields are around 1.5 wt% using BuOAc as solvent. The pretreatment of the imidazolium derivative and the cheap protic ionic liquid [$HNEt_3$]SO_4 gave comparable results. However it can be seen that when changing the solvent to dimethylcarbonate a decrease of extraction efficiency was observed, probably due to solubility issues. It should be mentioned that the aqueous solutions were also checked and no trace of betulinic acid (**70**) was found.

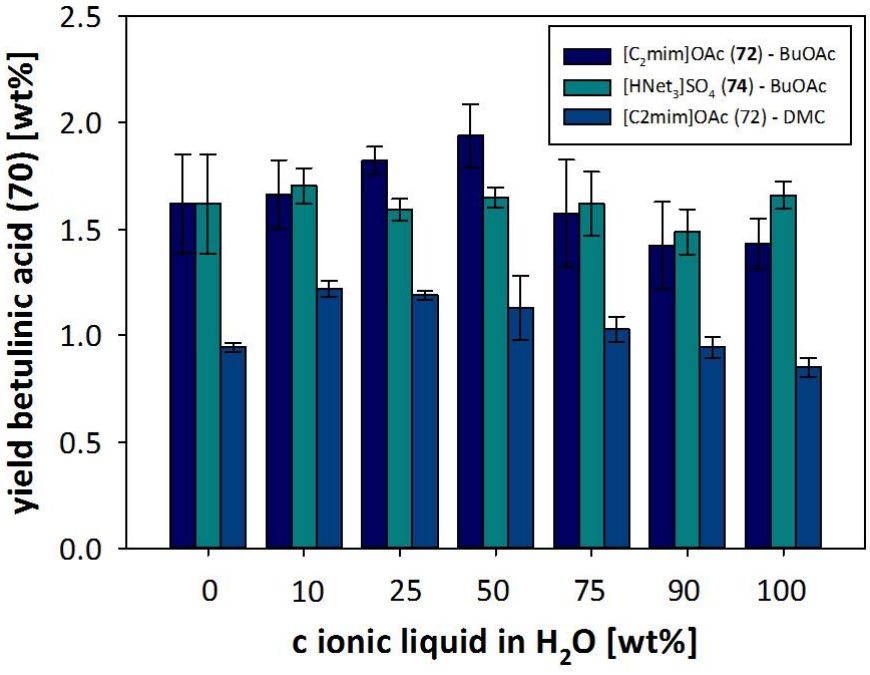


*Figure 84: Yield of betulinic acid (**70**) after pretreatment using aqueous solutions of [C_2mim]OAc (**72**) using BuOAc or DMC and [$HNEt_3$]HSO_4 (**74**) using BuOAc*

Since the pretreatment did not enable the enhancement of extraction yield, a second approach was chosen for the extraction of betulinic acid (**70**).

4.4.3.3 Two-phase extraction of betulinic acid (70) using hydrophilic ionic liquids

Another strategy based on a biphasic mixture of BuOAc and [C_2mim]OAc (**72**)/water was chosen as extraction solvent for the isolation of betulinic acid (**70**) from the bark of *platanus acerifola*.

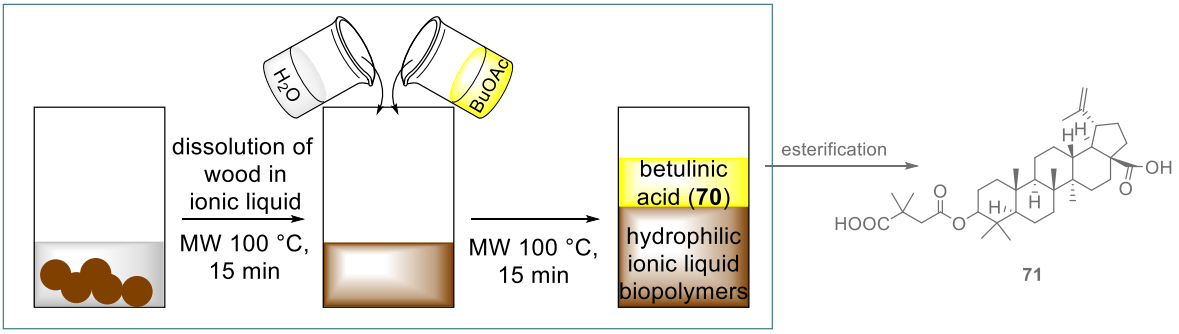


*Figure 85: Extraction of betulinic acid (**70**) using an ionic liquid/water/BuOAc system and further reaction to bevirimat (**71**)*

Wood was partially dissolved in (aqueous) ionic liquid solutions applying microwave irradiation. After the addition of water and BuOAc the active ingredient should be enriched in the organic layer. The organic layer should then be directly used for the esterification towards bevirimat (**71**). (Figure 85)

Table 24 shows the extraction yields of betulinic acid (**70**) obtained from plane bark. When dissolving plane bark in a 50:50 mixture of ionic liquid and water moderate yields of 1 wt% were obtained, if pure ionic liquid was used, the extraction yield remained the same. (Entry 1-2) The yield was increased to 2 wt% (entry 3) when a mixture of aqueous ionic liquid solution (**72**) and BuOAc was microwaved at the same time. Surprisingly best yields were obtained using pure BuOAc. (Entry 4) However a phase transfer problem seemed to be the reason, since if pure ionic liquid in combination with only BuOAc was used, no extraction yield was observed. (Entry 7)

*Table 24: Extraction yields of betulinic acid (**70**) using MW irradiaton*

Entry	MW 1, 100 °C, 15 min			Addition of solvents, MW 2, 100 °C, 15 min		Yield 70 [wt%]
	[C_2mim]OAc [mg]	water [mg]	BuOAc [mg]	water [mg]	BuOAc [mg]	
1	450	450			900	1.0 ± 0.2
2	900			900	900	1.1 ± 0.1
3	450	450	900			1.9 ± 0.1
4			900			2.6 ± 0.4
5		900				0.0
6	900					1.2 ± 0.4
7	900				900	0.0
8	450	450				0.0

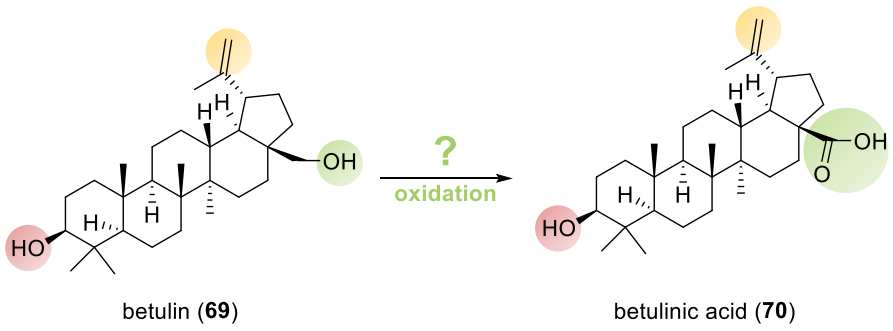
100 mg plane bark and [C_2mim]OAc (**72**)/water/BuOAC were heated under microwave irradiation at 80 °C for 15 min. Optionally water and BuOAc were added ant the solution heated again at 80 °C for 15 min. Analysis of the organic/aqueous layer *via* HPLC.

For further investigations the ionic liquid [P_{4444}]OH (**76**) represents a promising canditate, as this ionic liquid performed very well for the extraction of betulin (**69**) from birch bark. However only up to 3 wt% betulinic acid (**70**) are available in the plane bark[175] therefore the requirement of large amount of the waste product plane bark is necessary to obtain reasonable amounts of betulinic acid (**70**), the focus was set on the extraction of betulin (**69**) and its subsequent derivatization towards betulinic acid (**70**).

4.4.4 Synthesis of betulin derivatives

Since the extration of betulin (**69**) from birch bark worked particularly well, the focus was set on the *in situ* extraction and oxidation of betulin (**69**) from birch bark to betulinic acid (**70**).

The oxidation of betulin (**69**) to betulinic acid (**70**) provides a considerable synthetic challenge. As it can be seen from Figure 86 only the primary alcohol (green) group should be oxidized to a carboxylic acid group, whereas the secondary alcohol group (red) should remain untouched. Furthermore the oxidation sensitive double bond limits the choice of oxidation reagents.



betulin (**69**) betulinic acid (**70**)

*Figure 86: Functional groups of betulin (**69**)*

In order to develop reliable HPLC strategies betulin (**69**) and all possible oxidation products had to be synthesized (Figure 87), including not only the desired oxidation product betulinic acid (**70**) and its intermediate betulinic aldehyde (**78**, Lup-20(29)-en-28-al, 3β-hydroxy- (8CI)), but also betulonic aldehyde (**79**, Lup-20(30)-en-28-al, 3-oxo- (6CI,7CI)) and betulonic acid (**80**, lup-20(30)-en-28-oic acid, 3-oxo- (7CI)), as over-oxidation might occur or might be even desired in a first step.

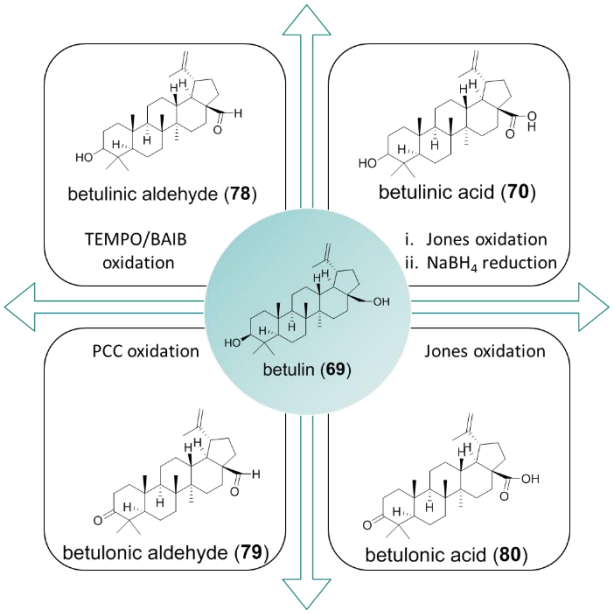


*Figure 87: Betulin (**69**) and its possible oxidation products*

The over-oxidation products betulonic acid (**80**) and betulonic aldehyde (**79**) were obtained using a chromium based oxidation. In case of betulonic acid (**80**) a Jones oxidation was applied whereas betulonic aldehyde (**79**) was synthesized using pyridinium chloro chromate (PCC, **81**).

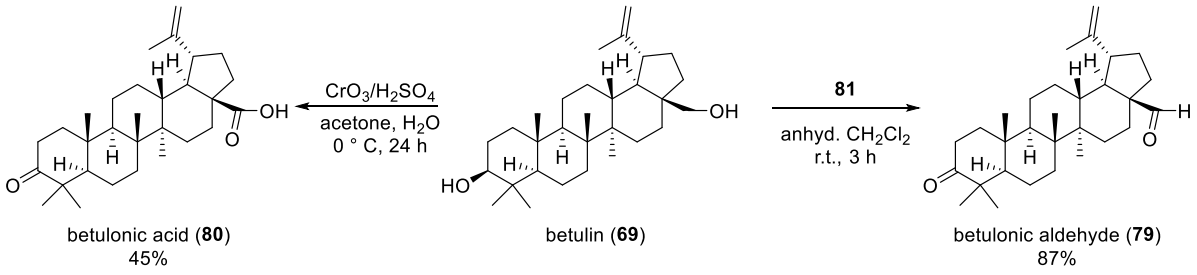


*Figure 88: Oxidation of betulin (**69**) to **80** via Jones oxidation and **79** with PCC (**81**)*

For the synthesis of betulonic acid (**80**) Jones oxidation was chosen, as with this oxidation reaction both alcohols groups can be oxidized. Although the reaction mechanism has not fully been elucidated yet, a suggestion is shown in Figure 89. For the Jones reagent chromium trioxid (VI) forms chromic acid (VI) with water and sulfuric acid. This species can then be attacked by an alcohol forming the chromate ester. In the next step the carbonyl compound is formed and the chrome is reduced to chrom-(IV)-acid that undergoes dispropotionation to $Cr(III)(OH)_3$ and chrom-(V)-acid. Chrom(V) is then reduced to Cr(III) *via* oxidation of the substrate. Therefore a colour change from orange (Cr(VI)) to green (Cr(III)) is observed.[247]

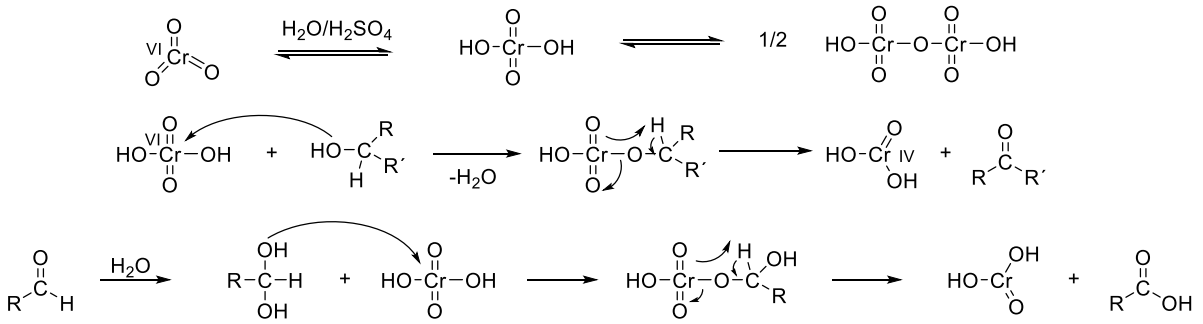


Figure 89: Top: formation of chromic acid, middle: oxidation of a secondary alcohol to ketone, bottom: oxidation of aldehyde to a carboxylic acid

The synthesis of betulonic acid (**80**) was conducted according to literature.[248] However 1.2 eq. of the Jones-reagents and the outlined 1.5 h stirring time at 0 °C did not lead to full conversion of the starting material. Therefore 3.6 eq. of Jones reagent and 24 h reaction time at 0 °C were necessary to gain full conversion of betulin (**69**). After purification only 45% yield of **80** were obtained. The synthesis of betulinic acid (**70**) will be discussed in the following chapter, while the synthesis of betulinal (**78**) will be mentioned in chapter 4.4.4.4.

4.4.4.1 Conventional oxidation of betulin to betulinic acid

Betulinic acid (**70**) can be conventionally synthesized from betulin (**69**) in two steps *via* Jones oxidation with CrO_3/H_2SO_4 to betulonic acid (**80**) followed by reduction to betulinic acid (**70**) using $NaBH_4$.[248, 249] (Figure 90) This is not only a two-step synthesis with only moderate yield and several purification steps that requires the waste use of organic solvents but also the use of stoichiometric Cr(VI) reagents that are extremely toxic and a serious safety and environmental concern. Chromic oxide adsorbed on silica gel was used to obtain betulinic aldehyde (**79**), which was further oxidized by potassium permanganate.[179]

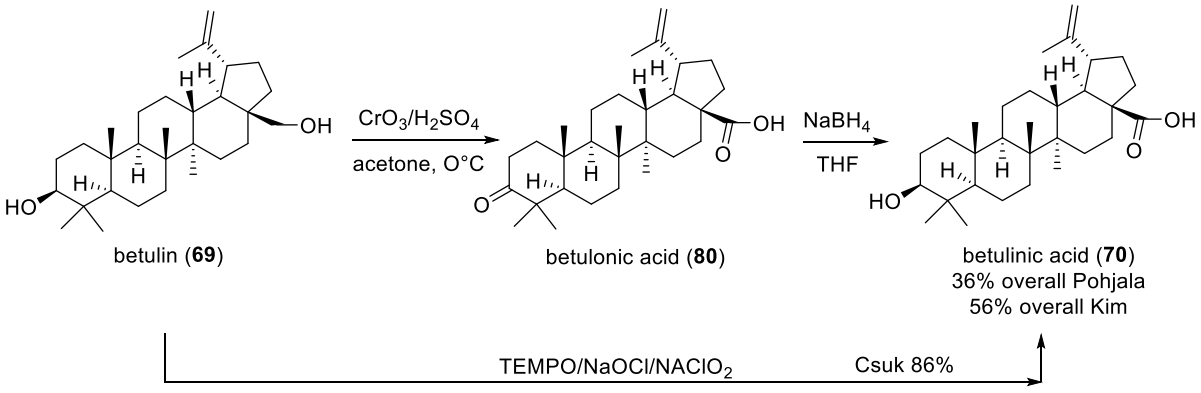


*Figure 90: Selected conventional syntheses of betulinic acid (**70**) and yields reported in literature*

An alternative synthetic pathway uses a catalytic oxidation with (2,2,6,6-tetramethylpiperidin-1-yl)oxy (TEMPO) or its derivatives in BuOAc in combination with substoichiometric amounts of NaOCl and stoichiometric $NaClO_2$ in a buffered system to obtain betulinic acid (**70**) in 86%.[250] Table 25 represent literature known oxidation reactions from betulin (**69**) to betulinic acid (**70**).

*Table 25: Literature survey of oxidation reactions of betulin (**69**) to betulinic acid (**70**)*

Entry	Oxidant	Co-oxidant	Over-oxidation[a]	Additional Oxidation[b]	Yield 70 [%]	Reference
1	CrO_3		yes		36 56	Pohjala[249] Kim[251]
2	CrO_3/SiO_2		no	$KMnO_4$	<50	Pichette
3	TEMPO	$NaOCl/NaClO_2$	no		86	Csuk[250]
4	TEMPO	Ru (II) or (VII) catalyst, O_2	no	Pinnick	10-22	Tulisalo[252]
5	TEMPO	TPAP, NMO	yes	Pinnick	- [c]	Tulisalo[253]
6	TEMPO	$NaClO_2$	no	Pinnick	50	Krasutsky[254]
7	e-chem.		no		70	Menard[255]
8	Pd (II)	ligand	yes	Pinnick	73-88%[d]	Tulisalo[256]
9	TEMPO	BAIB	no		90	Wickholm[257]

[a] To betulonic acid (**80**) or betulonic aldehyde (**79**) [b] Pinnick oxidation was performed to convert betulinic aldehyde (**78**) or betulonic aldehyde (**79**)to the corresponding acid. [c] Patent in Swedisch [d] Yield of betulonic acid (**80**).

A patent[252] describes the two step oxidation of betulin (**69**) to betulinic acid (**70**) using a Ruthenium catalyst e.g. $RuCl_2(PPh_3)_3$ and TEMPO under O_2 for the synthesis of betulonic acid (**80**) followed by reduction with $NaBH_4$. Remaining betulonic aldehyde (**79**) was further oxidized to betulonic acid (**80**) under Pinnick conditions, which had again been reduced to betulinic acid (**70**). Another approach was a three step synthesis using a TEMPO tetrapropylammoniumperruthenat (TPAP)/*N*-methylmorpholin-*N*-oxid (NMO) oxidation to obtain betulonic aldehyde (**79**), Pinnick oxidation[258] to betulonic acid (**80**) and finally the reduction to betulinic acid (**70**).[253]

The oxidation to betulinic aldehyde (**78**) was investigated using TEMPO and diluted bleach mixed with $NaClO_2$ in a mixture of aqueous KH_2PO_4 and *t*-BuOH. The aldehyde is further oxidized to betulinic acid (**70**).[254] Furthermore an electrochemical (e-chem) oxidation[255] and palladium based oxidations using a Pd (II) species such as $Pd(OAc)_2$ in combination with a ligand are described in literature. [256]

The oxidation method using [bis(acetoxy)iodo]benzene BAIB/TEMPO was already described in a patent that was published during ongoing research in this thesis. Up to 90% betulinic acid (**70**) could be obtained. However, no accurate information about the oxidation conditions are given.[257]

For the synthesis of reference material, betulin (**69**) was first oxidized to betulonic acid (**80**) with the classical Jones protocol described above in 45% yield. The ketone was consecutively reduced to the secondary alcohol to obtain betulinic acid (**70**). (Figure 90) This reduction was performed using $NaBH_4$, as this reagent is able to reduce only ketone or aldehydes in the presence of carboxylic acids.[247] The reactions mechanism is shown in Figure 91: A hydride attacks in a nucleophilic manner the carbonyl group and is later cleaved *via* acid hydrolysis.

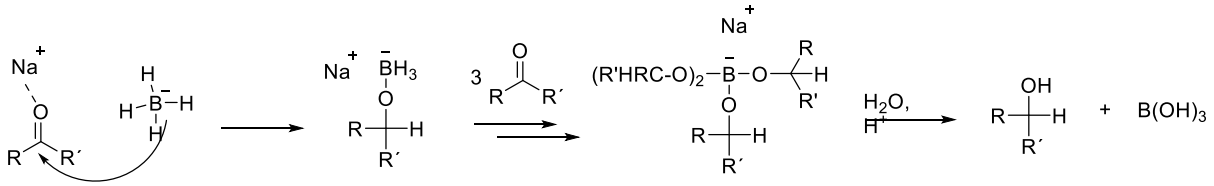


Figure 91: Reduction of a ketone using $NaBH_4$

Although those reductions are known to be fast, even after 24 h stirring starting material was still not fully converted. After recrystallization and purification *via* column chromatography only 29% betulinic acid (**70**) could be obtained in this conventional strategy.

Another synthetic approach for betulinic acid (**70**) was already shown before in Figure 90, a TEMPO mediated oxidation. This oxidation has the advantage that it selectively oxidizes primary alcohols to the corresponding aldehydes or carboxylic acids, whereas the secondary alcohol remains untouched. As the bulky oxoammonium salt derived from TEMPO is responsible for the selective oxidation, only sterically easily accessible primary alcohols are oxidized. The four methyl groups are shielding the oxidation reagent and sterically more demanding groups such as secondary alcohols cannot be oxidized.

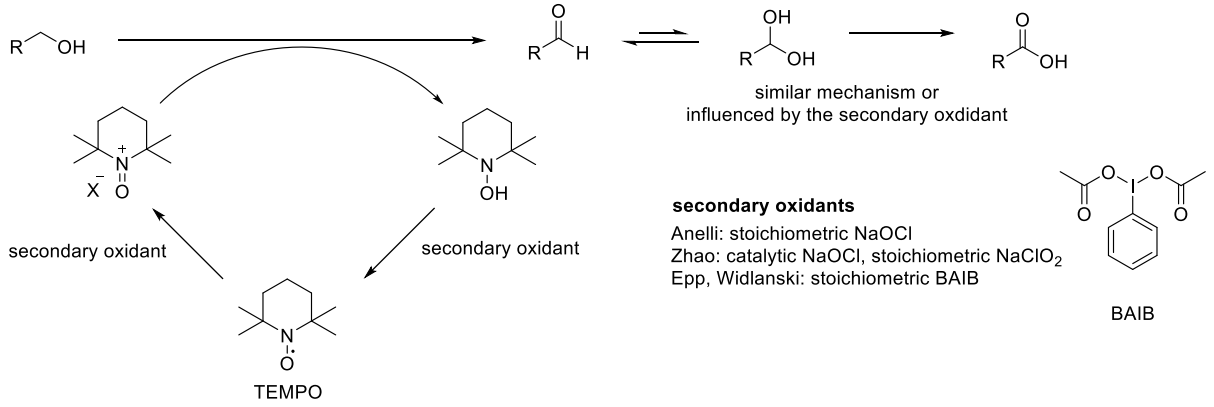


Figure 92: A simplification of the general mechanism of TEMPO mediated oxidations[259]

It should be stated, that the mechanism can be more complex with regard to the oxidation to the carboxylic acid.[259] However it is known that the oxidation to carboxylic acids rather is mediated by the oxoammonium salt. A number of co-oxidation systems using TEMPO and a secondary oxidant were applied for the oxidation of primary and secondary alcohols to their corresponding carbonyl compounds:

Anelli's oxidation protocol uses sodium hypochlorite as stoichiometric oxidant, however the tendency of NaOCl for chlorination might be challenging to sensitive compounds.[260] Therefore Zhao *et al.* modified this procedure and NaOCl was used in catalytic amounts in addition to the stoichiometric secondary oxidant $NaClO_2$.[261]

Furthermore the reaction is often run in a buffered system at pH values 6.8,[261] although the basic five-membered transition state is more compact and therefore the reaction rate are higher. It was also shown that under basic conditions a higher selectivity towards primary alcohols was observed. The reaction is slow at pH 13 (commercial bleach) and faster at a pH of 8.6 ($NaHCO_3$). At a high pH the

regeneration of the oxoammonoium salt becomes the rate determining step, and not the oxidation of the alcohol.

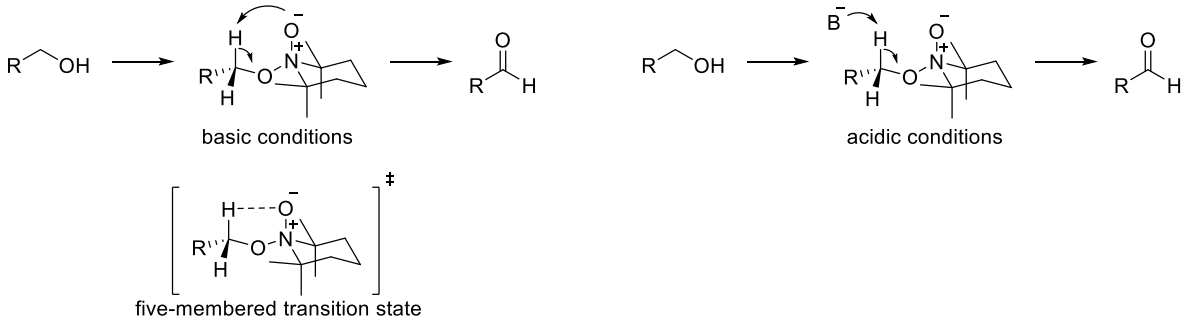


Figure 93: TEMPO oxidation under basic conditions (left) and acidic conditions (right)

4.4.4.2 Synthesis of betulinic acid (70) using TEMPO/NaOCl/$NaClO_2$ oxidation

According to a literature known procedure betulinic acid (**70**) should be prepared using a TEMPO mediated reaction.[261] (Figure 94)

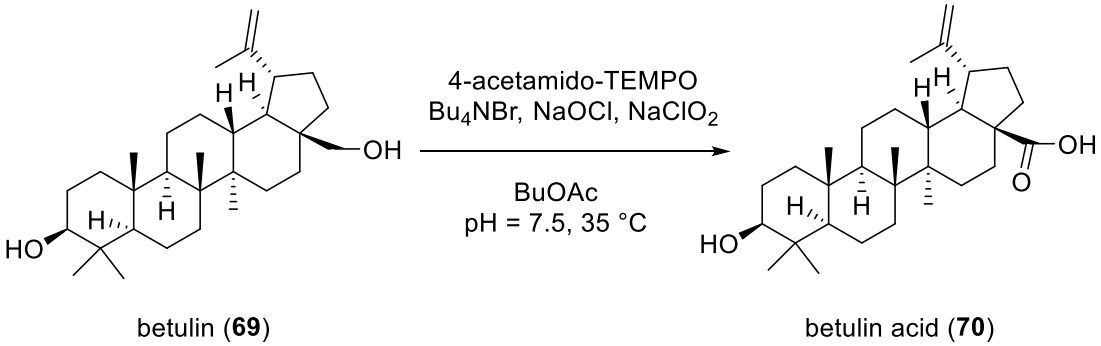


*Figure 94: Oxidation of betulin (**69**) to betulinic acid (**70**) according to Csuk[250]*

This reaction uses NaOCl in catalytical amounts to avoid chlorination side reactions. As a secondary oxidant $NaClO_2$ is used. (Figure 95)

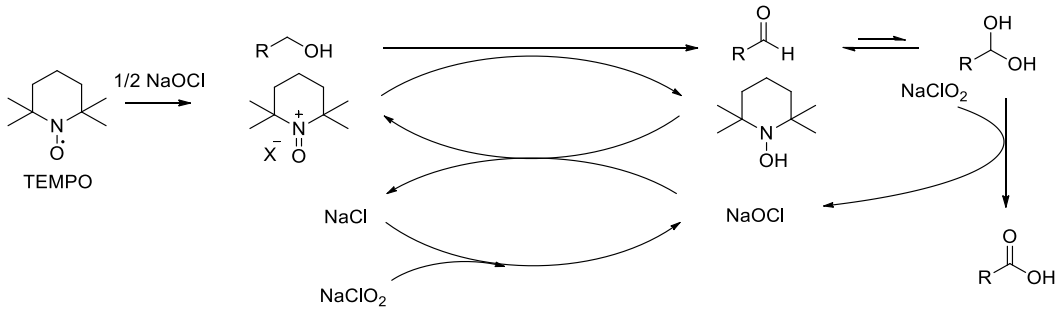


Figure 95: Mechanism of a TEMPO/NaOCl/$NaClO_2$ system

A selection of oxidation reactions using NaOCl and $NaClO_2$ is shown in Table 26. As it can be seen, betulinic acid (**70**) was only obtained in very low yields, as several side products were found according to HPLC. The reaction was as well run using a chlorine scavenger (results not shown) under the conditions of Csuk. However after 15 h at 50 °C starting material was still unreacted in the mixture.

NaOCl and $NaClO_2$ were added slowly and simultaneously, they were not mixed before adding to the reaction mixture. However, it is known that the oxoammonium salt is not stable at higher temperatures, but also room temperature did not lead to the desired product.

The concentration of starting material, especially in water soluble solvents e.g. ACN could also influence the oxidation reaction. Since betulin (**69**) is only poorly soluble in most organic solvents and insoluble in water, it immediately precipitates if water is added to water mixable solvent.

According to the procedure of Csuk *et al.*[250] a switch between betulinic aldehyde (**78**) and betulinic acid (**70**) should be possible by changing from TEMPO for the synthesis of betulinic aldehyde (**78**) to 4-acetamido-TEMPO for the synthesis of betulinic acid (**70**). Even with the use of 4-acetamido-TEMPO the synthesis of betulinic acid (**70**) was not possible. It should be mentioned that from a mechanistic point of view the switch from the aldehyde to acid is depending on the presence of water to form the hydrated species. According to the literature procedure both aldeyhde and acid were selectively formed using a biphasic system and a phasetransfer catalyst by only using two different TEMPO species.

However the protocol according to Csuk *et al*.[250] was further investigated using an chloride scavenger. (4 eq. 2-methyl-2-butene) Figure 96 shows that without scavenger an additional species is formed, since a second double bound signal appears. However, with or without scavenger the main product remained unreacted starting material. Up to double amounts of oxidation reagents were used, but no significant amounts of betulinic acid (**70**) were formed.

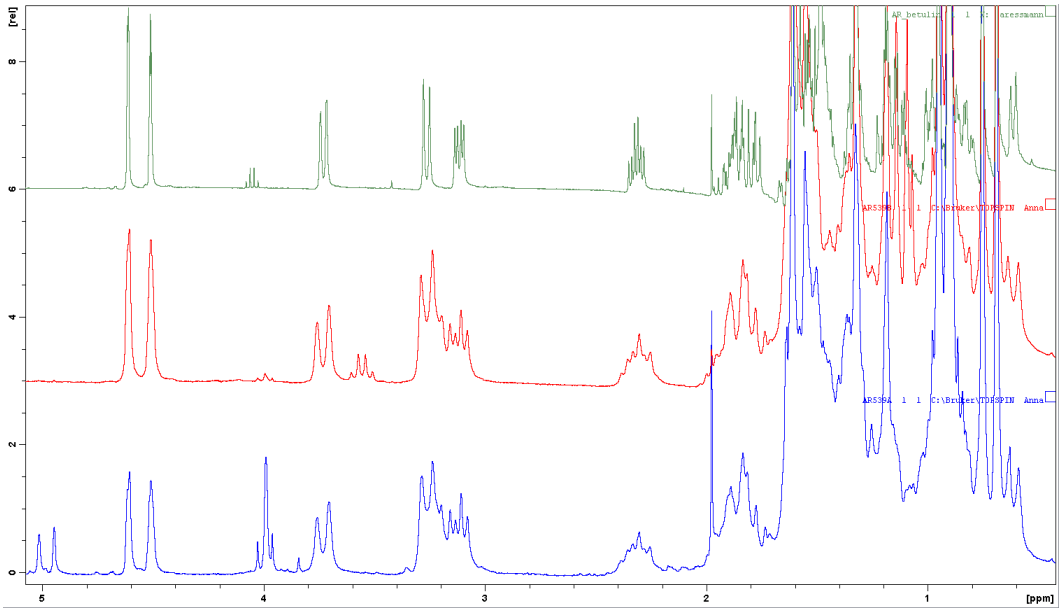


*Figure 96: Top betulin (**69**), middle NaOCl/$NaClO_2$ oxidation with chlorine scavenger, NaOCl/$NaClO_2$ oxidation without chlorine scavenger.*

However the reactions were also monitored using RP-HPLC. Surprisingly the more polar acid **70** is eluted after the less polar alcohol **69** using a mixture of methanol water-TFA. To an oxidation reaction according to the procedure by Csuk[250] betulin (**69**) and betulinic acid (**70**) were added to identify the peaks. In Figure 97 it can be seen that peak 1 is the internal standard, peak 2 and 3 are unidentified byproducts (cannot be the aldehyde **78** or **79**, since its retention time is significantly higher). Peak 4 was identified as betulin (**69**), since the area of betulin (**69**) compared to ISTD increased, when

additional betulin (**69**) was added. When betulinic acid (**70**) was added and additional peak appeared and confirmed that the product has not been formed.

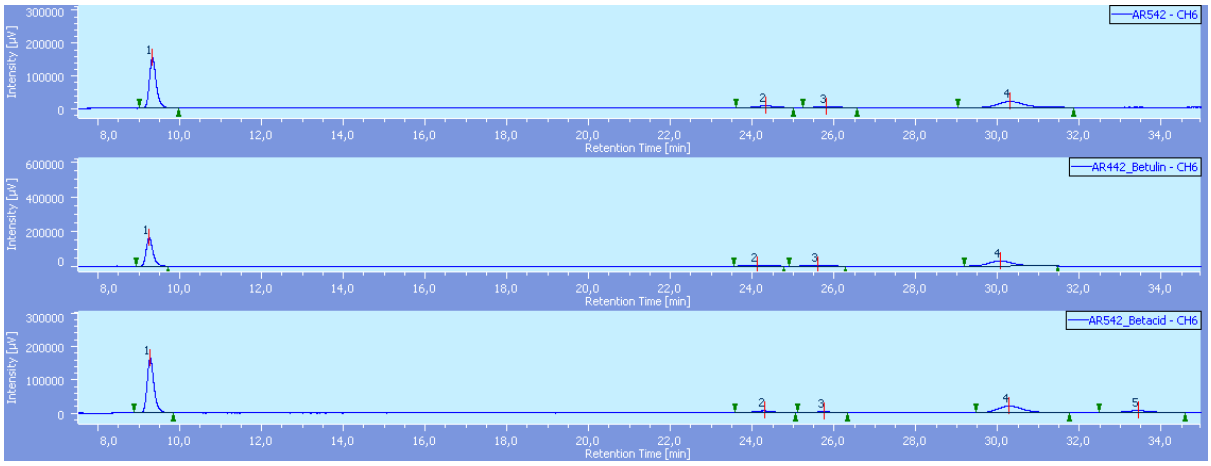


*Figure 97: Peak 1 internal standard, peak 2 and 3: unidentified, peak 4 betulin (**69**) and peak 5 betulinic acid (**70**)*

The addition of KBr accelerates the oxidation due to the formation of HOBr, which is a better oxidation for the regeneration of the oxoammonium salts than HOCl.[259] The mechanism is similar to those showed above and represented in Figure 98.

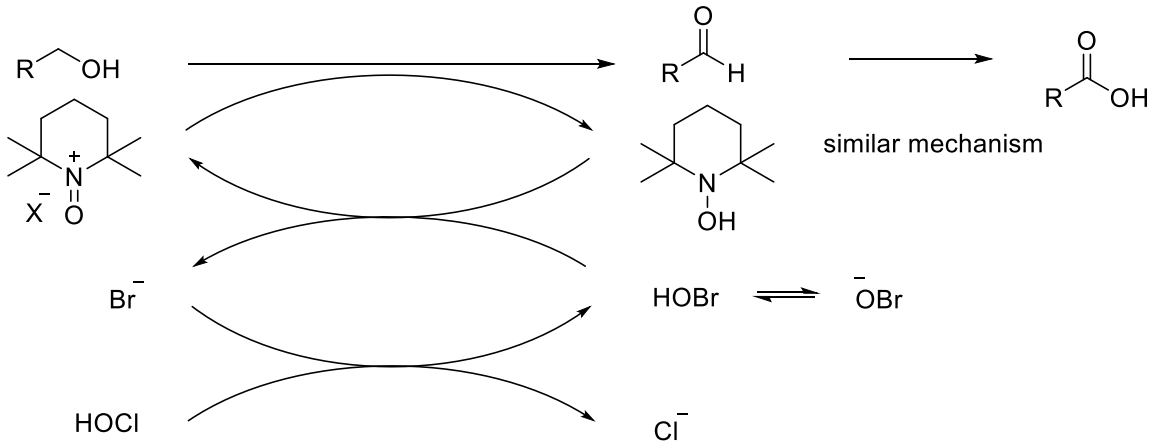


Figure 98: TEMPO oxidation using additional KBr

But also with the addition of KBr to the oxidation reaction did not lead to the desired product **70**. After testing a set of different oxidation agents, solvents, pH values and temperatures without obtaining the desired betulinic acid (**70**) it was decided to focus on different oxidation methods.

Table 26: Summary of oxidation reactions using NaOCl and $NaClO_2$

Entry	Procedure	Solvent	Aqueous phase	Co-Catalyst	Oxidation agents		PTC	reaction time [h]	T [°C]	Betulin (69) [a]	Betulinic Acid (70)[a]	Byproducts
					NaOCl	$NaClO_2$						
1	Zhao 2005[262]	ACN	buffer, pH 6.88	7 mol% TEMPO	7 mol%	2 eq.	Bu_4NBr	24	35	no conversion	-	-
2	Csuk 2006[250]	BuOAc	buffer, pH 6.88	7 mol% AA-TEMPO	7 mol%	2 eq.	Bu_4NBr	24	35	no conversion	-	-
3	Cusk 2006[250]	$CHCl_3$	buffer, pH 6.88	7 mol% AA-TEMPO	7 mol%	2 eq.	Bu_4NBr	48	35	no conversion	-	-
4	Cusk 2006[250]	BuOAc	buffer, pH 6.88	14 mol% AA-TEMPO	14 mol%	4 eq.	Bu_4NBr	24	35	full conversion	-	4
5	Zanka, 2003[263]	EtOAc	2 N NaOH, pH 8	4 mol% AA-TEMPO, 10 mol% KBr	1.25 eq.	1.25eq.	-	24	r.t	full conversion	-	3
6	Zanka, 2003[263]	EtOAc	2N HCl, pH 4	4 mol% AA-TEMPO, 10 mol% KBr	2.5 eq.	2.5 eq.	-	24	r.t	full conversion	0.40%	4
7	Zanka, 2003[263]	EtOAc	NaOH, pH 10	10 mol% TEMPO, 10 mol% KBr	2 eq.	-	-	24	r.t	conversion	1.50%	4
8	Zanka, 2003[263]	$CHCl_3$	NaOH, pH 10	4 mol% AA-TEMPO, 10 mol% KBr	1.25 eq.	1.25eq.	Bu_4NBr	24	r.t	full conversion	-	4
9	Zanka, 2003[263]	EtOAc	NaOH, pH 10	4 mol% AA-TEMPO, 10 mol% KBr	1.25 eq.	1.25eq.	Bu_4NBr	24	r.t	full conversion	-	3
10a	Zanka 2003[263]	EtOAc	buffer, pH 10	4 mol% AA-TEMPO, 10 mol% KBr	3 eq.	-	Bu_4NBr	24	r.t	full conversion	4%	5
10b	Zanka 2003[263]			+ 6mol%	+ 2eq.			24	r.t		4%	5
11	Noula 2002[264]	DCM	5% $NaHCO_3$	10 mol% AA-TEMPO 10 mol% NaBr	2.5 eq.		Bu_4NHSO_4	24	r.t	conversion	-	4
12	Cusk 2006[250]	BuOAc	buffer, pH=7.6	7 mol% AA-TEMPO	2	1.7	Bu_4NHSO_4	24	50	conversion	-	2
13	Cusk 2006[250]	BuOAc	buffer, pH=7.6	7 mol% AA-TEMPO	2	1.7	Bu_4NHSO_4	96	50	conversion	-	2
14	Cusk 2006[250]	BuOAc	buffer, pH=7.6	7 mol% AA-TEMPO	4	3.4	Bu_4NHSO_4	96	50	conversion	-	2

[a] Yields and conversion determined *via* HPLC using 1-methyl-1cyclohexene as internal standard. Apart from that control oxidation experiments using 1-pentanol and the conditions from Zanka et al.[263] were applied and valeric acid was obtained in 75% yield.

4.4.4.3 Synthesis of betulinic acid (70) using TPAP/NMO mediated oxidation

Since the oxidation using a TEMPO/NaOCl/NaO_2Cl system could not be performed in a reliable and successful manner, a ruthenium catalyzed oxidation was alternatively investigated. In a first attempt focus was set on an oxidation procedure using TPAP (tetrapropylammoniumperruthenat) and NMO (*N*-methylmorpholin-*N*-oxid) which is expected to yield betulonic acid (**80**) as main product that could then be reduced using $NaBH_4$ to the desired betulinic acid (**70**). This oxidation method was reported to give high yields of e.g. aliphatic carboxylic acids and showed a good tolerance to double bonds.[265] (Figure 99)

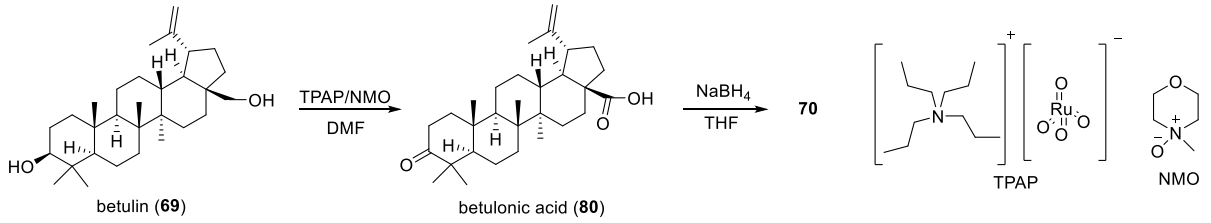


*Figure 99: TPAP/NMO oxidation of betulin (**69**) to betulonic acid (**80**) followed by the reduction to betulinic acid (**70**)*

It was shown that the TPAP catalyzed reaction worked in particular well due to dual role of NMO: NMO acts not only as regenerator of TPAP, but also as hydrate stabilizer, as it can be seen in the mechanism. (Figure 100)

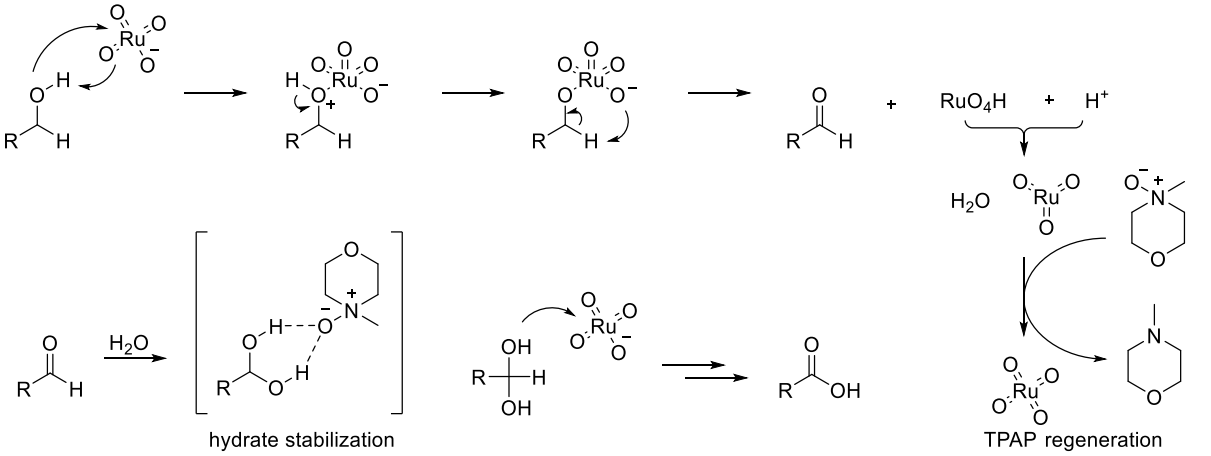


Figure 100: Mechanism of the TPAP/NMO oxidation reaction

Optimization of reaction parameters

After setting up a reliable HPLC strategy for the simultaneous determination of betulin (**69**), betulonic acid (**80**) and betulonic aldehyde (**79**) a time screening was initially performed. 20 eq. of NMO and 0.2 eq. of TPAP were used at the beginning, as best results in literature were reported using 10 eq. of NMO and 0.1 eq. of TPAP,[265] but two alcohol groups had to be oxidized in this case, so double amount of oxidation reagents was chosen. DMF was chosen since the solubility of betulin (**69**) is higher in DMF than in ACN. Full conversion of the starting material was observed after 1 h in DMF as solvent, using 20 eq. of NMO and 0.2 eq. of TPAP. Different reaction conditions such as room temperature, 50 °C and room temperature including the addition of water were tested. (Figure 101) Remarkable amounts of betulonic aldehyde (**79**), as well as small amounts of betulinic aldehyde (**78**) were detected as well,

explaining the unbalanced ratio of conversion and yield. As the yield of betulonic acid (**80**) increased with time, 24 h were chosen as reaction time. Best yields were obtained at room temperature and up to 53% betulonic acid (**80**) were formed. When adding 1 eq. of water to favor hydrate formation, a decrease in yield of betulonic acid (**80**) was obtained. The negative effect of water can be explained by the reduction of catalytic turnover caused by water due to the formation colloidal RuO_2.[266] Higher temperatures seemed to deactivate the catalytic system as well and therefore further investigations were performed at room temperature.

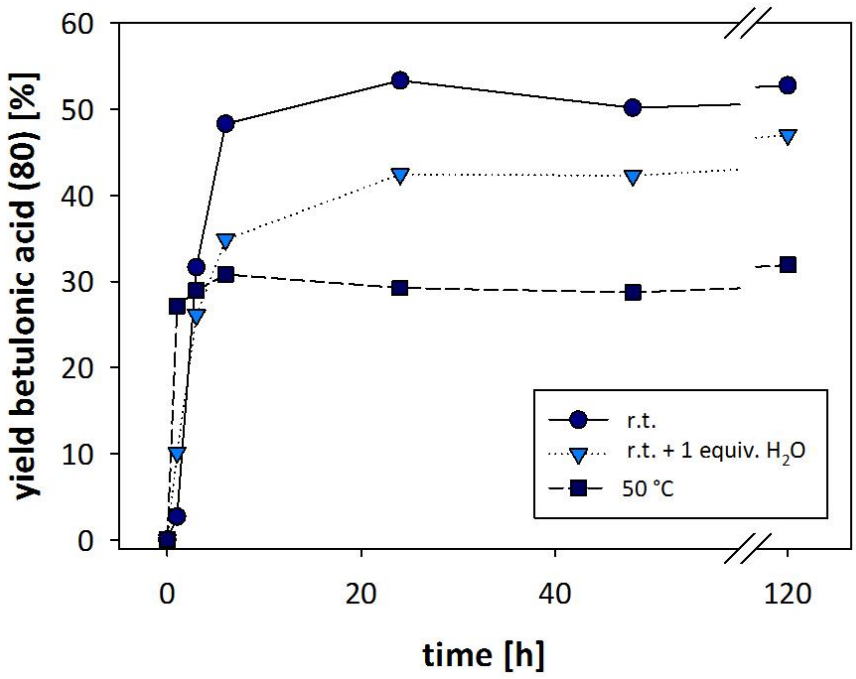


Figure 101: Time screening of the TPAP/NMO oxidation

In a next step the influence of the amount of NMO and TPAP was investigated: More than 50% betulonic acid (**80**) were obtained using a combination of 20 eq. of NMO and 0.2 eq. of TPAP. (Table 27, entry 5) If the amount of TPAP was reduced to 0.1 eq. (Table 27, entry 1) a tremendous decrease in yield was observed. When the amount of NMO was reduced the same trend was observed. With the addition 40 eq. of NMO (Table 27, entry 6) no improvement was observed compared to 20 eq. NMO. Neither subsequent addition of fresh NMO after 24 h led to an improvement (Table 27, entry 7, 9) nor could the addition of fresh TPAP (Table 27, entry 8). For further investigations 20 eq. of NMO and 0.2 eq. of TPAP were chosen.

Table 27: Variation of the amount of NMO and TPAP

Entry	NMO [eq.]	TPAP [eq.]	Yield Betulonic acid (**80**) [%][b]
1	20	0.1	36
2	2	0.2	4
3	5	0.2	24
4	10	0.2	37
5	20	0.2	53
6	40	0.2	48
7	20[a]	0.2	43
8	20	0.2[a]	46
9	40[a]	0.2	54

Performed using 10 mg betulin (**69**) in 1.5 ml DMF, stirring at r.t., 24 h. [a] 0.1 eq. TPAP added after 24 h, 48 h reaction time. [b] 100% conversion of betulin (**69**).

Influence of different solvents

After establishing the ideal conditions a closer look at different solvents, including several ionic liquids was taken. (Table 28 and Figure 102) Unfortunately the ionic liquids tested were not suitable for the oxidation. (Table 28, entry 1-4, 7) No conversion or only very low conversion were obtained. Only the hydrophilic ionic liquid $[C_2mim]Me_2PO_4$ (**4**) gave a moderate conversion of 58%, but did not yield betulonic acid (**80**). Organic solvents such as *t*-butylacetate (*t*-BuOAc), ACN, chloroform and dimethylcarbonate (DMC) gave low to moderate conversion. (Table 28, entry 5-6, 7 ,10) However it should be mentioned that in all cases except for dimethylcarbonate, ACN, BuOAc, 2-Me-THf and EtOAc the reaction was performed in a homogeneous manner.

*Table 28: Solvents tested for the oxidation of betulin (**69**) using a TPAP/NMO system*

Entry	Solvent	Conversion betulin (69) [%]	Yield betulonic acid (80) [%]
1	$[C_2mim]$DCA (**7**)	0	0
2	$[C_2mim]$OAc (**72**)	0	0
3	$[C_6mim]Cl$[a] (**82**)	0	0
4	ACN	15	0
5	*t*-BuOAc	16	0
6	$[C_2mim]N(Tf)_2$ (**9**)	31	9
7	$CHCl_3$	51	2
8	$[C_2mim]Me_2PO_4$ (**4**)	58	0
9	DMC	65	0

Performed using 10 mg betulin (**69**) in 1.5 ml/g solvent, 20 eq. NMO, 0.2 eq. TPAP, stirring at r.t., 24 h. [a] Performed at 50 °C

Conversion and yield was improved when using 2-Me-THF, BuOAc or *iso*-propylacetate as solvents, as it can be seen in Figure 102. Full conversion was obtained using DMSO, EtOAc in combination with $[C_2mim]N(Tf)_2$ (**9**), EtOAc and DMF. However reaction yields were rather low and only EtOAc with a yield of 45% was able to compete with DMF. Furthermore EtOAc in combination with the hydrophobic ionic liquid $[C_2mim]N(Tf)_2$ (**9**) in a ratio of 14:1 EtOAC:ionic liquid (1.4 g and 0.1 g)was also tested. The

reaction yield dropped to 31% when the ionic liquid was added, probably due to complex formation of the ionic liquid with the ruthenium catalyst.

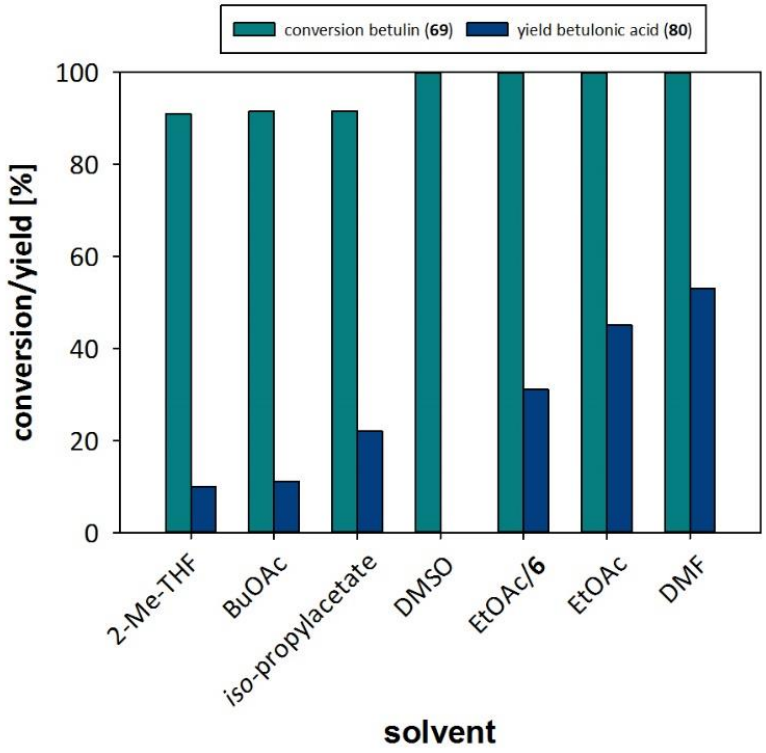


*Figure 102: Solvents tested for the oxidation of betulin (**69**) using a TPAP/NMO system*

In a last step the temperature and the reaction atmosphere were investigated. As it was already seen that higher temperatures were not beneficial for the formation of betulonic acid (**80**), lower temperature (0 °C) and oxygen atmosphere were investigated. Starting in DMF as solvent it could be seen that air-atmosphere gave a higher yield of betulonic acid (**80**) as argon atmosphere, probably due to moisture present and therefore a better hydrate formation. (Table 29, entry 1-2) When oxygen was used only a slight increase of reaction yield was observed. (Table 29, entry 3) A decrease of temperature did not improve the reaction yield, although the reaction was still performed homogeneously. A similar pattern was observed using EtOAc as solvent: The reaction yield was slightly increased using an oxygen atmosphere and lower temperature led to low conversion of betulin (**69**). (Table 29, entry 5-10)

Table 29: Influence of the atmosphere and lower temperature

Entry	Solvent	T [°C]	Atmosphere	Conversion betulin (**69**) [%]	Yield betulonic acid (**80**) [%]
1	DMF	25	air	100	53
2	DMF	25	argon	100	22
3	DMF	25	O_2	100	54
4	DMF	0	O_2	98	23
5	EtOAc	25	air	100	45
7	EtOAc	25	O_2	100	50
8	EtOAc	0	air	5	0
9	EtOAc[a]	0	O_2	16	0
10	EtOAc[b]	0	O_2	28	0

Performed using 10 mg betulin (**69**) in 1.5 ml solvent, 20 eq. NMO, 0.2 eq. TPAP stirring for 24 h.
[a] Starting material was not dissolved. [b] Performed in 3 ml solvent.

Since the amount of TPAP could not be reduced to 0.1 eq. without a loss of yield of betulonic acid (**80**) neither for DMF (31% yield) nor EtOAc (40% yield) this part of the project was closed.

4.4.4.4 Synthesis of betulinic acid (70) using hypervalent iodine mediated oxidation

In parallel to the investigations of the TPAP/NMO mediated oxidation reaction, a closer look was taken at the oxidation using hypervalent iodine compounds.

Hypervalent iodine (III) compounds are known to be very mild oxidant agents. It is possible to oxidize alcohols to aldehydes[267] or carboxylic acids[268] using stoichiometrical amounts of [bis(acetoxy)iodo]benzene (BAIB) and catalytic amounts of tetramethylpiperidinyl oxide (TEMPO).

Hypervalent iodine (V) compounds, such as the Dess-Martin periodinane (DMP) and *ortho*-iodoxybenzoic acid (IBX), which can be used as a precursor for DMP and hypervalent iodine (III) compounds such as BAIB or iodosobenzene[269, 270] have been frequently applied for the oxidation of alcohols to carbonyl compounds. Hypervalent iodine compounds have been widely used in total synthesis.[271]

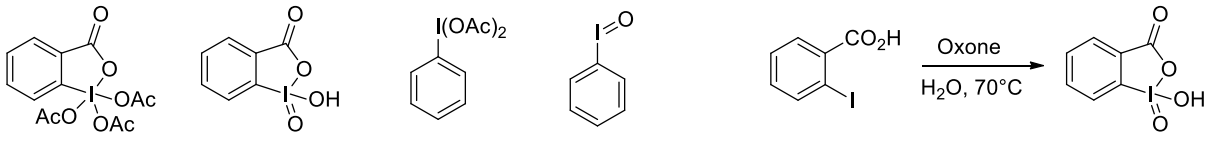


Figure 103: Hypervalent iodine (V) compounds (DMP, IBX) and hypervalent iodine (III) compounds (BAIB, iodosobenzene)

While DMP is known to be explosive, IBX is nowadays considered as a mild oxidation reagent, which can be simple prepared from *ortho*-iodobenzoic acid and Oxone® ($2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$).[272] However its low solubility in organic solvents is limiting the application to reactions run in DMSO. If ionic liquids have been used instead of DMSO, IBX could be applied for the synthesis of α,β-unsaturated carbonyl compounds.[270]

Hypervalent iodine (III) compounds are inexplosive and very mild oxidation reagents. Although it is difficult to oxidise aliphatic alcohols only with BAIB, a co-oxidation system of BAIB and catalytical

amounts tetramethylpiperidinyl oxide (TEMPO) guarantees access to either aldehydes[267] or carboxylic acids[268] depending on the solvent system. Benefical is hereby the retention of the stereogenic center in α position. According to the protocol of Epp and Widlanski[273] the hypervalent [bis(acetoxy)iodo]benzene in combination with TEMPO acts as secondary oxidant. Furthermore alcohols, such as benzylic alcohols, primary, secondary alcohols and diols were oxidised in water using a co-system of iodosobenzene, a hypervalent (III) compound and KBr.[274]

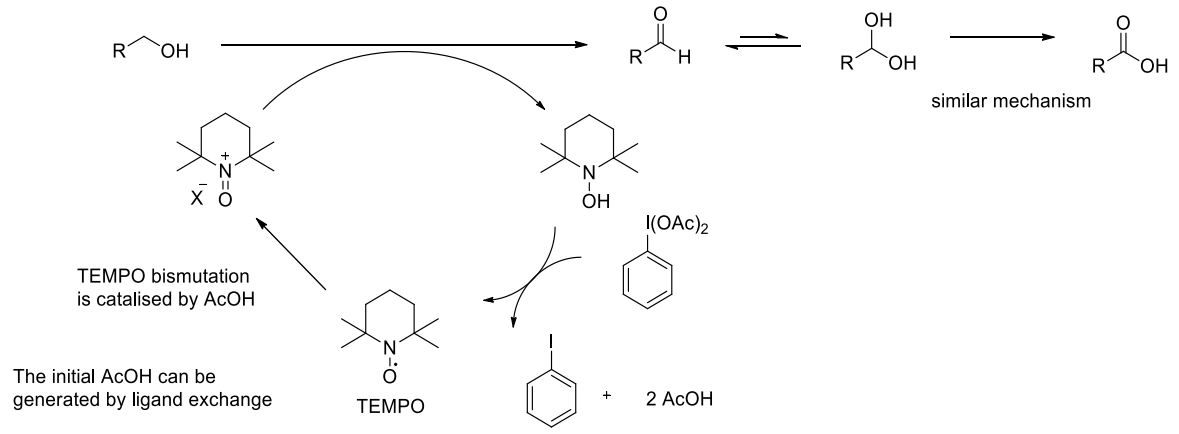


Figure 104: Mechanism for the oxidation using TEMPO and BAIB[273]

The TEMPO/BAIB oxidation method was chosen for the oxidation of betulin (**69**) to betulinic aldehyde (**78**). The reaction was performed in anhydrous dichloromethane under argon atmosphere. After 3 h the conversion of **69** was complete and the unstable aldehyde **78** was isolated after filtration over silica gel in 80% yield. (Figure 105)

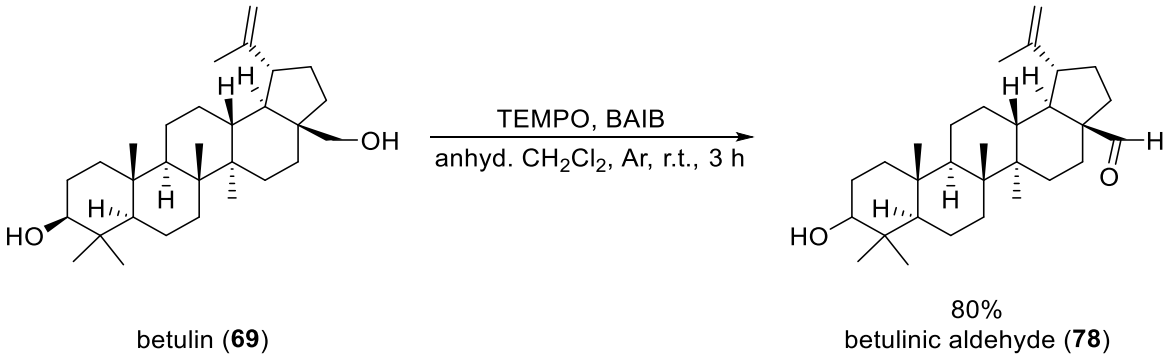
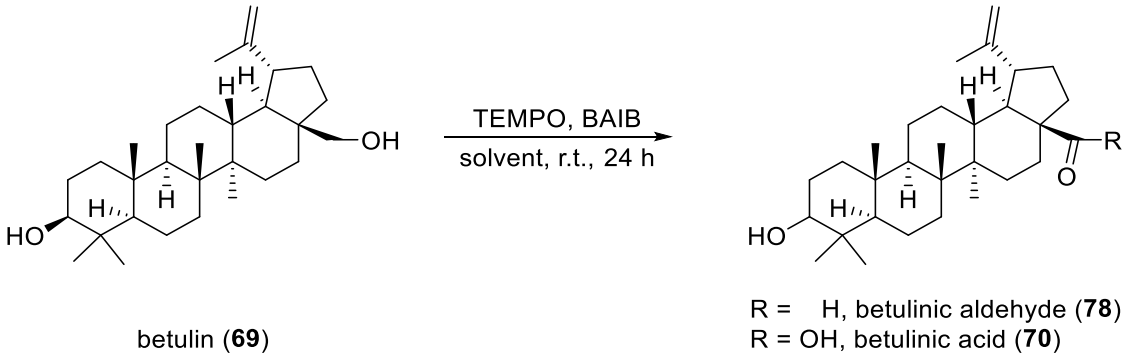


*Figure 105: Synthesis of betulinic aldehyde (**78**)*

To investigate the oxidation of betulin (**69**) to betulinic acid (**70**) a screening of reaction conditions was started using TEMPO/BAIB oxidation after setting an HPLC strategy for the simultaneous determination of betulin (**69**), betulinic aldehyde (**78**) and betulinic acid (**70**), since aldehyde **78** and acid **70** can be obtained. (Figure 106)



betulin (**69**)

*Figure 106: Synthesis of betulinic aldehyde (**78**) and betulinic acid (**70**) using TEMPO/BAIB oxidation*

In a first step, it was shown that a catalytic amount (0.1 eq.) of TEMPO and 3 eq. of BAIB in dichloromethane (DCM) did not lead to full conversion of betulin (**69**) (95%), whereas complete conversion of the starting material was observed using 1 eq. of TEMPO. The influence of an oxygen atmosphere was investigated showing a slightly higher conversion of 99% after 5 h. However after 24 h stirring time full conversion was observed in both cases and therefore air was chosen as reaction atmosphere.

Optimization of the reaction parameters

Investigations towards the optimum conditions were started with an equimolar amount of TEMPO and 3 eq. BAIB in different environmentally benign solvent. Figure 107 shows a comparison of all tested solvents after 24 hours reaction time. Diethyl carbonate (DEC) and butyl acetate (BuOAc) were considered as the best option. After 24 hours reaction time the conversion of betulin (**69**) was higher than 98% and surprisingly, relatively high amounts of betulinic acid (**70**) were already formed, although no water was added to the reaction. Still, residual water present in the solvents and air humidity might be responsible for the formation of the hydrate and therefore for the desired oxidation to betulinic acid (**70**).

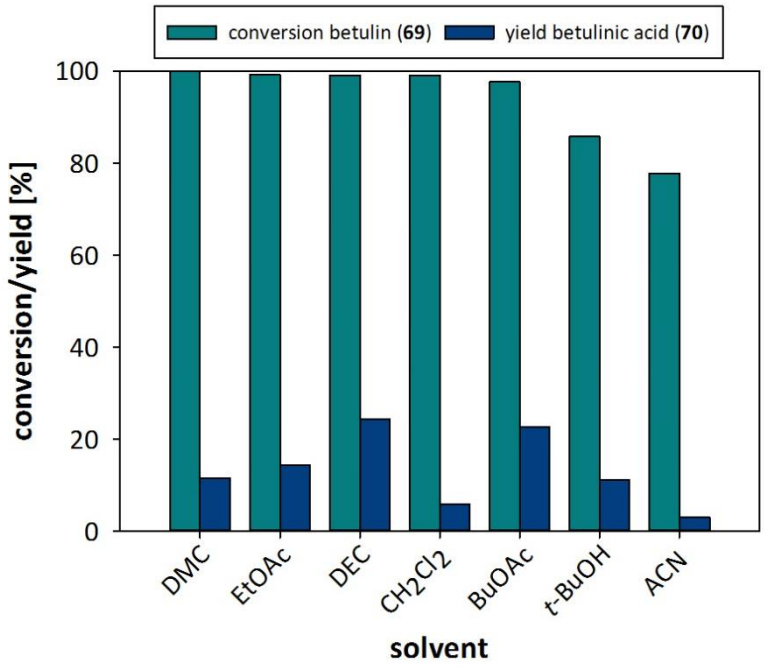


Figure 107: Comparison of the six tested solvents after 24 hours reaction time

EtOAc and dimethyl carbonate (DMC) showed good conversion of betulin (**69**), however the amount of betulinic acid (**70**) was lower as with DEC and BuOAc. In contrast, when performing the reaction in *t*-butanol (t-BuOH) and ACN a remarkable amount (>15%) of starting material was left. Therefore it was decided to investigate the reaction using DEC, BuOAc and DMC.

In a next step the influence of additional water was investigated. (Table 30) A higher amount of water would favour the formation of hydrate and therefore enable the oxidation towards the acid **70**. Using DMC or DEC as solvents the addition of water was not beneficial for the oxidation reaction. (Table 30, entry 1-4) In case of DMC, that is partially soluble with water, the additional water seems to precipitate betulin (**69**) and therefore conversion dropped if even only 10 eq. water were added. In case of DEC, conversion did not decrease in the presence of additional water. However the yield of betulinic acid (**70**) was not improved. In case of BuOAc as a solvent, the addition of water to the reaction mixture does not have a significant impact on the reaction. With increasing amount of water from 10 eq. to 50 eq. the yield of betulinic acid (**70**) slightly increased. Adding 245 eq. (100 µl), the reaction mixture turns into a biphasic system and becomes difficult to handle in terms of sampling. Furthermore unidentified byproducts were detected. Running the system in a 1:1 mixture of water:BuOAc (2500 eq., Table 30, entry 9) a low conversion of only 10% were obtained.

Moreover, using 50 eq. of water provided the best results of betulinic acid (**70**) in combination with the least formation of side products. Consequently, 50 eq. of additional water were used for all further screening reactions.

Table 30: Influence of the amount of water for the oxidation

Entry	Solvent	Water [eq.]	Conversion betulin (69) [%][b]	Yield betulinic acid (70) [%][b]
1	DMC	0	100	11.5
2	DMC	10	74	0
3	DEC	0	99	24.3
4	DEC	10	100[a]	12.3
5	BuOAc	0	95	22.7
6	BuOAc	10	99[a]	24.2
7	BuOAc	50	99[a]	26.1
8	BuOAc	250	99[a]	30.4[c]
9	BuOAc	2500	10	0

Performed using 10 mg betulin (**69**) in 1 ml solvent, 1 eq. TEMPO, 3 eq. BAIB. [a] Addition of 2 eq. *t*-BuOH. [b] According to HPLC c unidentified byproducts were detected

The amount of oxidations reagents was investigated for BuOAc (Table 31): While higher amounts of BAIB (4 eq.) did not improve the yield of betulinic acid (**70**), (Table 31, entry 1-2) a reduction of TEMPO to 0.5 eq. already improved the yield of betulinic acid (**70**) to 38%. When the amount of TEMPO is further reduced to 0.2 eq. and 0.1 eq. using 3 eq. BAIB, the yield of betulinic acid (**70**) is further increased to 41% and 45%. (Table 31, entry 5 and 8) However a slightly smaller amount of betulin (**69**) (3%) remained when using 0.1 eq. of TEMPO, whereas full conversion of betulin (**69**) was observed using 0.2 eq. TEMPO. If the amount of BAIB was increased to 4 eq. of BAIB did not improve the yield of betulinic acid (**70**), (Table 31, entry 6 and 9) whereas a reduction to 2 eq. of BAIB decreased the

conversion to 73% and 95%. (Table 31, entry 4 and 7) Therefor further investigations were performed using 0.2 eq. TEMPO (and 0.1 eq.), 3 eq. BAIB, 50 eq. water and 2 eq. *t*-BuOH as phase transfer catalyst.

Table 31: Variation of oxidation reagents

Entry	TEMPO [eq.]	BAIB [eq.]	Conversion betulin (69) [%]	Yield betulinic acid (70) [%]
1	1	3	99	26
2	1	4	99	27
3	0.5	3	99	38
4	0.2	2	73	16
5	0.2	3	100	41
6	0.2	4	100	43
7	0.1	2	97	39
8	0.1	3	98	45
9	0.1	4	95	38
10	0.05	3	95	37

Performed using 10 mg betulin (**69**), 0.2 eq TEMPO, 3 eq BAIB, 50 eq. water, 2 eq. *t*-BuOH at r.t. for 24 h. Yield was determined *via* RP-HPLC using 1-methyl-1-cyclohexen as internal standard.

The influence of the temperature was also investigated. Higher temperature (40 °C) dropped the yield from 41% to only 26% betulinic acid (**70**), probably due to the instability of the oxoammonium salt at elevated temperatures. Room temperature was chosen for further investigations, as lower temperatures would cause solubility and therefore concentration issues.

Apart from that the influence of the concentration of betulin (**69**) in the solvent was explored: As it is shown in Table 32 concentration of the starting material plays an important role during the oxidation process: The screening was started with a concentration of 10 mg **69**/ml BuOAc, as this seemed to be the maximum amount of betulin (**69**) soluble in BuOAc. With this concentration full conversion and a yield of 43% betulinic acid (**70**) were obtained. With a higher concentration of betulin (**69**)of 15 mg/ml solubility issues occurred, especially in the beginning of the oxidation. Betulin (**69**) was not completely dissolved in the beginning, but subsequently dissolved when more aldehyde was formed, as the aldehyde seemed to have higher solubility than betulin (**69**). However slightly lower yields of 39% of betulinic acid (**70**) (Table 32, entry 1) were obtained while having full conversion. If the concentration of betulin (**69**) was lowered to 7.5 mg/ml and 5 mg/ml incomplete conversion of 88% and 67% and poor yields of betulinic acid (**70**) were observed. (Table 32, entry 3 and 4)

Table 32: Concentration dependency of the starting material in BuOAc

Entry	Concentration betulin (69) [mg/ml]	Conversion betulin (69) [%]	Yield betulinic acid (70) [%]
1	15	100	39
2	10	100	43
3	7.5	88	28
4	5	67	13

Performed using 10 mg betulin (**69**), 0.2 eq TEMPO, 3 eq BAIB, 50 eq. water, 2 eq. *t*-BuOH at r.t. for 24 h. Yield was determined *via* RP-HPLC using 1-methyl-1-cyclohexen as internal standard.

To summarize, best conditions were 10 mg/ml betulin (**69**), 0.2 eq of TEMPO, 3 eq. BAIB, 2 eq. *t*-BuOH and 50 eq. water in BuOAc with a total yield of 43% betulinic acid (**70**) after a reaction time of 24 h at room temperature. Additionally, under these particular reaction conditions the expereiment was isolated without taking any HPLC samples. Up to 50% betulinic acid (**70**) with a purity of > 90 % could be isolated after performing flash column chromatography.

Further oxidation to Betulinic acid (**70**)

A time screening was accomplished for a more precise understanding of the TEMPO/BAIB oxidation of betulin (**69**). It could be seen that betulin (**69**) was (nearly) completely converted after 5 hours and the yield of betulinic acid (**70**) levelled off after this reaction time in case of using catalytic amounts of 0.1/0.2 eq. of TEMPO. (Figure 108)

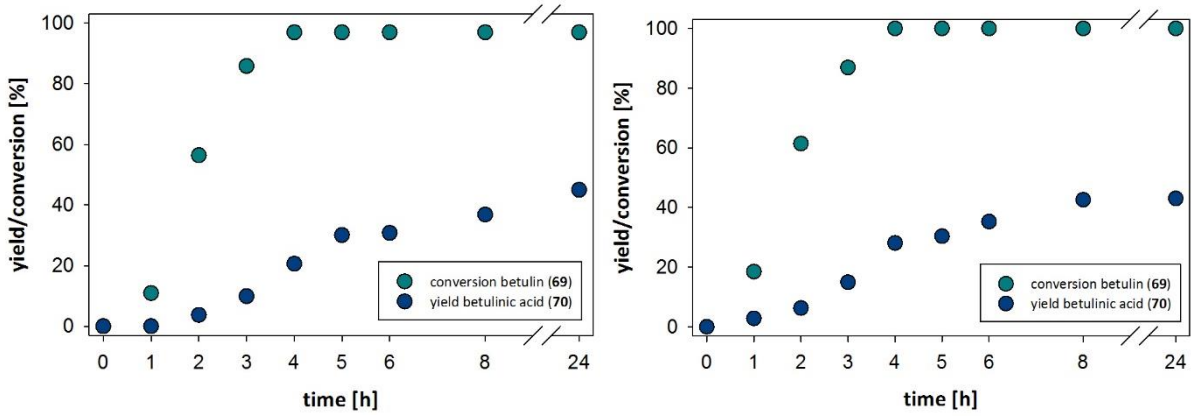


*Figure 108: Time screening of the TEMPO/BAIB oxidation of betulin (**69**) to betulinic acid (**70**) (left 0.1 eq. TEMPO, right. 0.2 eq. TEMPO)*

There are two approaches considered for the further oxidation of remaining aldehyde **78** to the acid **70**: (Figure 109) The upper reaction pathway represents the oxidation of betulinic aldehyde (**78**) to betulinic acid (**70**) using additional amounts of the hypervalent iodine and TEMPO, whereas the lower reaction pathway is the Pinnick oxidation,[275] which can selectively oxidize aldehydes to carboxylic acids.

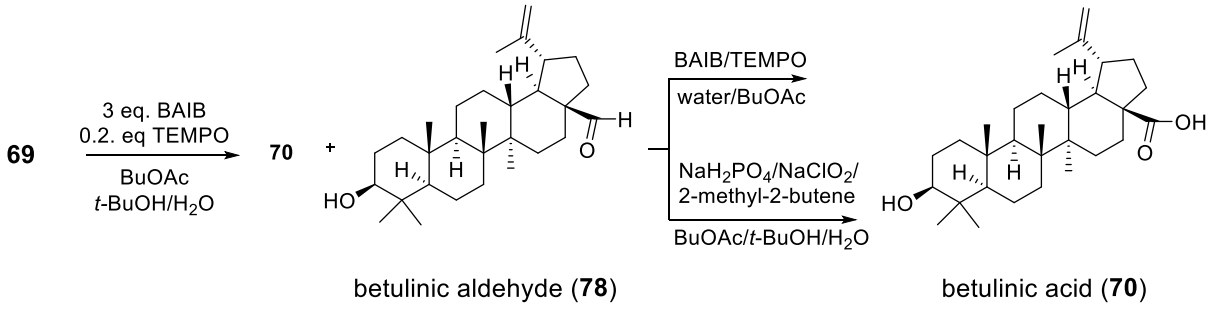


*Figure 109: Oxidation of betulin (**69**) to betulinic acid (**70**)*

Following the upper path, the yield of betulinic acid (**70**) was not improved by the addition of extra 0.2 eq. of TEMPO and/or 1 eq. BAIB after 5 hours, the time, when betulin (**69**) was fully converted. However aldehyde **78** remained unreacted and the maximum yield of **70** was 45%. Therefore it was decided to focus on a two-step strategy including the consecutive Pinnick oxidation of betulinic aldehyde (**78**).

Table 33: Addition of oxidation reagents after full conversion of starting material

Entry	TEMPO [eq.]	Additional TEMPO [eq.]	BAIB [eq.]	Additional BAIB [eq.]	Conversion betulin (**69**) [%]	Yield betulinic acid (**70**) [%]
1	0.2		3		100	43
2	0.2	0.2	3		100	43
3	0.2		3	1	100	43
4	0.2	0.2	3	1	100	45

Performed using 10 mg betulin (**69**), 0.2 eq TEMPO, 3 eq BAIB, 50 eq. water, 2 eq. *t*-BuOH at r.t. for 24 h. Yield was determined *via* RP-HPLC using 1-methyl-1-cyclohexen as internal standard.

In the Pinnick reaction, also known as the Lindgren oxidation, aldehydes can be selectively oxidized to carboxylic acids under mildly acidic conditions.[258] Functional groups are tolerated and even sterically demanding aldehydes can be reacted. This inexpensive reaction is usually applied to α,β-unsaturated aldehydes.

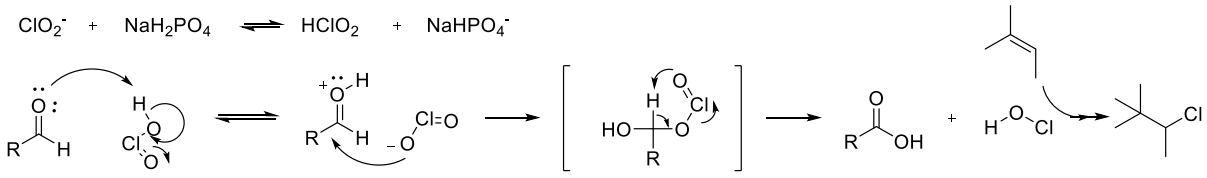


Figure 110: Reaction mechanism of the Pinnick oxidation

Chlorous acid is the active oxidation reagent, which is formed under acidic conditions. The aldeyhde is protonated and nucleophilic attacked by the chlorous acid. The chlorine group is released as hypochlorous acid, which needs to be scavenged as it can not only react with $NaClO_2$, but can also react with double bonds *via* a halohydrin formation reaction. Therefore 2-methyl-2-butene is provided as scavenger.

In the second approach (Figure 109) the TEMPO/BAIB oxidation and the Pinnick[276] oxidation were carried out without with sub sequential addition of the Pinnick reagents after 5 h. Therefore the first part of the reaction (TEMPO/BAIB oxidation) was performed using the optimized conditions with 3 eq. BAIB, 0.2 eq TEMPO, 2 eq. *t*-BuOH and 50 eq. water. After 5 hours reaction time, sodium chlorite ($NaClO_2$), sodium dihydrogen phosphate (NaH_2PO_4) and 2-methyl-butene were directly added to the reaction mixture. 2.6 eq. of $NaClO_2$ and NaH_2PO_4 are necessary to gain a full conversion of the intermediate aldehyde **78** to yield betulinic acid (**70**) in 75% isolated yield. As later discussed in detail, this yield could be further improved *via* the addition of acetic acid (**70**) (chapter 4.4.6) to an isolated yield of 89%.

Simultaneously addition of BAIB oxidation and Pinnick reagents gave a poor conversion of 26% with only 4% yield and byproducts were detected. Therefore it was decided to perform the BAIB oxidation using 0.1 eq. TEMPO, 3 eq. BAIB, 2 eq. t-BuOH and 50 eq. water with the addition of 2.6 eq. Pinnick oxidation reagents ($NaClO_2$ and NaH_2PO_4) and a chloride scavenger (4.6 eq.) after 5 h.

4.4.4.5 Conventional synthesis of bevirimat (71)

In parallel to the research on a benign oxidation strategies for betulin (**69**), the preparation of the antiviral drug bevirimate (**71**) from pure betulinic acid (**70**) was optimized.

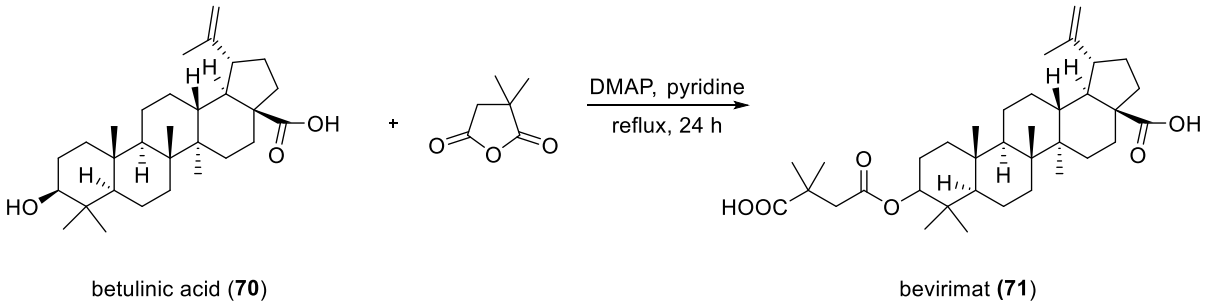


*Figure 111: Esterification of betulinic acid (**70**) to bevirimat (**71**)*

According to literature the synthesis of bevirimat (**71**) is performed *via* esterification of betulinic acid (**70**) using dimethylsuccinic anhydride under addition of DMAP (4-(dimethylamino)pyridine) in refluxing pyridine. (Figure 111)[277] A closer look at the reaction mechanism should be taken first, as the nucleophilic attack of DMAP will play an important role later:

DMAP is used in combination with anhydrides for the esterification of sterically demanding secondary or tertiary alcohols. DMAP attacks the carbonyl carbon of the anhydride at the steric less hindered site as it can be seen in Figure 112. The activated amide is then attacked by the alcohol and protonation occurs. DMAP is finally cleaved and recovered and the ester is formed.

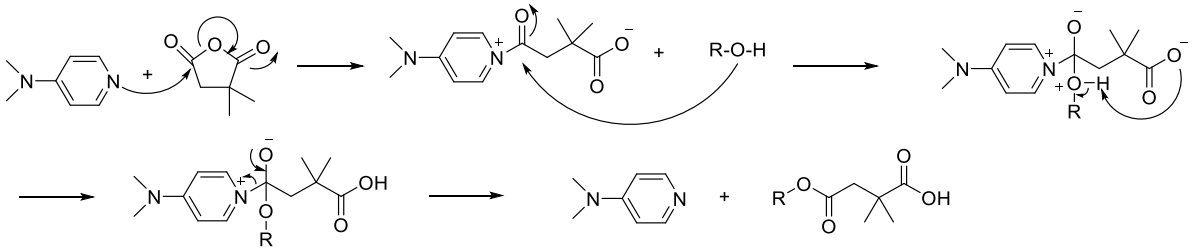


Figure 112: Reaction mechanism of the esterification using DMAP and anhydride

After developing an HPLC strategy for the determination of bevirimat (**71**) and betulinic acid (**70**) a screening regarding amount of anhydride, DMAP and solvent was performed, and an additional peak besides bevirimat (**71**) was observed. (Figure 113)

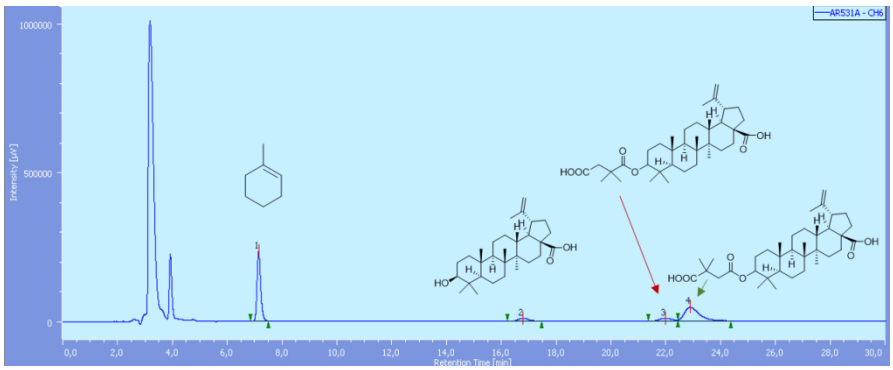


*Figure 113: HPLC chromatogram of esterification of betulinic acid (**70**): Peak 1: internal standard; peak 2: betulinic acid (**70**); peak 3: undesired regioisomer iso-(**71**); peak 4: bevirimat (**70**)*

As two possible nucleophilic attacks on the anhydride can occur, two regioisomers can be formed. However the attack of the less hindered carbonyl group (green) is more likely than the sterically demanding carbonyl group (red). (Figure 114)

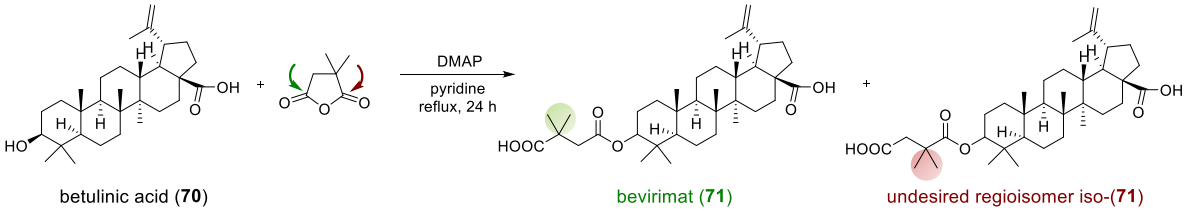


*Figure 114: Synthesis of bevirimat (**71**) and the undesired regioisomer iso-(**71**)*

4.4.4.5.1 Structure elucidation

To confirm the previous assumption HPLC-MS, chrystal structure and NMR techniques (^{1}H, ^{13}C, long range methods) were applied.

In a first step HPLC-MS was performed, showing that the mass of the two products was the same of 585 corresponding to [M+H^{+}] (MW =584 g/mol) as it can be seen in Figure 115.

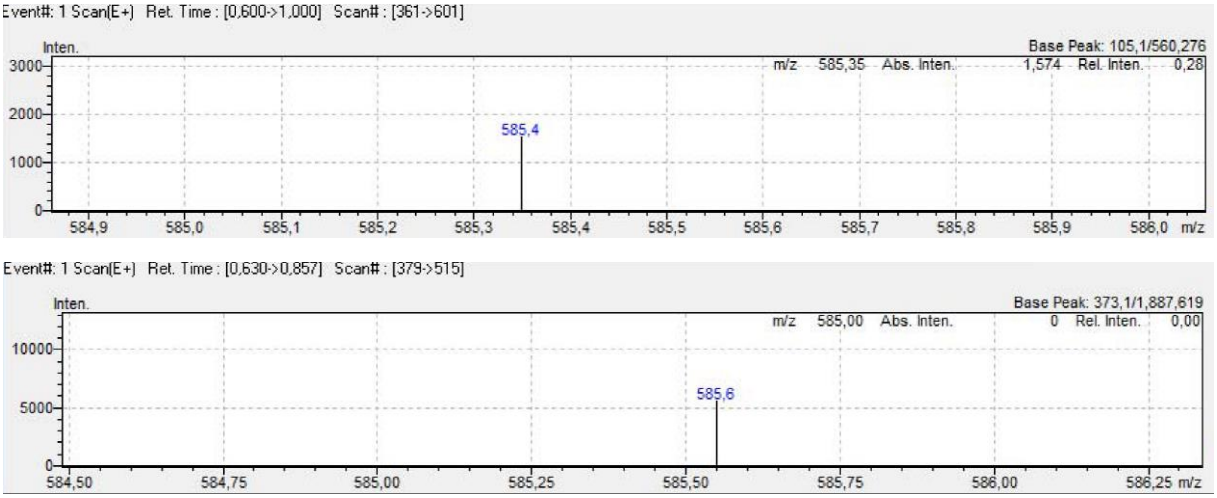


*Figure 115: HPLC-MS spectra for bevirimat (**71**) (top) and its regioisomer iso-(**71**) (bottom), measured in positive mode*

For a better understanding a HMBC NMR (Heteronuclear Multiple Bond Correlation)-Experiment was recorded. It can be seen in Figure 116 the important signals from the proton spectra are marked in orange, those from the carbon in blue. A strong correlation from the carboxy carbon (A) can be seen to the two methyl groups (3), whereas this is not the case for the ester carbon (B). Furthermore B shows a correlation to the proton 1. Worth to mention is also the different solubility in NMR solvents: while bevirimat (**71**) was soluble in chloroform, its regioisomer iso-**71** was not.

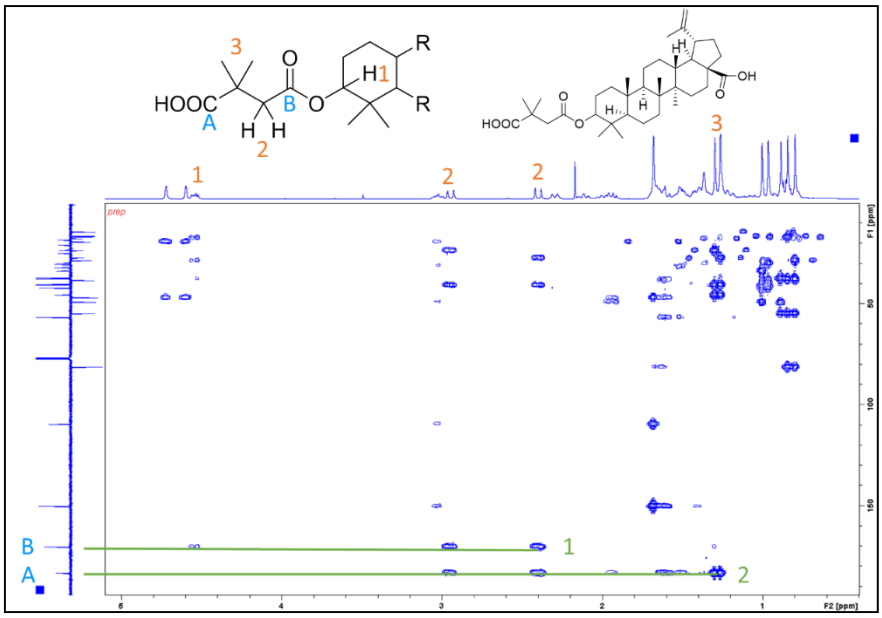


*Figure 116: 2D-NMR of bevirimat (**71**)*

In contrast, the 2D-NMR for the regioisomer iso-**71** is shown in Figure 117. As it can be seen, the A and B signals are shifted, probably due to a solvent effect, since the regioisomer iso-**71** was only soluble in DMSO. Proton 1 proofs the shifting, as it has to correlate to B. The ester B also shows a correlation to the two methyl groups 2, which is not the case for carboxylic acid A. Therefore the structure of iso-**71** is confirmed.

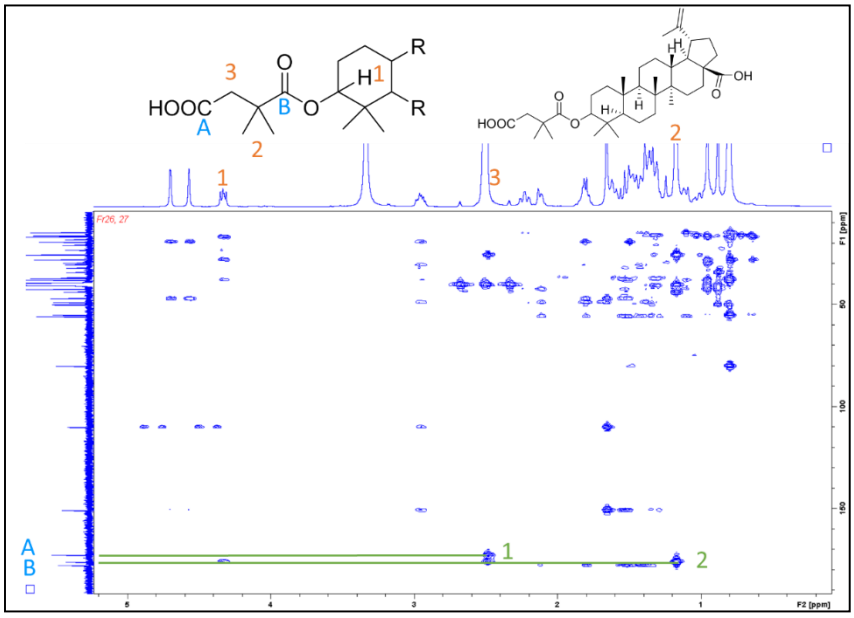


*Figure 117: 2D-NMR of the undesired regioisomer iso-(**71**)*

Since bevirimat (**71**) was crystallized from methanol, its crystal structure was also analyzed confirming the assumption that the main regioisomer is bevirimat (**71**).

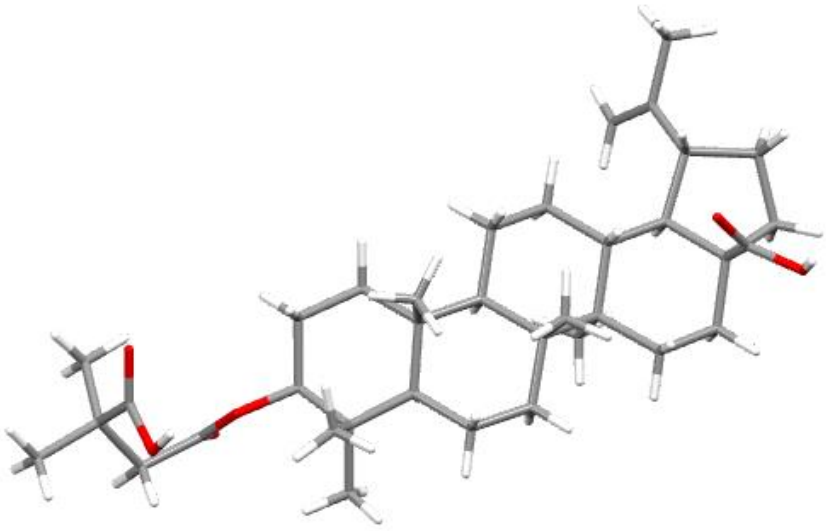


*Figure 118: Crystall structure of bevirimat (**71**)*

4.4.4.6 Optimization of the esterification of betulinic acid (70)

After establishing a reliable HPLC setting the screening was started with 1 eq. betulinic acid (**70**) and different ratios of DMAP and anhydride in refluxing pyridine. (Table 34) It could be seen that the yield of bevirimat (**71**) was increased if the amount of anhydride was increased from 1 eq. to 3 eq. and 6 eq. (Table 34, entry 1-3) keeping the amount of DMAP constantly at 1 eq. Up to 65% bevirimat (**71**) were obtained.

*Table 34: Variation of reagents and time for the esterification of betulinic acid (**70**)*

Entry	Time [h]	DMAP [eq.]	Anhydride [eq.]	Conversion betulinic acid (70) [%]	Yield regioisomer iso-(71) [%]	Yield bevirimat (71) [%]
1	24	1	1	41	4	37
2	24	1	3	45	3	41
3	24	1	6	71	6	65
4	24	2	3	72	6	66
5	48	2	3	96	10	85
6	72	2	3	100	12	88

Performed using 15 mg (1 eq.) betulinic acid (**70**), 0.5 ml pyridine, reflux. Yields were determined *via* HPLC.

The reflection of the reaction mechanism explains the necessity of 2 or more eq. of DMAP: although DMAP is recovered during the reaction there are several carboxylic acid groups present where protonation can occur, one in the starting material betulinic acid (**70**), two in the product bevirimat (**71**) and one in the anhydride after ring opening.

In a next step different solvents were evaluated. Solutions were refluxed for 24 h and 72. While DCM and triethylamine showed no conversion (Table 35, entry 1-4), $CHCl_3$ and methyl-THF performed only slightly better with conversion of appr. 10% after 24 h or 72 h. (Table 35, entry 5-8) A more suitable solvent is represented by DMC, however approximately 60% of starting material remained unreacted after 72 h. (Table 35, entry10) Changing the solvent to EtOAc resulted in an increase of conversion and 67% of betulinic acid (**70**) was converted after 72 h. (Table 35, entry 12) The best results so far were

obtained with BuOAc (Table 35, entry 13, 14) although the conversion of approximately 90% was slightly lower than obtained with pyridine. (Table 35, entry 15, 16) However regarding the environmental aspect, BuOAc is more favorable than pyridine.

*Table 35: Variation of the solvent for the esterification of betulinic acid (**70**)*

Entry	Solvent	Time [h]	Conversion betulinic acid (70) [%]	Yield regioisomer iso-(71) [%]	Yield bevirimat (71) [%]
1	CH_2Cl_2	24	0	0	0
2		72	0	0	0
3	Triethylamine	24	0	0	0
4		72	0	0	0
5	$CHCl_3$	24	0	0	0
6		72	9	9	
7	Methyl-THF	24	9	9	
8		72	11	11	
9	Dimethylcarbonate	24	11	11	
10		72	37	2	34
11	EtOAc	24	38	4	34
12		72	67	6	61
13	BuOAc	24	61	7	54
14		72	87	12	75
15	Pyridine	24	41	6	66
16		27	100	12	88

15 mg betulinic acid (**70**), 2 eq. DMAP, 3 eq. anhydride, reflux. Yields were determined *via* HPLC.

Application of microwave irradiation

When applying microwave irradiation conversion was not improved using pyridine as solvent at 125 °C for 1 h, and unreacted starting material remained. The focus was set on BuOAc and increased the temperature of microwave irradiation to 150 °C, 160 °C and 170 °C. As it can be seen in Figure 119, the highest conversion of betulinic acid (**70**) and therefore the highest formation of the product was obtained when using 160 °C after 1 h only. The prolongation of the reaction did not influence the yield significantly.

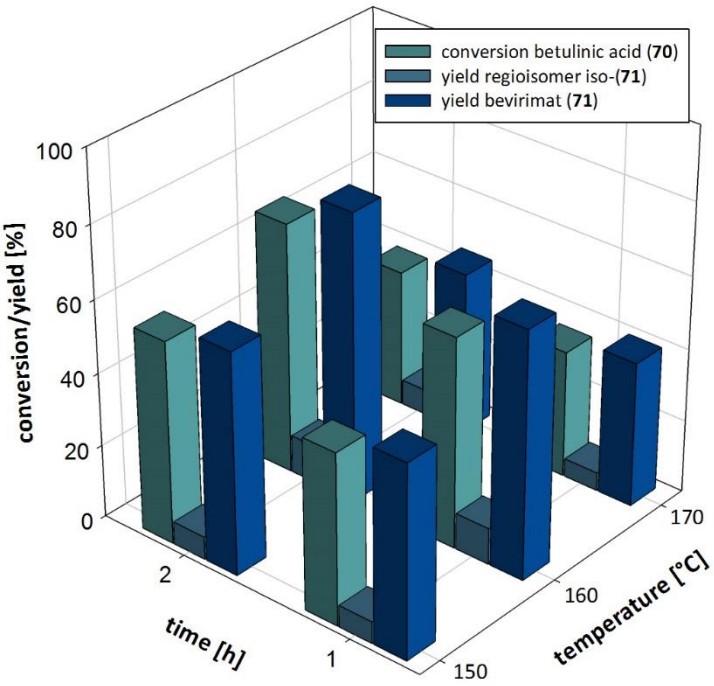


*Figure 119: Esterification of betulinic acid (**70**) applying MW irradiation*

Again the influence of the amount of DMAP and anhydride should be investigated. The most efficient product formation was observed using again 2 eq. of DMAP and 3 eq. of anhydride. (Table 36, entry 1) A control experiment without DMAP was performed and no conversion of betulinic acid (**70**) was observed. (Table 36, entry 4)

Table 36: MW irradiation and variation of the esterification reagents

Entry	DMAP [eq.]	Anhydride [eq.]	Conversion betulinic acid (70) [%]	Yield regioisomer iso-(71) [%]	Yield bevirimat (71) [%]
1	2	3	68	10	58
2	1	3	62	7	55
3	3	2	28	4	24
4	0	3	0	0	0

Performed using 15 mg (1 eq.) betulinic acid (**70**) in 0.5 ml BuOAc. 160 °C, MW, 1 h. Yields were determined *via* HPLC using 1-methyl-cyclohexene as ISTD.

In a last step the influence of the concentration of the starting material present was, showing a strong concentration dependency (Table 37). This is problematic for the *in situ* extraction and esterification of betulinic acid (**70**) from biomass were larger volumes of solvents are required. The screening was run with 15 mg betulinic acid (**70**) and 0.5 ml of solvent, and a homogenous solution was obtained under heating. (Table 37, entry 1) If the concentration of betulinic acid (**70**) was increased to 60 mg/ml an increase in conversion of betulinic acid (**70**) and product formation was observed. (Table 37, entry 2) A further increase to 150 mg/ml resulted in 95% conversion and 84% product formation. (Table 37, entry 3) Similar results were obtained using dimethylcarbonate and pyridine as solvent. (Table 37, entry 4, 5) Interestingly the reaction could be performed on a small scale without solvent with high

conversion and 76% product yield, although problems due to insufficient mixing are expected on larger scale. (Table 37, entry 5)

*Table 37: Concentration dependency of betulinic acid (**70**) for the esterification towards bevirimat (**71**)*

Entry	c 70 [mg/ml]	Solvent	Conversion betulinic acid (70) [%]	Yield regioisomer iso-(71) [%]	Yield bevirimat (71) [%]
1	30	BuOAc	45	4	41
2	60	BuOAc	52	5	46
3	150	BuOAc	95	11	84
	150	DMC	94	13	81
4	150	pyridine	92	11	81
5	-	neat	88	11	76

Performed using 15 mg **70**, 2 eq. DMAP, 3 eq. anhydride, MW, 160 °C, 1 h. Yields were determined *via* HPLC including ISTD.

4.4.5 Reactive dissolution of plane bark

4.4.5.1 Basic ionic liquids for the reactive dissolution of plane bark

After successful evaluation of esterification with pure starting materials, the attention was turned towards the extraction from biomass and *in situ* reaction for the synthesis of the desired compound bevirimat (**71**). Two approaches starting from different types of biomass were chosen: The first one was the direct extraction and conversion of betulinic acid (**70**) to bevirimat (**71**) from plane bark. (Figure 120) The second one (chapter4.4.2) starts with the extraction of betulin (**69**) from birch bark, followed by its oxidation to betulinic acid (**70**) and the esterification towards bevirimat (**71**).

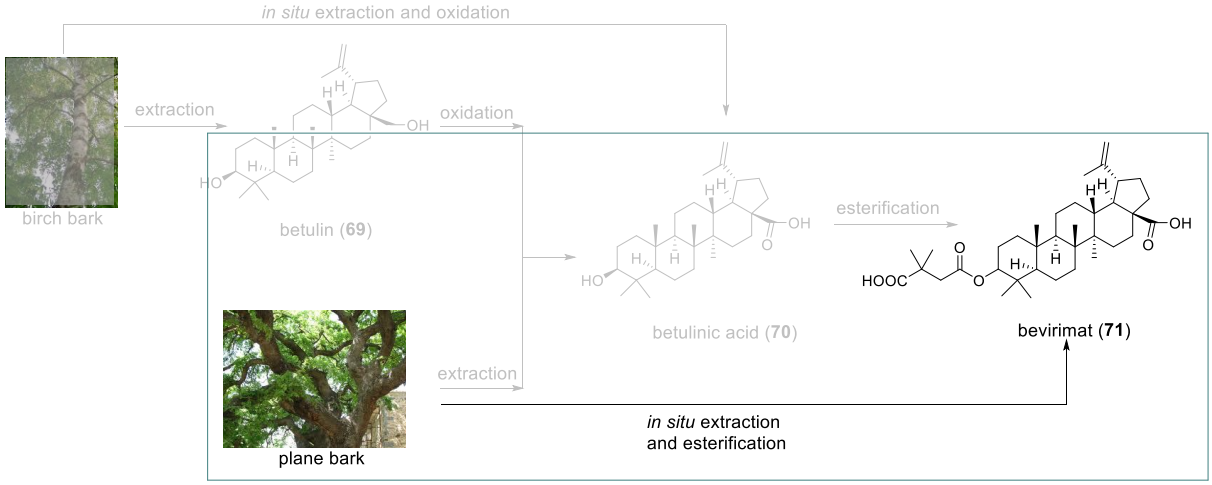


*Figure 120: In situ extraction and esterification of betulinic acid (**70**) towards bevirimat (**71**)*

Initially the reactive dissolution of betulinic acid (**70**) using basic ionic liquids was investigated. Since betulinic acid (**70**) is naturally occurring in the bark of *platanus acerifola* up to 3 wt%[175] the possible dual role of ionic liquids that could act not only as solvent but also as catalyst and reaction media for the transformation towards bevirimat (**71**) was explored. (Figure 121)

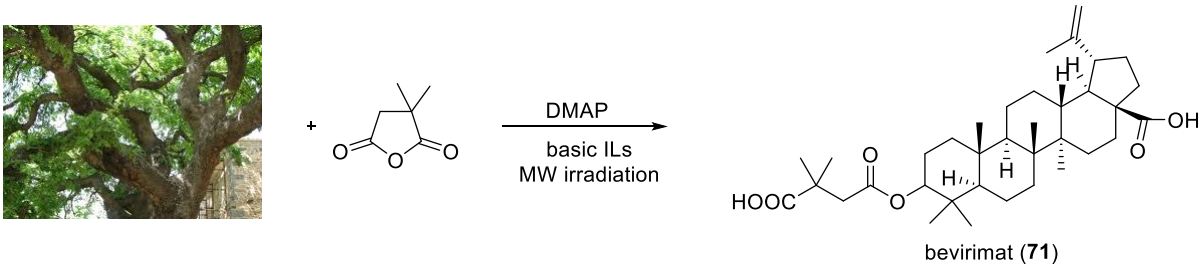
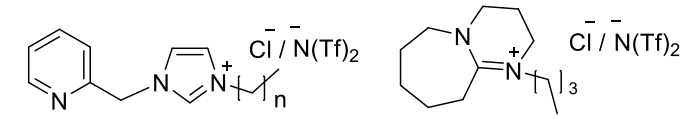


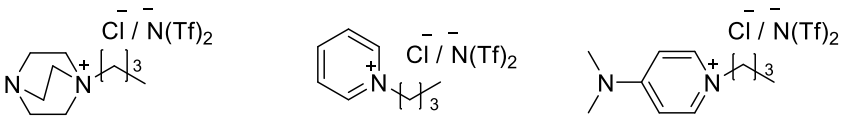
Figure 121: Reactive dissolution of plane bark

4.4.5.1.1 Choice of ionic liquids

As the literature reaction is performed in pyridine and the esterification is catalyzed by a base, a set of basic ionic liquids was chosen. They should play a dual role and should serve as additional base for the esterification of betulinic acid (**70**) and as solvent for the dissolution of plane bark. Chloride and bistriflimide ionic liquids based on imidazolium-pyridine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 1,4-diazabicyclo[2.2.2]octane (DABCO), 4-dimethylaminopyridine (DMAP) and pyridine were synthesized for this purpose. (Figure 122)



n = 0, [C_1impyr]Cl (**20**), 98 %
n = 0, [C_1impyr]N(Tf)$_2$ (**21**), 96 % [C_4DBU]Cl (**11**), 87%
n = 3, [C_4impyr]Cl (**23**), 84% [C_4DBU]N(Tf)$_2$ (**15**), 96%



[C_4DABCO]Cl (**12**), 48% [C_4Pyr]Cl (**13**), 82% [C_4DMAP]Cl (**14**), 67%
[C_4DABCO]N(Tf)$_2$ (**16**), 99% [C_4Pyr]N(Tf)$_2$ (**17**), 94% [C_4DMAP]N(Tf)$_2$ (**18**), 96%

Figure 122: Basic ionic liquids used for esterification

4.4.5.1.2 Esterification of betulinic acid (70) using basic ionic liquids

Since it is known that ionic liquids have the ability to efficiently absorb microwave, the microwave assisted esterification of betulinic acid (**70**) using basic ionic liquids was considered a promising strategy. With the optimized conditions obtained from esterification a screening using hydrophilic basic chloride ionic liquids and hydrophobic basic bistriflimide ionic liquids was performed. 15 mg betulinic acid (**70**) were irradiated under microwave conditions for 1 h at 160 °C in 0.1 g ionic liquid/solvent with the addition of 2 eq. DMAP and 3 eq. anhydride.

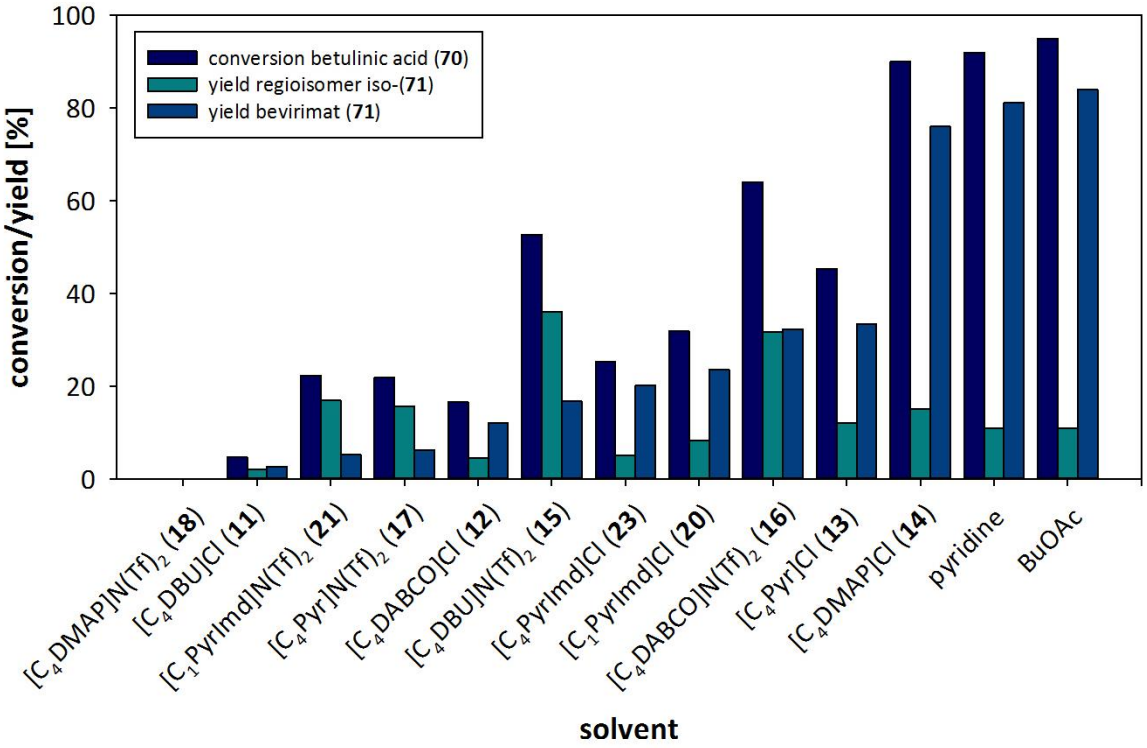


*Figure 123: Basic ionic liquids for the esterification of betulinic acid (**70**)*

As it can be seen in Figure 123 ionic liquids gave generally lower yields of bevirimat (**71**) compared to the organic solvents. Interestingly the bistriflimide ionic liquids preferably yielded the regioisomer iso-**71** instead of bevirimat (**71**). This effect is particularly pronounced in case of $[C_4DBU]N(Tf)_2$ that gave twice the yield of the regioisomer iso-**71** compared to bevirimat (**71**). Highest conversion was obtained using the ionic liquid $[C_4DBU]N(Tf)_2$, however only 32% of both products was obtained. The chloride ionic liquids yielded bevirimat (**71**) preferably. Only the DMAP based chloride ionic liquid gave similar yields compared to organic solvents such as pyrdine or BuOAc. Since the ionic liquid esterification did not lead to desired enhancement of the yield of bevirimat (**71**) this part of the project was closed.

4.4.6 *In situ* extraction and oxidation of betulin (69)

The last approach towards the synthesis of betulinic acid (**70**) and bevirimat (**71**) is presented in Figure 124. The combination of established extraction condition to obtain betulin (**69**) from birch bark (chapter 4.4.2.2) and the optimized oxidation to betulinic acid (**70**) (chapter 4.4.4.4) should be combined.

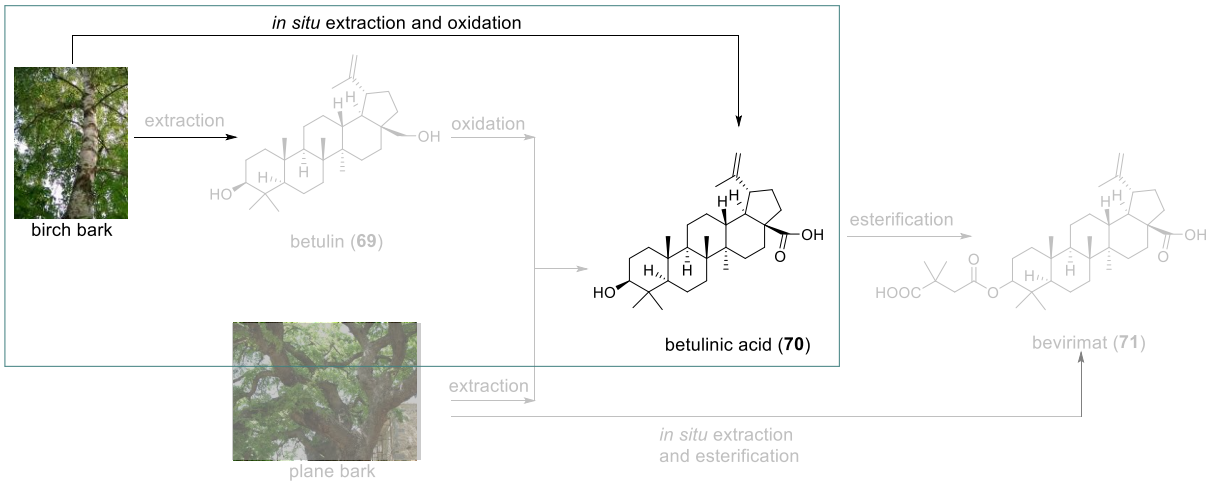


*Figure 124: In situ extraction and oxidation of betulin (**69**) towards betulinic acid (**70**)*

After establishing the ideal extraction and oxidation conditions these two aspects were combined into the direct oxidation of the crude biomass-derived betulin (**69**) extract in BuOAc. (Figure 125) As ionic liquid a 40 wt% solution of [P_{4444}]OH was chosen, since this ionic liquid showed superior extraction efficiency compared to other solvents (chapter 4.4.2.2).

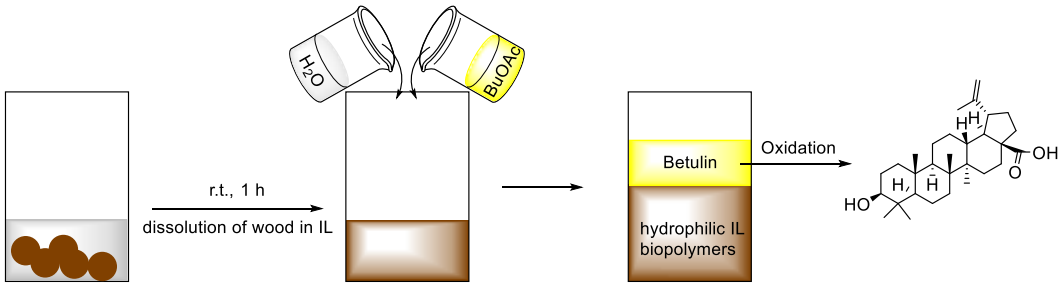


*Figure 125: Extraction of betulin (**69**) using an ionic liquid/water/BuOAc system and further reaction to betulinic acid (**70**)*

However, difficulties were faced when oxidizing the crude betulin (**69**), as conversion and yield remained significantly below the values of obtained for pure starting material. The amount of oxidation reagents was increased up to 1 eq. TEMPO and 6 eq. BAIB, however the maximum betulin (**69**) conversion was 12% with a yield of betulinic acid (**70**) below 1%.

Part of these problems could be traced to the presence of small amounts [P_{4444}]OH (**76**) in the BuOAc layer, as the oxidation of pure betulin (**69**) was inhibited when [P_{4444}]OH (**76**)was present. When only 0.5 eq. of the ionic liquid were added in regard to betulin (**69**), conversion dropped from 100% to 88%, whereas no conversion was observed when 2 eq. of the phosphonium salt **76** were present. With the addition of 0.5 eq. of [P_{4444}]OH (**76**) a conversion of 95% could be reached when the double amount of oxidation reagents was used. Therefore a closer look was taken at the reaction mechanism. (Figure

104) As acetic acid (HOAc) is released from the hypervalent iodine species to activate TEMPO and a basic ionic liquid is used as extraction medium, the idea of neutralizing the basic ionic liquid with HOAc was raised. The oxidation with the addition of 2 eq. ionic liquid was repeated with a 10 fold excess of HOAc. Conversion of 100% was reached and betulinic acid (**70**) was obtained in 50% according to HPLC. The influence of HOAc on the oxidation reaction was investigated.

Addition of HOAc to the oxidation of betulin (**69**) *to betulinic acid* (**70**)

A time screening was performed in order to check the influence of the HOAc on the conversion of the reaction. First time screening was performed with 0.1 eq TEMPO, as incomplete conversion was previously observed without HOAc. It can be seen that the addition of 1 eq. of HOAc shifted the conversion of betulin (**69**) from 97% to 100%. However with already 5 and 10 eq. HOAc a slightly faster increase of conversion was observed, while adding 50 eq. of HOAc lead to full conversion already after 2 hours. With higher amounts of HOAc the conversion of betulin (**69**) was slower, but still reached 100% after 24 h. The yield of betulinic acid (**70**) was not significantly influenced by the addition of 0 to 50 eq. of HOAc. Again with the addition of 100 and 250 eq. HOAc the formation of betulinic acid (**70**) was slower, but reached similar yields after 24 h. The slow reaction using 100 or more eq. of HOAc can be explained by the larger solvent volume and therefore a lower concentration of betulin (**69**). The influence of HOAc with a constant volume will be investigated later. (Figure 131)

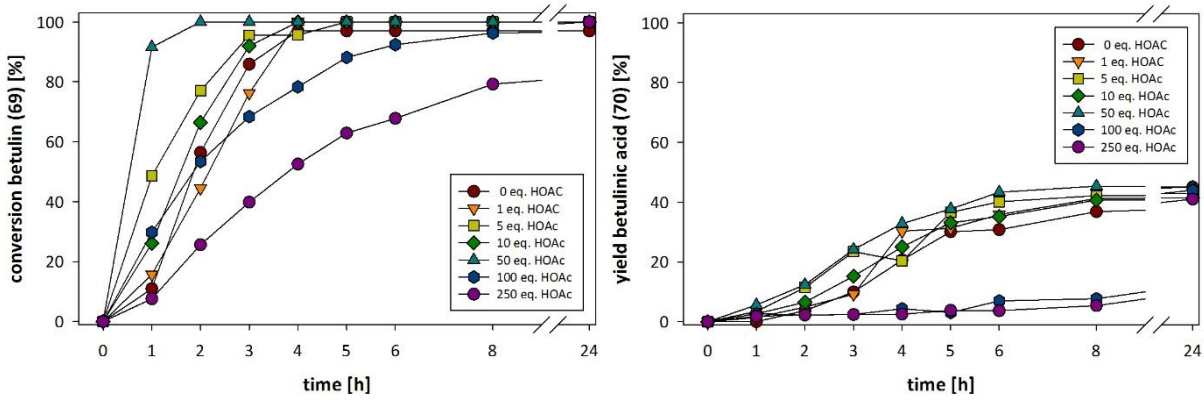


*Figure 126: Time screening using 0.1 eq TEMPO (left conversion betulin (**69**); right yield betulinic acid (**70**))*

In case of 0.2 eq. of TEMPO the influence of HOAc was not as strong as with 0.1 eq. of TEMPO. With the addition of low amounts of HOAc no significant acceleration of the reaction was observed, whereas with the addition of 100 eq. of HOAc a similar trend was obtained as before with 0.1 eq. of TEMPO. (Figure 127) For the formation of betulinic acid (**70**) already 50 eq. of HOAc slowed the formation. However a significant increase up to 58% with 100 eq. of HOAc was observed.

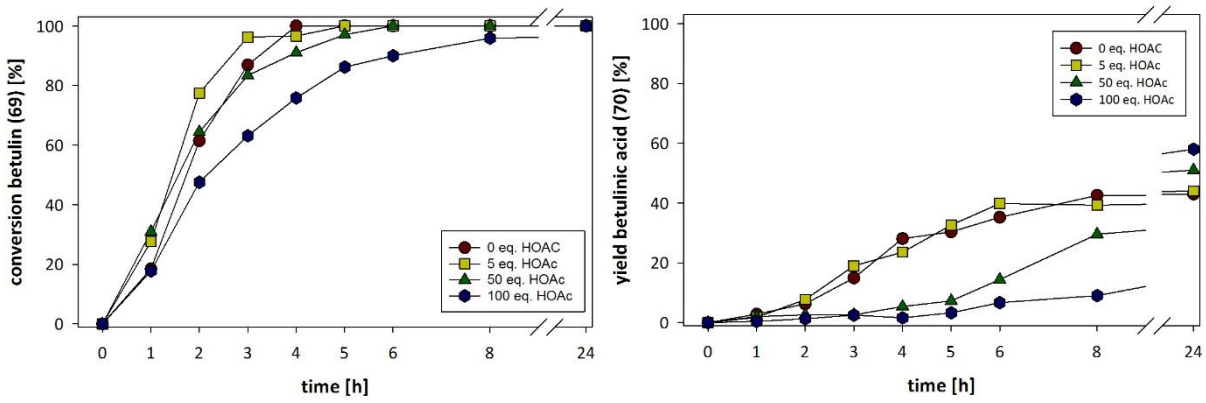


*Figure 127: Time screening using 0.2 eq. TEMPO (left conversion betulin (**69**); right yield betulinic acid (**70**))*

The TEMPO/BAIB oxidations were repeated with the addtion of HOAc and the yield of betulinic acid (**70**) was investigated after 24 h reaction time. (Figure 128) It can be seen that at concentrations from 1 to 10 equivalents HOAc the reaction yield was not affected by the added HOAc. However with higher amounts the yield of betulinic acid (**70**) was increased in case of 0.2 eq. of TEMPO and up to 58% yield betulinic acid (**70**) were obtained. With higher concentrations of 250 eq. of HOAc the yield dropped again, probably due to a dilution effect, as mentioned before.

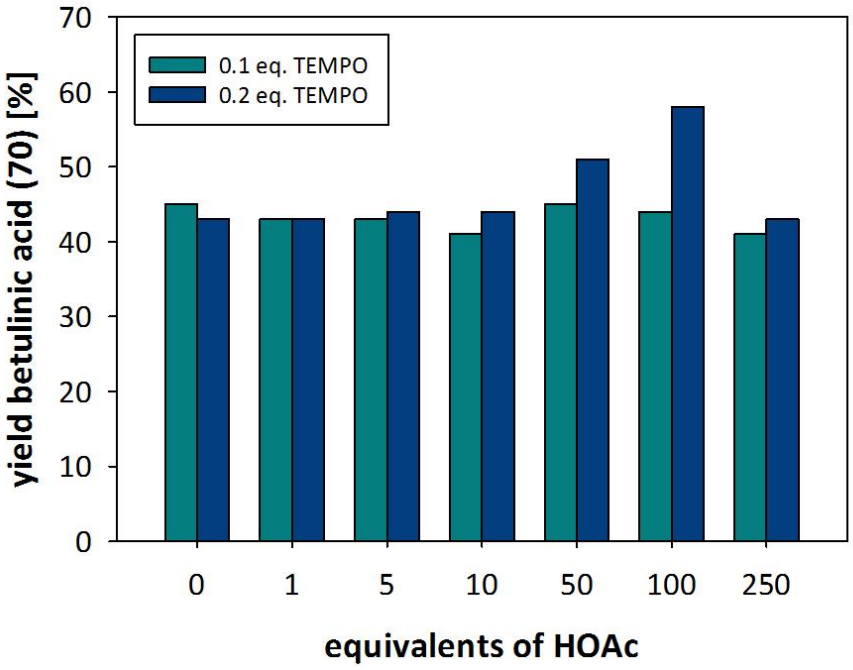


*Figure 128: Comparison of the yield of betulinic acid (**70**) between 0.1 eq. TEMPO and 0.2 eq. TEMPO with the addition of HOAc*

To evaluate the beneficial influence of HOAc, the amount of BAIB was varied. Unfortunately it was not possible to reduce the amount of BAIB, since a reduction to only 2 eq. resulted in incomplete conversion of the starting material.

Table 38: Variation of the amount of BAIB in combination with HOAc

Entry	BAIB [eq.]	Conversion betulin (69) [%]	Yield betulinic acid (70) [%]
1	3	100	58
2	2	70	22
3	1	16	1

Performed using 10 mg betulin (**69**), 0.2 eq. TEMPO, 100 eq. HOAc and a mixture of 1 ml BuOAc, 50 eq. water and 2 eq. *t*-BuOH.

Apart from that the Pinnick oxidation was also tested with the addition of HOAc. It was shown that with the addition of 100 eq. HOAc the yield of betulinic acid (**70**) was increased from 75% to 91%. However, it seems that the influence of HOAc is mostly based on the TEMPO/BAIB oxidation.

Influence of different work-up

After evaluating the influence of the HOAc, the attempt of oxidizing the crude extract with the addition of 100 eq. of HOAc failed, although the amount of oxidation reagents was doubled and TEMPO was even added in a stoichiometric amount. Therefore the crude extract was treated in different ways and the influence of different work-up strategies were investigated in details. First, the crude brown extract was washed with water to remove any residual ionic liquid present in the BuOAc layer. The organic layer was then filtered over Na_2SO_4/SiO_2 to obtain a slightly yellow liquid. Removal of excess solvent ensured a concentration of betulin (**69**) of 10 mg/ml. To evaluate the influence of each work-up, all work-up steps were performed separately as well.

In order to have a closer look at the inhibition of the oxidation reaction ^{1}H, ^{13}C and ^{31}P-NMRs were recorded from the crude extracts. Table 39 shows that in case of no work-up and only washing with water a phosphor signal was found indicating that traces of the ionic liquids were enriched in the organic layer. In case of filtration over silica gel no phosphor signal was detected.

Table 39: ^{31}P-NMR investigations of different work-ups

Entry	Work-up	^{31}P signal found
1	No work-up	+
2	Washing with water	+
3	Filtration over SiO_2	-
4	Washing with water and filtration over SiO_2	-

Interestingly two phosphor signals were detected and comparison to a phosphor spectrum of $[P_{4444}]OH$ (**76**) showed that the second signal might be derived from $[P_{4444}]Br$ (**75**), an impurity in the commercial solution that serves as precursor for the synthesis of $[P_{4444}]OH$ (**76**). Figure 129 shows a comparison of the crude extract (top), $[P_{4444}]OH$ (**76**) with a second signal and $[P_{4444}]Br$ (**75**) and a mixture of the two phosphonium salts.

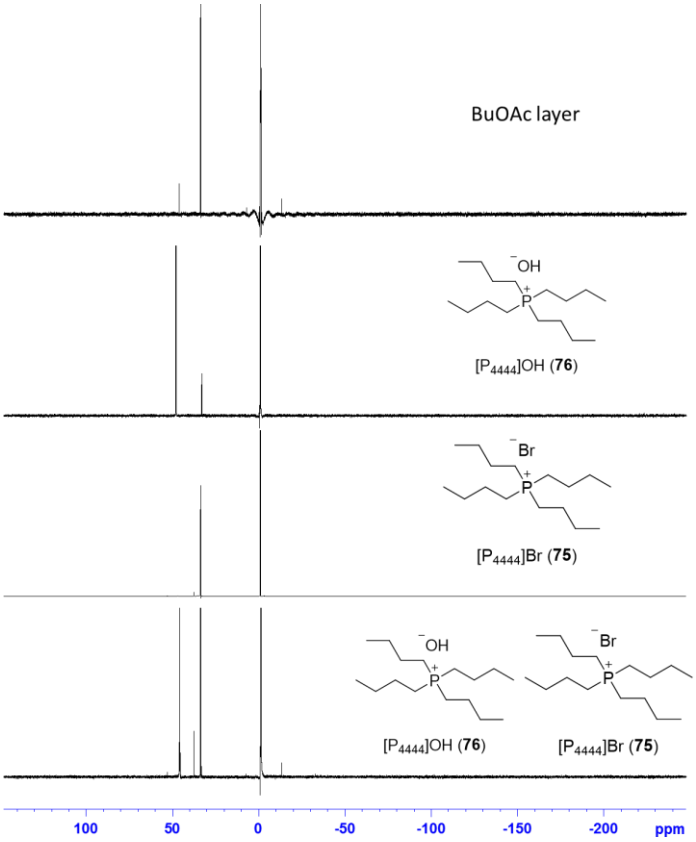


*Figure 129: BuOAC (top) phase after stirring with [P_{4444}]OH (**76**) and the reference materials (phosphoric acid as external standard)*

The phosphor NMRs were also recorded in D_2O and compared to the signals obtained in d_6-DMSO: As it can be seen in Figure 130 on the left, two signals were obtained for [P_{4444}]OH (**76**) measured in d_6-DMSO (plus phosphoric acid as external standard), whereas only one signal was obtained when D_2O was used as solvent. A solvation effect seemed to shift the phosphor peaks in DMSO. However in the very polar deuterated water the same chemical shift was observed for both phosphonium salts. (Figure 130)

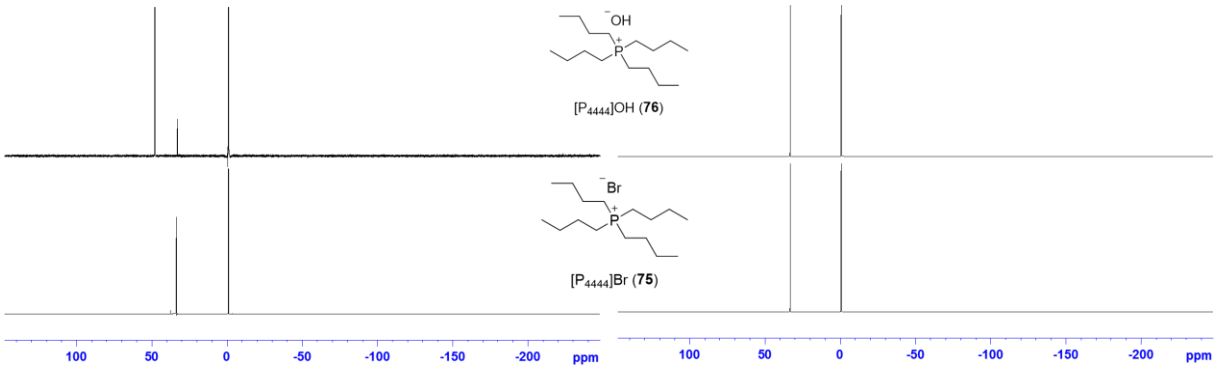


*Figure 130: ^{31}P-NMRs of [P_{4444}]OH (**76**) and [P_{4444}]Br (**75**) in d_6-DMSO (left) and in D_2O (right) using phosphoric acid as external standard*

The oxidation was also performed with the addition of [P_{4444}]Br (**75**) and more unidentified byproducts were detected. With the addition of 10 mol% [P_{4444}]Br (**75**) the conversion of betulin (**69**) dropped to 90%, whereas only 70% of betulin (**69**) were converted with the addition of 50 mol% [P_{4444}]Br (**75**). Therefore it is important to avoid any traces of [P_{4444}]Br (**75**) and [P_{4444}]OH (**76**).

Furthermore the influence of HOAc on the oxidation of crude extracts was evaluated. Table 40 shows that without the addition of HOAc, no conversion was obtained, although “full” work-up including washing with water, filtration over silica gel and concentration was performed. (Table 40, entry 1) However with the addition of 100 eq. HOAc a conversion of 94% was observed. (Table 40, entry 2) If the work-up was simplified and the water washing excluded the conversion dropped to 84%. If the solution was not filtered over silica gel and only concentrated only 4% conversion was observed. (Table 40, entry 3,4) Keeping in mind that untreated biomass was used higher amounts of HOAc were added, since some unidentified coextracted products might be neutralized. As it can be seen in entry 9-13 additional HOAc had a good influence on the reaction and up to 64% conversion of betulin (**69**) were reached, if only evaporation was applied for the work-up. The ideal amount of HOAc was 300 eq. An even higher conversion was reached when evaporation and filtration over silica gel were combined. (Table 40, entry 14-18) With 200 eq. and 300 eq. even 100% conversion was obtained. However with increasing amount of HOAc the conversion dropped. Similar behavior was also observed for the combination of water washing and evaporation. (Table 40, entry 19-22) However yields were significantly lower as for the silica filtration.

Table 40: Oxidation of the extract using different methods (important results highlighted)

Entry	H_2O washing	SiO_2 Filtration	Evaporation	HOAc [eq.]	Ratio BuOAc:HOAc	Converison betulin (**69**) [%]	Yield betulinic acid (**70**) [%]
1	•	•	•	-		0	0
2	•	•	•	100	6:1	94	52
3		•	•	100	6:1	84	4
4			•	100	6:1	4	0
5		•		100	6:1	0	0
6	•			100	6:1	0	0
7				300	1.5:1	6	2
8				500	1:2	0	0
9			•	200	3:1	53	3
10			•	300	1.5:1	64	5
11			•	400	1:1.5	60	4
12			•	500	1:2	58	7
13			•	600	1:4	33	6
14		•	•	200	3:1	100	54
15		•	•	300	1.5:1	100	39
16		•	•	400	1:1.5	92	33
17		•	•	500	1:2	79	16
18		•	•	600	1:4	50	9
19	•		•	200	3:1	83	8
20	•		•	300	1.5:1	81	7
21	•		•	400	1:1.5	77	18
22	•		•	500	1:2	54	16

Crude extract derived from an extraction using $[P_{4444}]OH$ (**76**) and BuOAc with a content of 10 mg betulin (**69**) was subjected to oxidation using 0.2 eq. TEMPO, 3 eq. BAIB in a mixture of BuOAc/*t*-BuOH/water.

In order to investigate the reaction medium BuOAc/HOAc in more details, oxidation of pure betulin (**69**) was performed. In contrast to previous experiments where the addition of rather low amounts of HOAc was investigated (up to 100 eq.) the addition of high amounts HOAc was investigated. With optimized conditions (0.2 eq. TEMPO, 3 eq. BAIB, a concentration of 10 mg **69**/ml and 2 eq. *t*-BuOH and 50 eq. water) oxidations were performed. (Figure 131) It should me mentioned that in all cases

complete dissolution of starting material was observed. However, BuOAc seems to be the better solvent, since the conversion of betulin (**69**) dropped in all cases with increasing amount of HOAc added. It can also be seen that only evaporation and a combination of water washing and evaporation were not sufficient to obtain full conversion. The additional water washing however had a good influence, probably due to removal of coextracted phosphonium salts, and higher conversion was observed. In case of filtration and evaporation the same conversions as for oxidation of pure betulin (**69**) was observed.

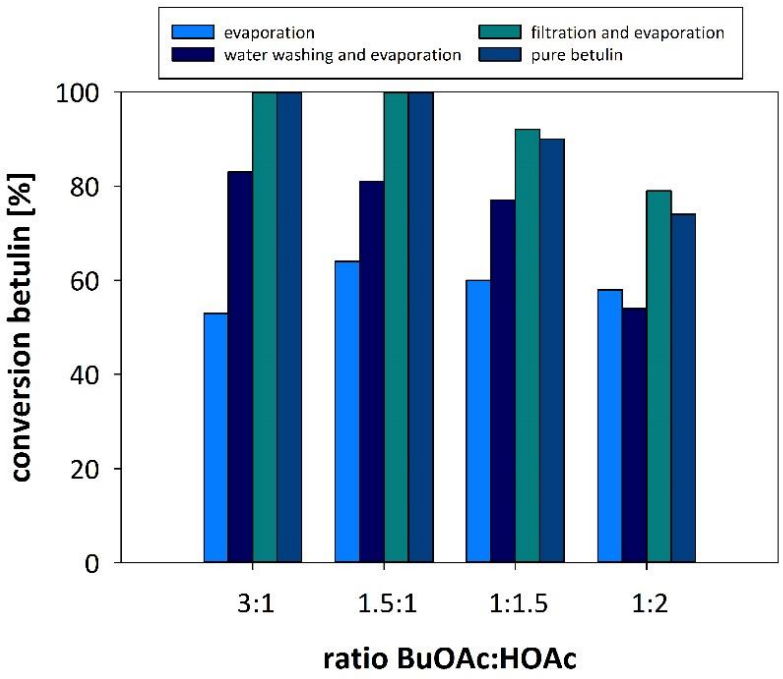


*Figure 131: Oxidation from crude extracts with differents work-ups and pure betulin (**69**) with the addition of high amounts of HOAc*

4.4.6.1 Isolation of betulinic acid (70)

After optimization of the extraction of betulin (**69**) from birch bark and the oxidation of pure betulin (**69**) to betulinic acid (**70**) and the combination thereof, betulinic acid (**70**) was isolated using following strategy: (Figure 132) Birch bark was dissolved in the phosphonium ionic liquid [P_{4444}]OH (**76**) at room temperature for a short dissolution time of 1 h. After the addition of water and BuOAc the solution was stirred for another hour. After separation of the organic layer, filtration over SiO_2 the solution was concentrated to obtain a 10 mg/ml solution of betulin (**69**) in BuOAc. The oxidation reagents were added, starting with TEMPO/BAIB reagents. After 5 h reaction time Pinnick reagents were added and the solution stirred for 19 h. After purification using column chromatography 18 wt% in reference to used birch bark were obtained.

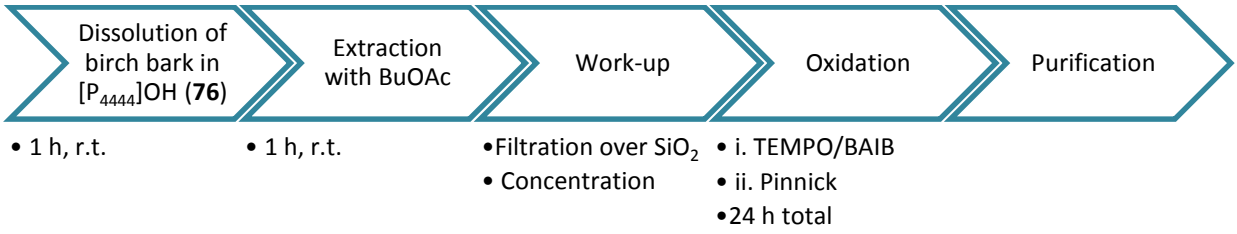


*Figure 132: Isolation strategy for betulinic acid (**70**) from birch bark*

One byproduct was obtained in 3 wt% yield and was identified as lupeol (**77**). Lupeol (**77**) is structurally very similar to betulin (**69**) as it can be seen in Figure 133. The content of lupeol (**77**) in birch bark varies between 1-4 wt% according to Rizhikovs *et al*.[246]

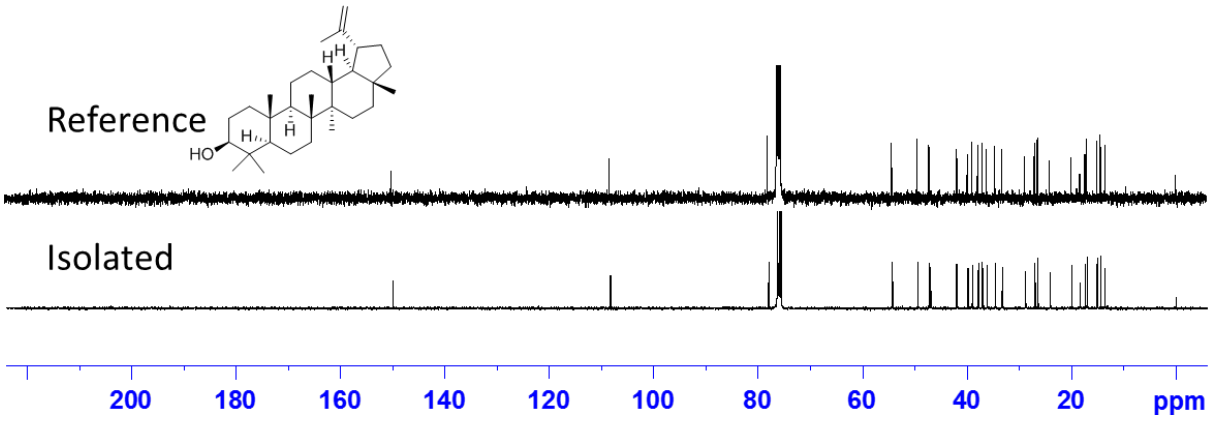


*Figure 133: ^{13}C NMR of reference lupeol (**77**) (top) and isolated lupeol (bottom) after column chromatography*

So far a biomass loading of 200 mg was used. A small scale-up was performed and 500 mg starting material was used. To get an impression on the beneficial impact of the ionic liquid a control experiment using water/BuOAc as extraction solvents. Furthermore the obtained betulinic acid (**70**) should be directly crystallized from the organic layer after performing a work-up. As methanol/water was a suitable crystallization solvent for betulinic acid (**70**), this mixture was applied. However this crystallization steps cannot separate betulinic acid (**70**) and lupeol (**77**). In case of the ionic liquid-based extraction 21.9 wt% betulinic acid (**70**) (including **77**) with high purity were obtained and a second fraction of 7 wt% **70** (including **77**) with a purity <90%. For the ionic liquid free process only 15.5 wt% of betulinic acid (**70**) (including **77**) in high purity and again a second fraction of 6 wt% **70** (including **77**) in lower purity was obtained. Compared to the conventional approaches for the isolation or synthesis from e.g. synthesis or isolation the developed ionic liquid strategy implies some tremendous improvement for the isolation of betulinic acid **70**. (Table 41) Not only the use of chrome was avoided, but also the yield was tremendously from 56% to 89% yield. Furthermore long reflux times for the extraction of betulin (**69**) using toxic dichloromethane was replaced by a 1 h process run at room temperature using a cheap aqueous ionic liquid solution and the environmentally benign BuOAc.

*Table 41: Comparison of conventional and ionic liquid approach for the synthesis of betulinic acid (**70**)*

Entry	Strategy	Conventional approach	[P_{4444}]OH strategy
1	Synthesis from betulin (**69**)	CrO_3	Chromium free
2		2 step synthesis	One pot synthesis
		36-56% yield **70** [248, 249]	89% yield **70**
3	Isolation from bark	Dichloromethane	BuOAc
4		Reflux, long hours	Room temperature, 1 h
		3 wt% (plane)	18 wt% (22 wt%)[a]
		15 wt% (birch)[a]	(birch)

[a] Isolated in this study after recrystallization

4.5 Isolation of limonene and *in situ* multistep biocatalysis reaction[vi]

The versatility of applications of ionic liquids led to a cooperation with the research group of Prof. M. D. Mihovilovic (TU Vienna). The combination of biomass extraction assisted with ionic liquids and a multistep biocatalytic reaction was investigated based including a novel one pot strategy on the industrial waste product orange peel.

Oranges reached with 68 million tons appr. 9% of the global fruit production in the year 2014.[vii] More than a half of oranges are used for juice production, and up to 60% of the initial biomass end up as waste.[278, 279] A major part of this waste (44%) is represented by their peels.[280] Since the amount of wasted orange peels typically reaches between 6 to 11 tons/year, their valorization represents an important issue. One of the major components of orange peels is (*R*)-(+)-limonene (**83**) that is also naturally occurring in the peel of different other citrus fruits, such as lemons. It has an orange-like smell. Its enantiomer (*S*)-(-)-limonene smells like turpentine and its natural abundance is in peppermint oil.

The major aim of this study was based on a waste to value strategy to produce a highly valuable chiral polymer building block starting from limonene (**83**) containing orange peels: Initially the biomass should be either dissolved in pure ionic liquids or limonene was extracted continuously in the presence of an aqueous ionic liquid mixture. Subsequently, the extracted the extracted (*R*)-(+)-limonene (**83**) *via* a multistep biocatalysis reaction to a chiral lactone **87**, which can further be converted to a thermoplastic biopolymer.[281] (Figure 134) While the extraction and isolation of limonene (**83**) from orange peels was already successfully performed by Bica *et al.*,[195] a part from the cascade reaction starting from carveol (**84**) to the corresponding lactone **87**in *E. coli* was investigated by Oberleitner *et al in vivo* and *in vitro*.[282, 283]Figure 134 outlines the direct conversion of orange peel to a valuable thermoplastic biopolymer using different solvents:

1. Water
2. Organic solvents
3. Ionic liquids

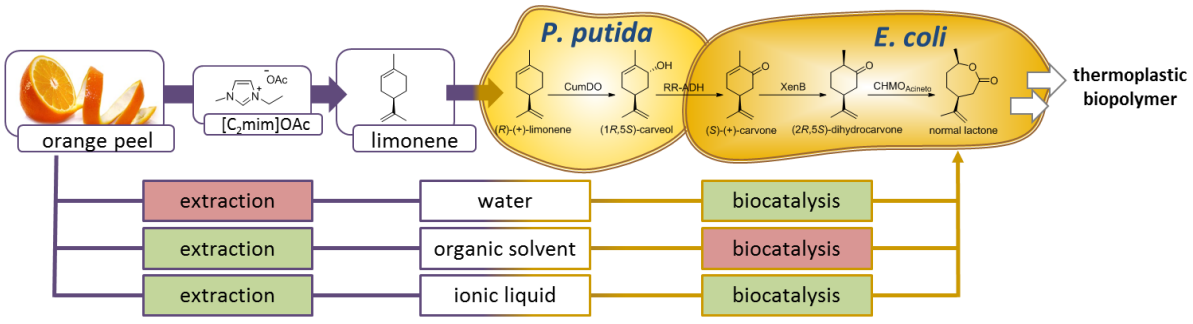


Figure 134: Combination of extraction and biocatalytic multistep reaction

Ionic liquids represent the ideal medium for a biocatalytic process for the production of value-added products from orange peels compared to conventional solvents: While water is not a suitable extraction medium for apolar compounds as limonene (**83**), it is the natural medium for biocatalytic

vi In cooperation with research group Mihovilovic, TU Vienna.

vii FAO, Food and Agriculture Organization, 2014, http://www.faostat.org/.

reactions. One the one hand organic solvents e.g. dichloromethane, EtOAc are suitable solvents for the extraction of valuable ingredients from biomass, on the other such solvents have a massive impact on biocatalytic reactions either by denaturation of proteins or extensive cell lysis in whole cell processes.[284]

Three different strategies were designed and tested for the investigation of a biocompatible and efficient extraction method. First, the simplest approach is based on a one-pot process by just mixing orange peels with aqueous ionic liquid solutions and resting cells. (Approach I) For this purpose aqueous ionic liquid solutions have to be suitable for the extraction and the biocatalysis. For economic reasons, the concentration of the ionic liquids should be kept as low as possible. Approach II starts with the dissolution of biomass in pure ionic liquids, followed by a dilution step to obtain aqueous ionic liquid solutions for the biocatalysis. Although the second approach requires an additional handling step, higher extraction yields compared to approach I might be expected, therefore justifying the more complicated procedure. The last strategy will use hydrophobic ionic liquids to form a biphasic mixture. (Approach III) Beneficial is hereby the role of the ionic liquid as reservoir preventing intensive contact of organic compounds with the cell membranes. Furthermore, the ionic liquid could be recycled as described in literature the possibility of recovery of the ionic liquid.[285] (Figure 135)

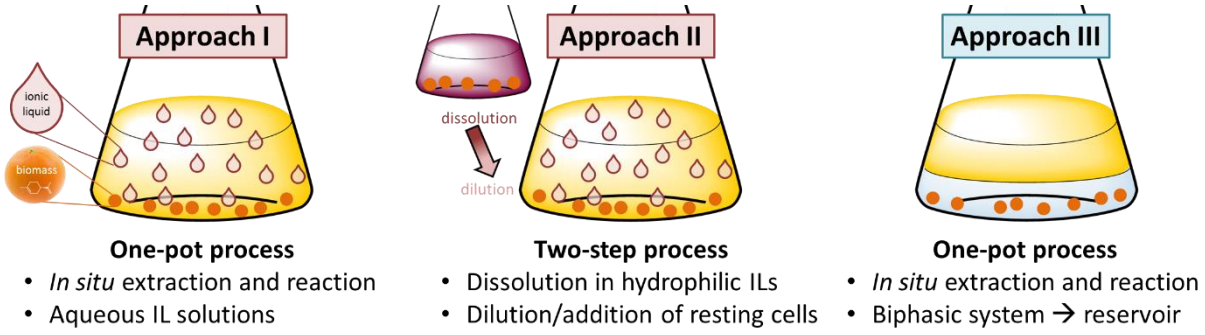


*Figure 135: Different approaches for the synthesis of lactone **87** from oranges*

4.5.1 Biocatalysis in ionic liquids

The use of enzymes has gained attraction during the past decades due to their efficient and clean chemical syntheses. Two main approaches can be distinguished, the use of isolated enzymes (*in vitro*) and the use of whole cells (*in vivo*). The first approach is less complex resulting in an easier control of reaction conditions. The optimization of reaction parameters such as enzyme concentrations, pH-value, or temperature can be investigated systematically. Furthermore the storage of cell free extracts or purified enzymes is more robust compared to living cells. Despite the addition of expensive cofactors the use of an *in vitro* system is more appreciated by chemists since no special equipment is needed. In contrast *in vivo* whole cell systems provide better enzyme stability due to their natural environment and an integrated cofactor regeneration system. Apart from that no enzyme purification is necessary.[286]

Ionic liquids and biocatalysis share a long story as the first biocatalytic reaction conducted in ionic liquids was reported in 2000.[287-289] The application of ionic liquids as unconventional solvents addresses the replacement of volatile organic solvents in biocatalytic reactions that are used to improve the solubility of hydrophobic compounds and/or products, since the natural environment of

enzyme catalyzed reactions is water. Biocatalytic reactions have been performed either as whole cell reaction or with isolated enzymes. The activity and stability of enzymes in neat ionic liquids or aqueous solutions thereof have already been well investigated.[290]

4.5.1.1 Enzyme compatibility and enzyme catalyzed reactions with ionic liquids

Since the early 1980s it is known that enzymes can be used in hydrophobic organic solvents[291, 292] though with a reduced reaction rate.[284] The use of ionic liquids for enzyme catalyzed reaction was particularly interesting, since improvement of the low turnover rates was expected. Aqueous ionic liquid solutions were able to show an activating effect, e.g. low concentrated aqueous mixtures of $[EtNH_3]NO_3$ activated the alkaline phosphatase from *E. coli*.[293] However at higher concentration of the ionic liquid the activity was tremendously decreased until a concentration of 80% of ionic liquid in water, where the activity was irreversibly lost. To predict and order the properties of ionic liquids for the stabilization of enzymes, the position of the anion in Hofmeister series is applied.[24] A stabilizing effect is predicted for kosmotropic (kosmotropic molecules enhance the stability of water-water interactions) anions, whereas a destabilizing effect is associated with chaotropic (a chaotropic reagent can disrupt hydrogen network bonding in aqueous systems resulting in an entropic increase) anions. Since it is known that (thermal) stability of enzymes can be better in organic media than in aqueous solution, especially at low water activity, a similar effect for nearly anhydrous ionic liquids was assumed. In case of the sweet protein monellin unfolding temperature changed from 40 °C in water to 105 °C in $[C_4mpyro]N(Tf)_2$.[294]

Several examples in literature demonstrate that the stability of enzymes in ionic liquids, neat or as aqueous solution can be improved, as well as the activity. However it is hard to predict the compatibility based on general properties of the ionic liquid.

In the same year when Cull *et al*. published the first whole-cell biotransformation in an ionic liquid[287] the first biotransformation using an isolated enzyme in combination with an ionic liquid was reported.[288] *Z*-Aspartame was synthesized using the enzyme thermolysin with the hydrophobic ionic liquid $[C_4mim]PF_6$ containing 5 % (v/v) water. (Figure 136) The activity of the enzyme was similar to observed values in organic solvents, however the stability of the enzyme in the ionic liquid was excellent compared to EtOAc. The ionic liquid was recovered by dilution with water and by dissolving starting material and product, decantation and evaporation. However, traces of substrate and product remained in the ionic liquid.

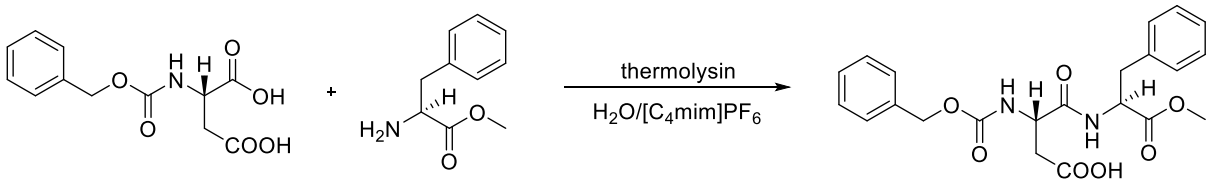


Figure 136: Synthesis of Z-aspartame[288]

However, ionic liquids seem to be suitable for various kinds of biotransformations: they have been intensively used with lipases and esterases, but also with proteases. They have been applied for dynamic kinetic resolution reactions for the formation of chiral alcohols, as well as for redox enzyme systems and for lyases.[290]

4.5.1.2 Whole cell biotransformations in ionic liquids

Although most studies of biocatalysis in ionic liquids focused on the use of isolated enzymes the first reported biocatalytic reaction in ionic liquids was a whole cell biotransformation.[287] A biphasic system containing water and $[C_4mim]PF_6$ was used for a nitrile hydrolysis with a nitrile hydratase from *Rhodococcus* R312. (Figure 137) It was shown that the specific activity was higher in the water-ionic liquid system than in water-toluene. The substrate was better soluble in the ionic liquid, which acted as reservoir. Due to the increase of activity a slightly higher yield was reached.

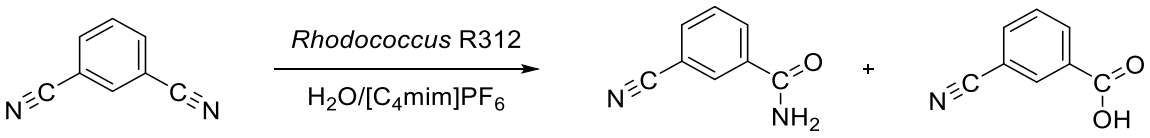


Figure 137: The first whole cell biotransformation in ionic liquids[287]

Only small amounts of water or no water was used for beakers's yeast[295], *Rhodococcus* R312 and *E. coli*[290] while maintaining the activity in the ionic liquid $[C_4mim]PF_6$. However, due to solubility issues whole cell biotransformations were mostly performed in a biphasic system, since a widespread number of organic compounds is barely soluble in water. Apart from that the storage in a second phase avoids contact with the cell membranes and therefore intoxication by organic compounds. Ionic liquids such as $[C_4mim]PF_6$, $[C_4mim]N(Tf)_2$ and $[N_{1888}]N(Tf)_2$ preserved the cell membrane integrity of *Lactobacillus kefir* better than solvents such as decane, octanol and methyl *tert*-butyl ether. An asymmetric reduction of 4-chloroacetophenone towards the alcohol was investigated. The ionic liquid $[C_4mim]N(Tf)_2$ was used as reservoir resulting in enhancement of product yield from 46% to 93%.[296] In case of the cell viability of *E. coli* and *S. cerevisiae* the same ionic liquids were harmless. Again, an asymmetric reduction of ketones was investigated and the use of ionic liquids resulted in higher yields of the chiral alcohol from 50% to 80-90% while the *ee* increased from 62% to 84%. It was stated that the use of ionic liquid was beneficial as reservoir, but also for the appropriate distribution coefficients for substrate and product.[297] The yield of an *E. coli* catalyzed reaction was improved, as well as the *ee* with the use of $[C_n mim]PF_6$ and $[C_n mim]N(Tf)_2$ with n = 4,6 and the corresponding methylpyrrolidinium bistriflimides.[298] An elegant strategy was presented by Dennewald *et al.*: 1-Hexyl-1-methylpyrrolidinium bistriflimide was recovered 25 times without loss of productivity (full conversion, enantiopure products) according to the scheme outlined in Figure 138.[285]

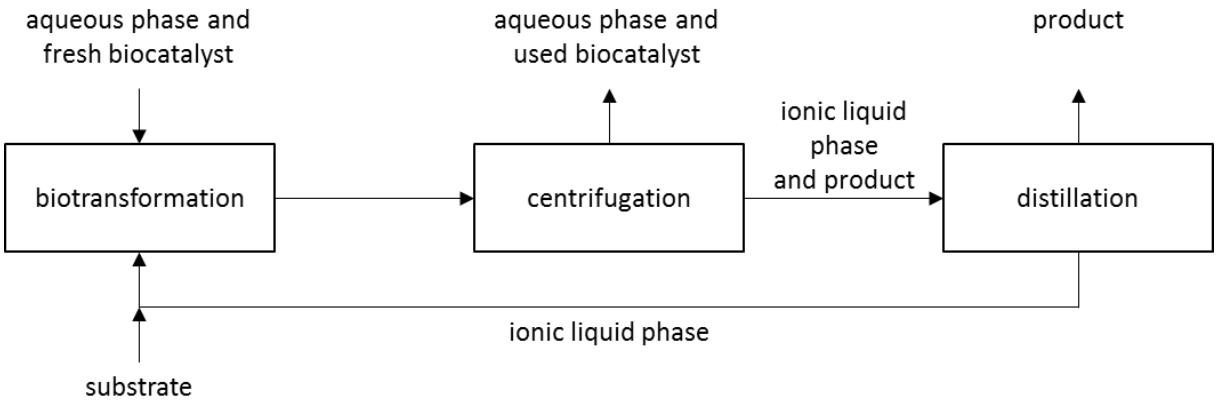


Figure 138: Approach for a recycling system for whole cell biotransformation in biphasic ionic liquid water systems[285]

Furthermore, hydrophobic bistriflimide ionic liquids were used for nucleoside acylation[299] and tetramethylammonium based ionic liquids with a cysteine anion for an asymmetric reduction.[300] Hydrophobic bistriflimide dimethylimidazolium, phosphonium and pyrrolidinium ionic liquids were tested for a *cis*-dihydroxylation of haloarenes with a *P. putida* bacterium as well as the hydrophilic ionic liquids [C_8m_2im]Br and [C_8mpyrro]Br. Compared to the hydrophobic derivatives the hydrophilic ionic liquid showed similar effects on the biotransformation.[301]

A more detailed overview of whole cell biotransformation can be found in literature.[302] Generally it can be stated that mostly hydrophobic ionic liquids were used for whole cell biotransformation resulting in a beneficial influence on reactions yields and enantiomeric excess due to their role as reservoir.

4.5.2 Choice of ionic liquids

After discussing ionic liquids in combination with biocatalysis, ionic liquids were chosen for the *in situ* extraction and conversion of limonene (**83**). The choice of ionic liquids was difficult, since several requirements had to be fulfilled: they should not only be tolerated by the two bacteria stains *Escherichia coli* (*E. coli*) and *Pseudomonas putida* (*P. putida*) and therefore being suitable for the cascade reaction, but also be able to extract limonene (**83**) from orange peels.

A short look in literature[303] showed that long-chain ionic liquids are usually toxic for bacteria. This was confirmed by some pre-experiments. Therefore ionic liquids with a maximum chain length of 6 carbon atoms were chosen. Imidazolium cations with hydrophilic and hydrophobic anions were chosen, as well as the environmentally benign choline ionic liquids with variable chain length from formate to hexanoate. (Figure 139)

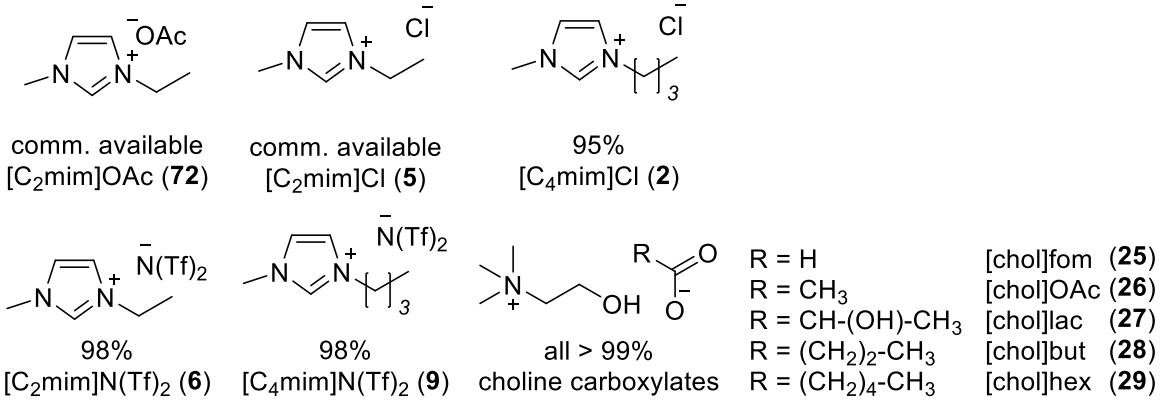


Figure 139: Ionic liquids used for the limonene project

The chosen ionic liquids were first tested in growth curves for the evaluation of the tolerance of bacteria towards the ionic liquids. In a second step biocatalysis should be investigated in the presence of the ionic liquids. With the selected ionic liquids extraction experiments should be performed. Finally extraction and biocatalysis should be combined.

4.5.3 Growth of *E. coli* and *P. putida* in the presence of ionic liquids

One simple and fast method for the evaluation of the toxicity of ionic liquids on bacterial strains is the determination of bacterial growth. While the ionic liquid [C_4mim]BF_4 delayed the growth of *E. coli*, *Pichia pastoris* and *Bacillus cereus* at a 1% concentration, the growth was completely inhibited at a

concentration of 4%. [C_4mim]PF_6 was toxic towards *E. coli* at a concentration of 1% already, well tolerated by *Pichia pastoris* up to a concentration of 10%, but decreased the growth of *Bacillus cereus* at higher concentration than 1%.[304] The same two ionic liquids were used for investigation with baker's yeast showing a good tolerance of 10%.[305]

A set of 90 hydrophilic and hydrophobic ionic liquids was investigated towards the toxicity of *E. coli*. *E. coli* was chosen as it is frequently used for biotransformations. Imidazolium, pyridinium, ammonium, alkanolammonium and phosphonium derivatives containing different anions, e.g. halogenides, sulfate and sulfonate anions were tested. They used an agar diffusion test for the screening of the ionic liquids and inhibition zones were determined. They stated that imidazolium derivatives with short alkyl chain lengths were relatively non-toxic, especially in combination with alkyl sulfate anions. However a short alkyl chain or the use of a bistriflimde anion seemed to lower the toxicity. The most promising class was represented by hydrophilic ionic liquids based on alkanolammonium cations in combination with alkyl sulfate anions. The same cations were used with docusates to form hydrophobic non-toxic ionic liquids.[303]

Figure 140 shows selected growth curves for *E. coli* in the presence of different ionic liquids tested in a concentration of 100 mM (appr. 1% ionic liquid in water). For comparison reasons growth curves of cell cultures without ionic liquid (circle in dark blue) as well as pure medium (not shown) were performed. Pure medium did not show any growth. While [chol]lac (**27**) showed similar growth behavior as the control value [chol]fom (**25**) showed only a slight decrease of bacterial growth. The imidazolium ionic liquid [C_2mim]Cl (**5**) retarded the growth of *E. coli* and [C_4mim]Cl (**2**) completely inhibited the growth.

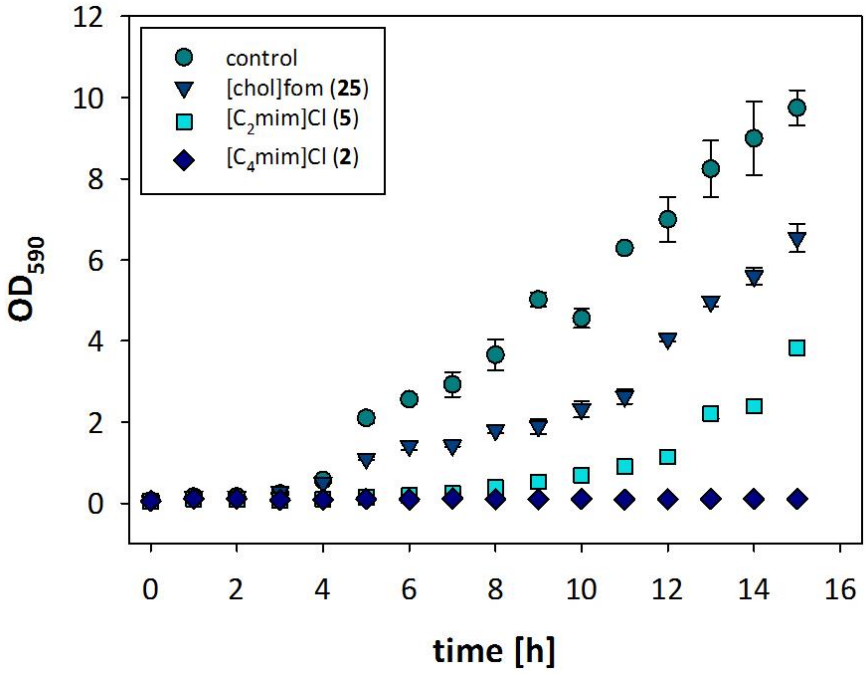


Figure 140: A selected growth curve of E. coli using a 100 mM ionic liquid concentration

In order to investigate the influence of the chosen ionic liquids on the viability on the two bacterial strains *P. putida* and *E. coli*, growth curves in the presence of ionic liquids in two different concentrations – 50 mM and 100 mM – were performed. Starting with the evaluation of *P. putida* since

it can be seen that this bacterium was very sensitive to the contact of ionic liquids. (Figure 141) Only two choline derivatives were well tolerated at a 50 mM concentration [chol]fom (**25**) and [chol]lac (**27**). Choline derivatives with higher chain length and with an acetate anion inhibited the growth. The acetate anion seemed to have a negative effect, since no growth was observed with the imidazolium derivative as well. If the anion was changed from acetate to chloride a significant difference was observed and the chloride ionic liquid only slightly reduced the growth of *P. putida*. This is in accordance with literature where full inhibition of yeast growth and biofuel production with the presence of only 0.25% of [C_2mim]OAc (**72**) was observed, whereas [C_2mim]Cl (**5**) influenced yeast growth to a lesser extent.[85] Again, elongation of the side chain length of the ionic liquid resulted in a lower growth. The same trend was observed by Wood *et al*.[303] The biphasic bistriflimide ionic liquids **6** and **9** were not tolerated at all, which is again in accordance with literature. It was shown that imidazolium bistriflimide ionic liquids inhibit the bacterial growth[303] and affect membrane integrity.[306, 307] The concentration of the ionic liquid had a significant influence on the bacterial growth. Greater inhibition was observed in all cases when increasing the concentration to a 100 mM. The influence of the concentration was comparable in all cases.

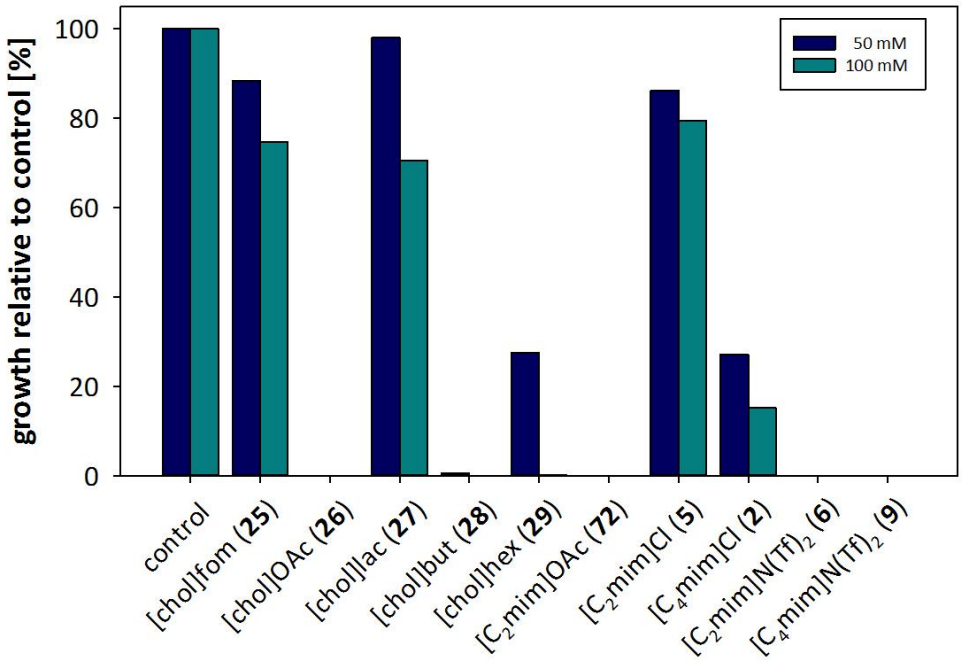


Figure 141: Growth of P. putida in the presence of ionic liquids relative to control

Apart from that the growth of *E. coli* was also investigated: Compared to *P. putida E. coli* showed higher tolerance towards the addition of ionic liquids. (Figure 142) Especially the choline derivatives were very well tolerated and showed insignificant growth inhibition. A good tolerance was shown towards the butyrate derivative and an increase of growth inhibition was observed with [chol]fom (**25**). Hence, an increase of the chain length of the carboxylate anion to 6 carbons fully inhibited the growth the growth of *E. coli* cells. Apart from the choline derivatives the 1-ethyl-3- methylimidazolium derivatives were well tolerated. No or poor growth was observed in case of [C_2mim]N(Tf)$_2$ (**6**), [C_4mim]N(Tf)$_2$ (**9**)and [C_4mim]Cl (**2**), which is comparable to *P. putida*. Some ionic liquids, such as [chol]hex (**29**) led to a massive cell lysia and bacterial growth was retarded. Again in nearly all cases the increase of ionic liquid concentration resulted in a stronger inhibition of growth.

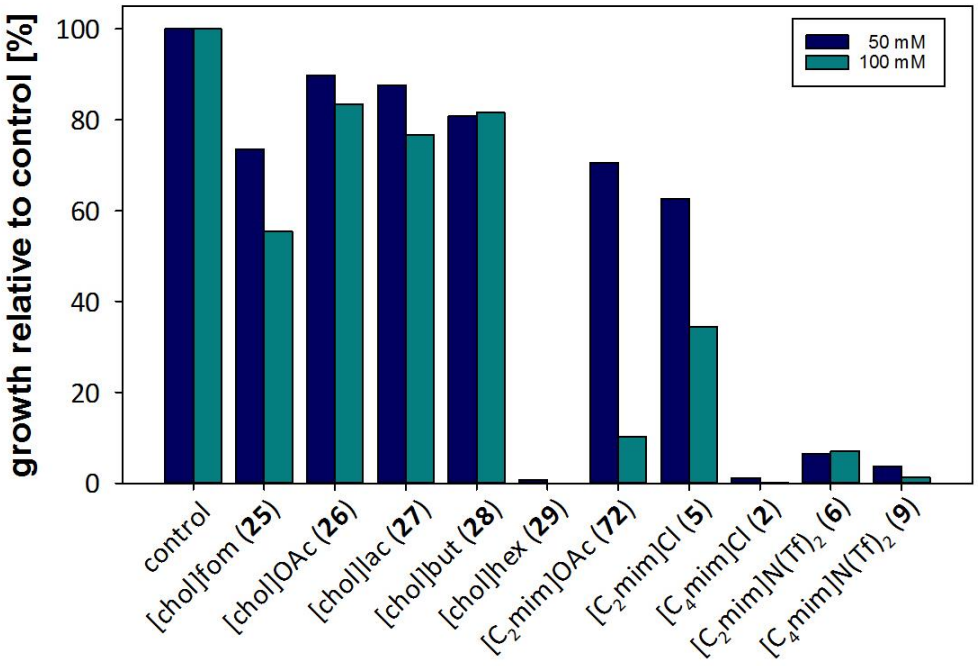


Figure 142: Growth of E. coli in the presence of ionic liquids relative to control

After performing growth curves a first trend towards the compatibility of the chosen ionic liquids and the bacteria can be estimated. However, biocatalytic cascade reaction have to be performed for a precise evaluation.

4.5.4 Multistep enzyme catalyzed reaction in the presence of ionic liquids

Mimicking nature multistep enzymatic cascade reactions emerged to a valuable tool for the one pot synthesis of organic compounds. Naturally a living cell optimized its multistep reactions catalyzed *via* different enzymes by evolution.[308] Compared to conventional synthesis multistep enzyme cascade reaction avoid several purification and isolation steps and make the whole process more efficient. (Figure 143) As there is no need for purification, operating time, costs and waste are reduced, atom economy and overall yield are improved.

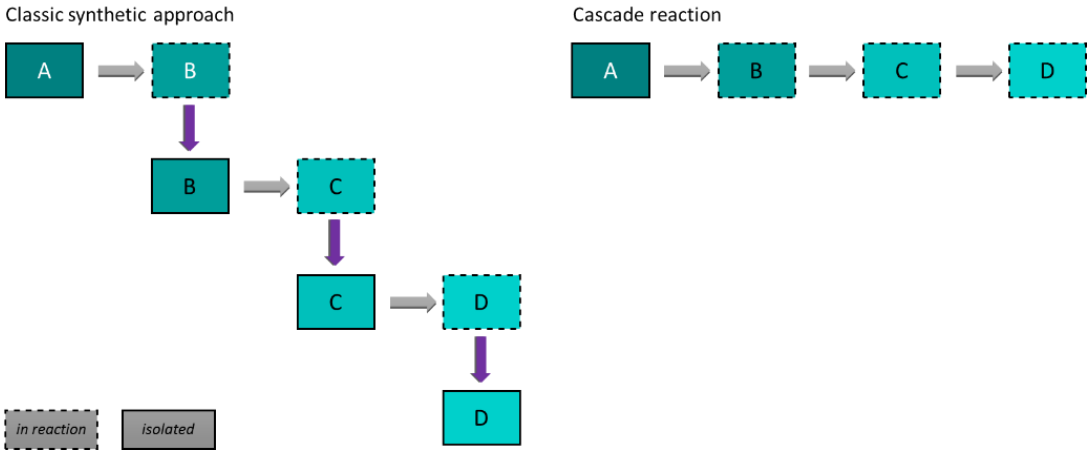


Figure 143: Comparison of classical synthetic approach and cascade reaction[309]

In contrast to chemical one pot reactions where different reaction conditions are needed, biocatalytic cascade reactions benefit from similar reactions milieus. One famous *in vitro* cascade reaction was

published by Sattler *et al.*[310] They used an alcohol dehydrogenase (ADH) from *Bacillus stearothermophilus* in combination with a ω-transaminase from *Chromobacterium violaceum* and L-alanine dehydrogenase for the recycling of the amine donor in a non buffered system. (Figure 144) Nearly full conversion was reached of 50 mM 1-hexanol to the amine derivative. They could also expand their method to diols.

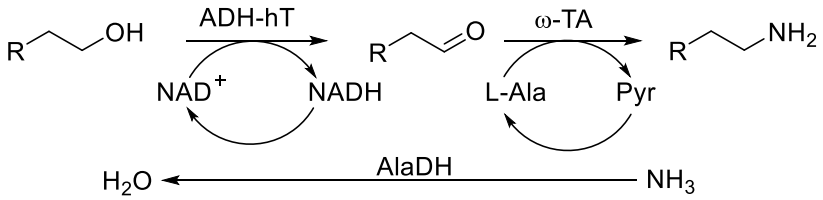


Figure 144: Cascade reaction investigated by Sattler et al.

Multistep biocatalysis reaction are not limited to *in vitro* experiments, *in vivo* cascades are reported as well. However the use of whole cells in cascade reactions is quite challenging: Side reactions can occur caused by other enzymes present in the host. Host background is not the only problem to be faced. Growth deficiency due to competition with the cell's metabolism for cascade precursors can occur. Expression levels are harder to control compared to *in vitro* experiments. The topic enzymatic cascade reactions reactions has recently been reviewed.[311]

Limonene (**83**) represents a cheap chiral building block for polymers, which can be obtained by a four step cascade reaction that was investigated recently.[282, 283] (Figure 145) In a first step limonene (**83**) is converted to (1*R*,5*S*)-carveol (**84**, carveol) *via* specific hydroxylation by the cumene dioxygenase CumDo derived from *Pseudomonas putida* PWD32 (originally called *Rhodococcus opacus* PWD4) expressed in *P. putida* S12. An already established artificial 'minipathway' in *E. coli* was implemented for the formation of chiral carvolactones: The allylic alcohol is further oxidized *via* the RR-ADH from *Rhodococcus ruber*[312] to the α,β-unsaturated ketone (*S*)-(+)-carvone (**85**, carvone). This oxidation is followed by a selective reduction of the double bond to the saturated ketone (2*R*,5*S*)-dihydrocarvone (**86**, dihydrocarvone) using an enoate reductase (ERED), OYE1 derived from *Saccharomyces sp*.[313] The last step is the application of a Baeyer-Villiger-monooxygenase $CHMO_{Acineto}$ (*Acinetobacter calcoaceticus*)[314] to form carvolactone (**87**, lactone).[282]

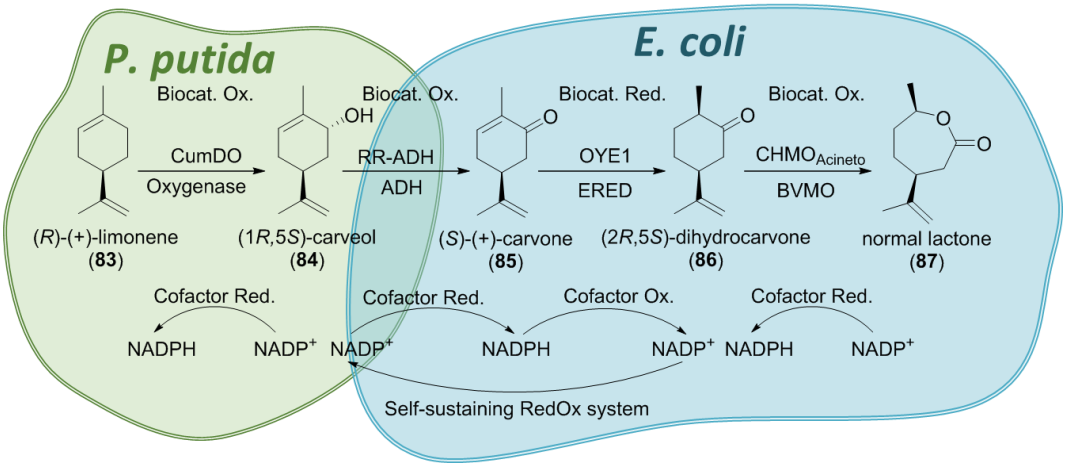


Figure 145: Developed cascade by Oberleitner et al.

The polymerisation from the lactone has been recently investigated.[281] Knight *et al.* used a ring-opening trans-esterification polymerization (ROTEP) for that purpose. Furthermore the olefinic sidechains can be modified.

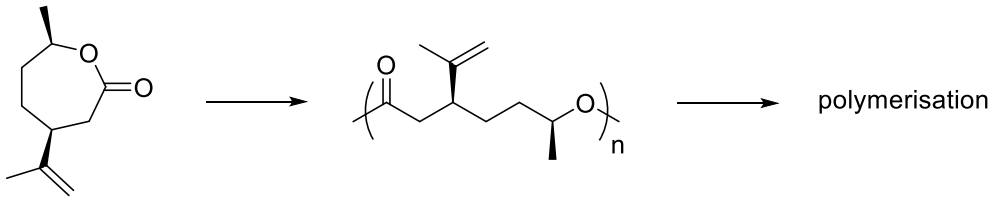


*Figure 146: Transformation of lactone **87** towards biopolymer*

After having a first impression on the compatibility of the tested ionic liquids with both bacterial strains by performing growth curves, biocatalysis was performed with some selected ionic liquids. Again *P. putida* and *E. coli* were tested separately. In a first attempt resting cells of *P. putida* expressing CumDO, were supplemented with limonene (**83**) and variety of choline and imidazolium ionic liquids, to investigate the hydroxylation reaction to carveol (**84**) in their presence. Generally ionic liquids were added in a concentration of 50 mM. Interestingly [C_2mim]OAc (**72**) which showed no to low compatibility with growing cells, emerged as the best candidate in resting cell biocatalysis. For a better understanding of this phenomena more investigations are necessary. The choline derivatives gave lower yields than the imidazolium derivative.

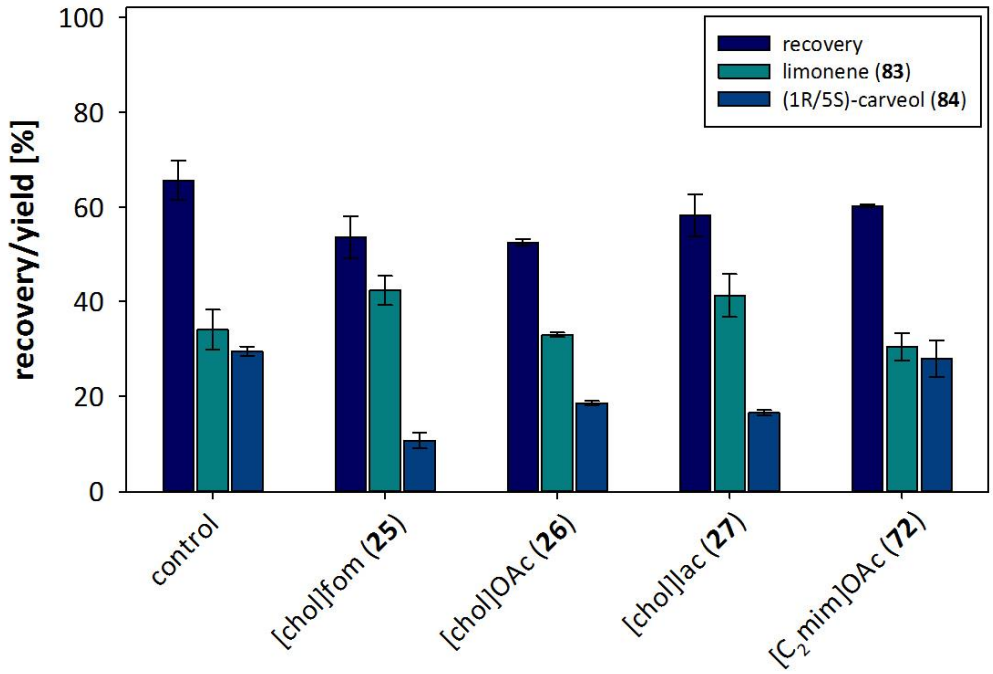


*Figure 147: Influence of the addition of ionic liquids on the transformation of limonene (**83**) to carveol (**84**)*

As [C_2mim]OAc (**72**) showed best compatibility with the biocatalysis reaction of CumDO in *P. putida* it was tested also in concentrations of 100 mM and 250 mM. Unfortunately the reaction was inhibited with increasing concentrations of the ionic liquid **72**. (Figure 148)

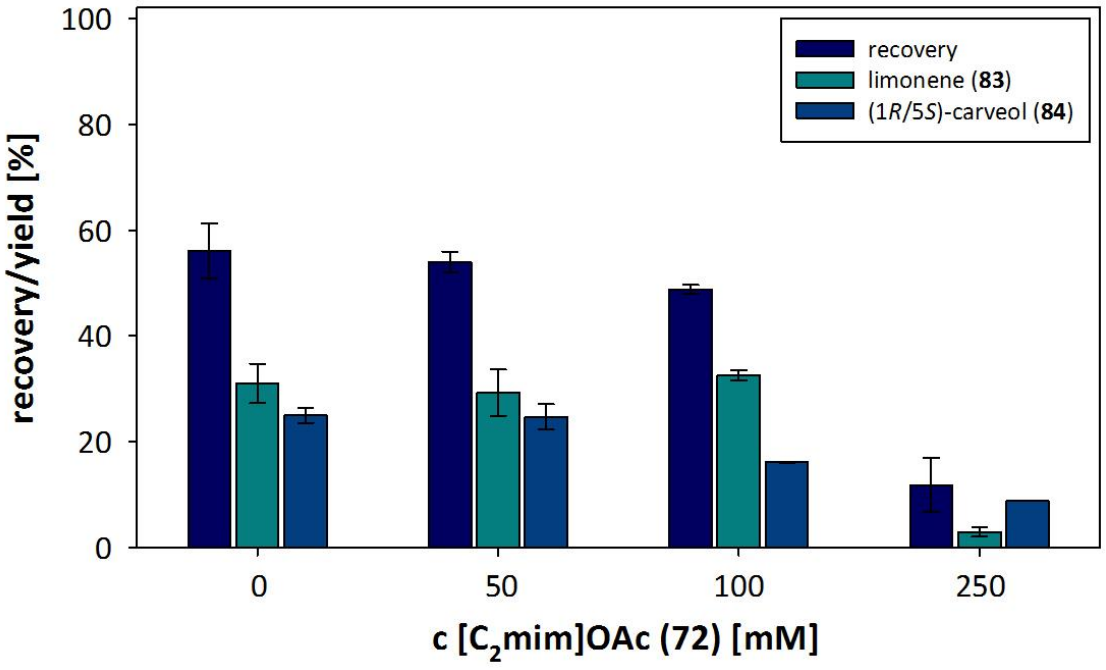


*Figure 148: Influence of the concentration of [C₂mim]OAc (**72**) on the formation of carveol (**84**)*

A similar trend was observed when testing [chol]fom (**25**) with *P. putida*. (Table 42) A concentration of the ionic liquid higher than 50 mM resulted in no product formation. The concentration of 50 mM was the maximum concentration tolerated for the cascade reaction.

Table 42: Variaton of the concentration of a choline derivative

Entry	[chol]fom (25) [mM]	Recovery [%]	Limonene (83) [%]	Carveol (84) [%]
1	control	70	29	39
2	50	72	33	37
3	100	51	51	0
4	250	47	47	0

OD 15, 4 mM limonene (**83**), 30 °C, 200 rpm, 12 h.

The bistriflimde ionic liquids **6** and **9** were also tested in different concentrations for the conversion to carveol (**84**). Different concentrations were used. Apart from testing concentrations of 50 mM and 100 mM a visible biphasic system was also applied, since those systems are reported to be beneficial for biocatalytic reactions due to the role of the ionic liquid as reservoir.[296] Table 43 shows a summary of the two bistriflimide ionic liquids **6** and **9** tested with *P. putida*. Unfortunately carveol (**84**) formation was in all cases low for the hydrophobic ionic liquids. With increasing ionic liquid concentration the yield of carveol (**84**) decreased for both bistriflimide ionic liquids. (Table 43, entry 2-4 and 5-7) A higher limonene (**83**) loading resulted in a better recovery, but not in better yield, indicating that the limonene (**83**) was accumulated in the hydrophobic layer.

Table 43: Influence of hydrophobic ionic liquids on the biocatalytic reaction using P. putida

Entry	ionic liquid/control	C ionic liquid	Recovery	Limonene (**83**)	Carveol (**84**)
1	control		56	3	53
2	$[C_2mim]N(Tf)_2$ (**6**)	50 mM	28	16	11
3		100 mM	35	27	8
4		20 v/v%	80	75	5
5	$[C_4mim]N(Tf)_2$ (**9**)	50 mM	54	25	29
6		100 mM	71	49	22
7		20 v/v%	85	80	5
8	control[a]		28	17	11
9	$[C_2mim]N(Tf)_2$[a] (**6**)	20 v/v%	93	92	1

4 mM limonene (**83**) concentration used. [a] 20 mM limonene (**83**)

The impact of the addition of ionic liquids on the performance of the enzymatic cascade, consisting of RR-ADH, OYE1 and $CHMO_{Acineto}$, was tested on resting *E. coli* cells expressing these enzymes. In this first attempt single experiments unfortunately showed low conversions for the best candidate $[C_2mim]OAc$ (**72**) from the *P. putida* and extraction studies. However the short chained choline carboxylates showed nearly no interference with the enzymatic cascade.

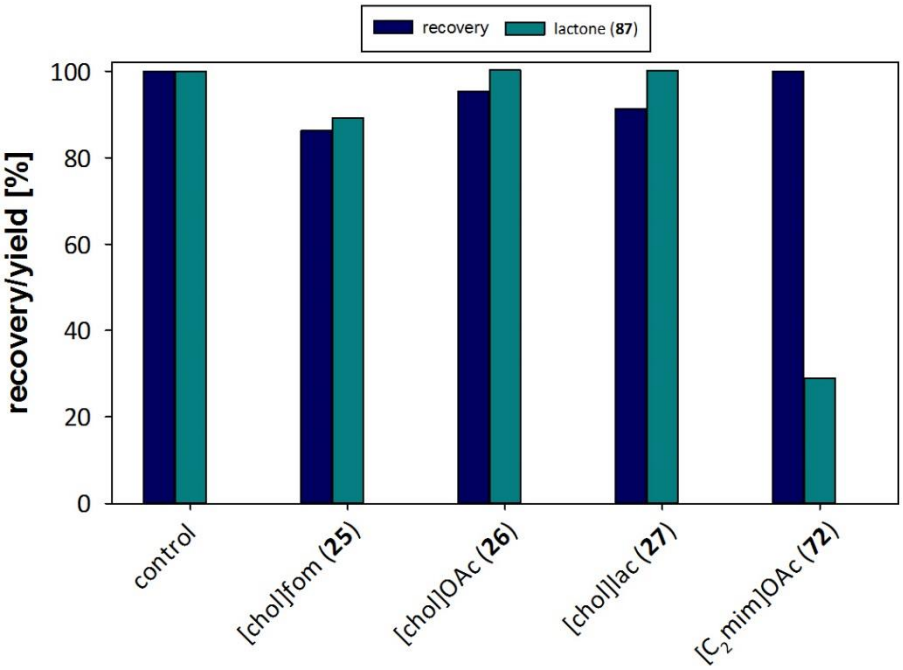


*Figure 149: Ionic liquids tested for the transformation of carveol (R)-(**84**)[viii] to lactone (**87**)*
Values relativ to control experiment = 100

4.5.5 Extraction of limonene (83) from orange peels

After performing growth curves and biocatalysis the set of ionic liquids was reduced to 3 promising candidates: [chol]fom (**25**), [chol]lac (**27**) and $[C_2mim]Cl$ (**5**). Extraction should mainly be investigated

[viii] Performed using (1*R*,5*R*)-carveol since limonene derived (1*R*,5*S*)-carveol was not available.

using those 3 ionic liquids. To comparison, [C_2mim]OAc (**72**) should also be used for the extraction, since this ionic liquid performed particularly well in the extraction of limonene (**83**) from orange peels reported by Bica *et al*. Choline ionic liquid **26** was chosen, since its performance in previous biocatalysis experiments was good. Furthermore the hydrophobic ionic liquid [C_2mim]N(Tf)$_2$ (**6**) was also investigated, since hydrophobic ionic liquids are reported to be suitable (co)solvents for whole-cell biocatalysis. (Chapter 4.5.1.2)

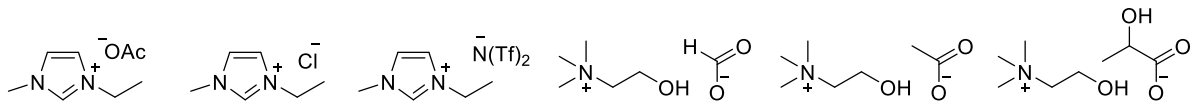


Figure 150: Ionic liquids used for extraction experiments

After setting up reliable GC conditions, which seemed to be the appropriate analytical tool for the determination of limonene (**83**), a time screening was started at room temperature to evaluate a suitable extraction time. To obtain homogenous biomass samples, orange peels were finely ground with a common kitchen grater. For comparison reasons and to eliminate the influence of different limonene (**83**) contents of different oranges, all values are related to an EtOAc value. For every newly used orange, an EtOAc experiment was performed. As it can be seen in Table 44 the ideal extraction time was 3 h. (entry 2) Compared to pure water the 100 mM [C_2mim]OAc (**72**) solution gave only slightly better extraction yields. This might be due to mechanical extraction of limonene (**83**) while grinding.

*Table 44: Extraction of limonene (**83**) from ground orange peels*

Entry	Solvent	Conditions	Yield 83[rel.%]
1	100 mM [C_2mim]OAc (**72**)	r.t., 1 h	67 ± 2
2	100 mM [C_2mim]OAc (**72**)	r.t., 3 h	74 ± 1
3	100 mM [C_2mim]OAc (**72**)	r.t., 5h	69 ±9
4	100 mM [C_2mim]OAc (**72**)	r.t., 24 h	42 ± 3
5	water	3 h	62 ± 1
6	EtOAc	3 h	100 ± 3

The work-up was also optimized. It was shown that centrifugation and filtration were not suitable for the analysis of limonene (**83**) due to its volatility. A sample of 100 µl of the aqueous phase was taken and extracted with an excess of 5 ml EtOAc containing the ISTD methyl benzoate. After intensively seeking for the optimum conditions, including variation of different biomass loading, the ionic liquids shown in Figure 150 had no significant improvement compared to the water value. The water obtained approximately 75% extraction efficiency compared to the aqueous ionic liquid solutions, questioning the necessity of them. Since the extraction power of water relies on the mechanical extraction due to the grinding, a switch to bigger pieces of the orange peel was decided.

Starting with a time screening, 100 mg of orange peels cut into pieces in 900 µl solvent, corresponding to a 10 wt% solution were stirred at room temperature. A sample of 100 µl was taken and extracted

using 5 ml of EtOAc containing ISTD methyl benzoate. The time screening was performed using EtOAc, water and aqueous solutions of [C_2mim]Cl (**5**) with concentrations of 100 mM and 250 mM, since **5** was already successfully tested for the extraction of limonene (**83**).[195] Only EtOAc gave moderate yields of up to 3 wt% with increasing extraction time. Water and aqueous ionic liquid solutions gave maximum extraction yields of 0.1 wt% corresponding to an extraction efficiency of 3% compared to EtOAc. No significant difference of water and aqueous ionic liquid solutions was observed. Further extraction processes were performed for 24 h at room temperature.

In order to investigate the influence of pure ionic liquids on the extraction of limonene (**83**), an HPLC strategy was set up. The screening was started at room temperature and the solution was stirred for 24 h. Room temperature extraction was not efficient for all solvents. (Table 45, enty 1-6) Furthermore results were neither reliable nor reproducible for water, 1 M [C_2mim]Cl (**5**) and choline ionic liquids **25**-**27** (Standard deviation > 50%; triplicates, repeated several times). For EtOAc moderate yields of 2.3 wt% were obtained. However, when literature reported conditions[195] of 80 °C for 1.5 h were applied a significant increase in extraction yield was observed. (Table 45, entry 7)

*Table 45: Room temperature experiments for the extraction of limonene (**83**)*

Entry	Extraction solvent	T [°C]	Time [h]	Yield 83 [wt%]	Yield 83 [rel.% to EtOAc]
1	EtOAc	25	24	2.3 ± 0.4	100
2	Water[#]	25	24	0.3 ± 0.	12
3	[C_2mim]OAc	25	24	1.6 ± 0.3	68
4	1 M [C_2mim]Cl[*,#]	25	24	0.1 ± 0.1	6
5	[chol]fom[#]	25	24	0.3 ± 0.2	12
6	[chol]lac[#]	25	24	0.3 ± 0.1	13
7	[C_2mim]OAc	80	1.5	4.2 ± 0.2	184

100 mg orange peels were dissolved in 900 µl of solvent. Yields were determined using RP HPLC. * Solid at room temperature; # Standard deviation > 50%, all experiments were carried out in triplicates, experiments repeated at least 2 times

Since the extraction at room temperature did not lead to satisfying results the temperature was switched to 80 °C and an extraction time of 1.5 h. The biomass loading was doubled according to Bica *et al*. and different solvents were tested. Figure 151 shows the extraction yields obtained with EtOAc, water and the different ionic liquids. All values were related to the value obtained with [C_2mim]OAc (**72**), since this ionic liquid performed particularly well in the extraction process as expected. Biomass was completely dissolved after a short time of 1.5 h. Comparable results were obtained with [chol]fom (**25**). A slight decrease was obtained for the other two choline derivatives [chol]lac (**27**) and [chol]OAc (**26**). While a moderate performance was observed for EtOAc, poor extraction yields were obtained with water and the 1-ethyl-3-methyl-imidazolium derivatives, the hydrophilic chloride **5** and the hydrophobic bistriflimide **6**. The low extraction efficiency with chloride slats might be explained by the high melting point and therefore by the high viscosity of this solution.

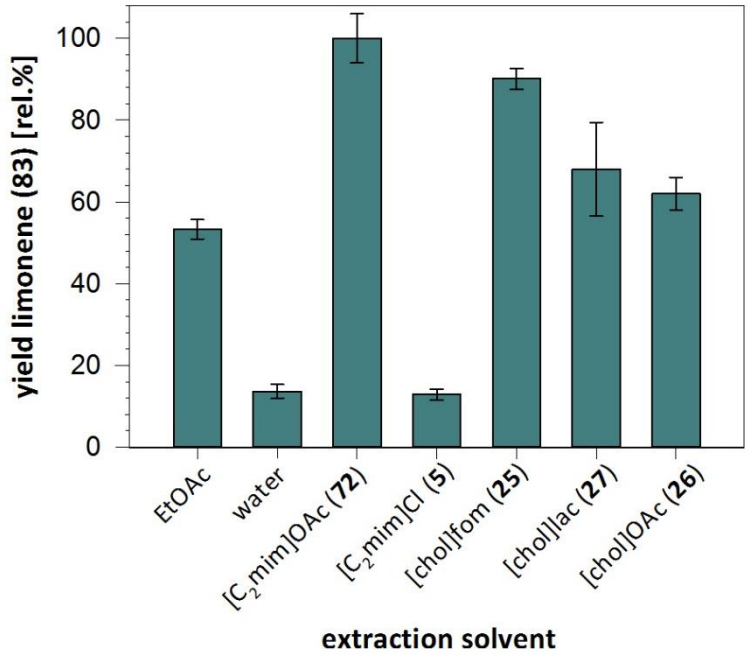


*Figure 151: Different solvents used for the extraction of limonene (**83**) from orange peels*

The extraction using $[C_2mim]N(Tf)_2$ **6** was also investigated at 36 °C for 12 h simulating the biocatalytic process. An extraction yield of 26% compared to the value of $[C_2mim]OAc$ (**72**) at 80 °C was obtained. Disappointingly aqueous solutions of ionic liquids gave only low yields and results were not reproducible.

In a next step the biomass loading was investigated using $[C_2mim]OAc$ (**72**), an extraction time of 1.5 h and a temperature at 80 °C. The biomass loading of 20 wt% orange peels in ionic liquids gave the highest extraction yield of approximately 6 wt% limonene (**83**) referred to biomass. (Table 46, entry 1) This value was significantly higher than the value obtained in previous experiments under the same conditions. Responsible for that phenomena might be the different content of limonene (**83**) in orange peels, since a new biomass pot was used for this experiment series. With increasing biomass loading the extraction yield decreased significantly. The uptake of the ionic liquid seemed to be limited to a 20 wt% where complete dissolution of biomass was observed. In contrast only partial dissolution of orange peels in $[C_2mim]OAc$ (**72**) was obtained with higher biomass loading.

*Table 46: Different biomass loading tested with $[C_2mim]OAc$ (**72**)*

Entry	Biomass loading [wt%]	Extraction yield 83 [wt%]	Rel. extraction efficiency [%]
1	20	5.8 ± 0.4	100
2	25	4.9 ± 0.7	83
3	30	2.1 ± 0.2	37
4	35	1.7 ± 0.3	29

200 mg orange peels, ionic liquid amount varied.

In order to test different orange peels on their content of limonene (**83**), peels from different oranges were dissolved as a 20 wt% solution in $[C_2mim]OAc$ (**72**). The solution was again stirred for 1.5 h at

80 °C until nearly full dissolution of biomass was observed. As it can be seen in Figure 152 the content of limonene (**83**) in oranges differed significantly. The amount of extracted limonene (**83**) varied between 5 mg **83**/200 mg orange peels and 12 mg **83**/200 mg orange peels. Due to the volatility of limonene (**83**) it is assumed that older oranges contain less **83**. Furthermore, other criteria, such as the sort of orange, their state of mellowness, etc.,… might be responsible for a different limonene (**83**) content.

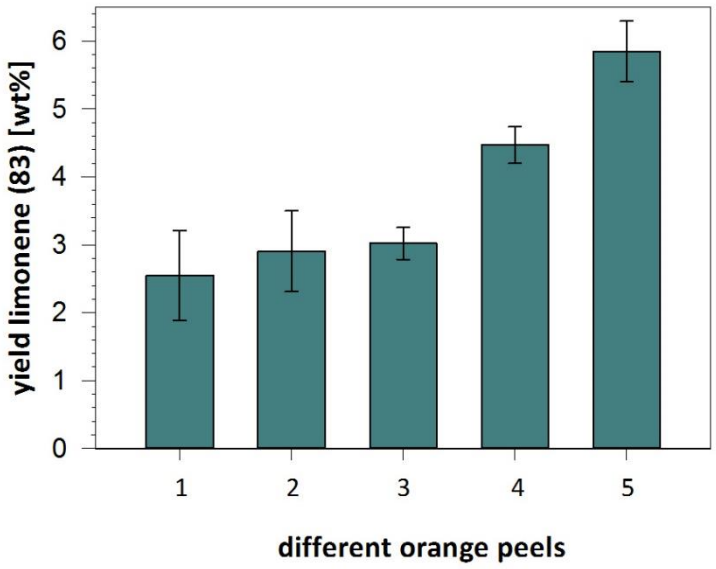


*Figure 152: Different batches of orange peels tested with [C₂mim]OAc (**72**)*

Since the results of the extraction using ionic liquids were very promising, the next step, the combination of extraction and cascade reaction was investigated.

4.5.6 *In situ* extraction and cascade reaction

After intensively evaluating the toxicity of ionic liquids towards bacteria, their performance in biocatalysis and in extraction experiments, the results are summed up in Table 47. The best performing experiment is scaled as 100 and all other values are relative to it.

Table 47: Evaluation of ionic liquids for the in situ use

Entry	Solvent/ionic liquid (50 mM)	Growth		Biocatalysis		Extraction
		P. putida	***E. coli***	***P. putida***	***E. coli***	
1	water/medium	100	100	100	100	14
2	[chol]fom (**25**)	88	74	65	89	90
3	[chol]OAc (**72**)	0	90	85	100	62
4	[chol]lac (**27**)	98	88	63	100	68
5	[C₂mim]OAc (**26**)	0	71	90	29	100
6	[C₂mim]Cl (**5**)	86	63	n.a.	n.a.	13
7	[C₂mim]N(Tf)₂ (**6**)	0	7	21	n.a.	19

Growth medium respectively aqueous buffer performed best in growth experiments and biocatalysis but water failed in extraction experiments. Short chain choline derivatives were generally well tolerated in bacterial growth with exception for the acetate derivative. However they gave moderate to good extraction yields and performed very well in biocatalysis. Very promising was the ionic liquid [C_2mim]OAc (**72**) despite the low tolerance in bacterial growth. Except for the performance in the *E. coli* catalyzed mini pathway this ionic liquid was superior in extraction and excellent in the first reaction of the cascade. The hydrophic bistriflimide ionic liquids failed and were not compatible with bacterial growth or the biocatalytic reaction. Furthermore only poor extraction yields were obtained.

For approach I difficulties for the extraction were expected, as the extraction yield of limonene (**83**) was very poor using aqueous ionic liquid solutions. However the circumstance that the extracted limonene (**83**) is directly converted to carveol (**84**) and therefore slowly consumed, might be beneficial for the subsequent extraction of it.

The second approach seemed to fulfil the requirements. Excellent extraction yields were obtained by dissolution of the orange peels in the pure ionic liquid. Although the mandatory dilution is an additional step, this method seemed to be most promising.

Approach III was not considered to take in account, since not only the extraction power of the bistriflfimide ionic liquids was only moderate, but also the biocatalysis did not lead to satisfying yields.

Starting with some preliminary experiments from orange peels using approach I, approach II and control experiments immediately showed that only poor yields of carveol (**84**) can be obtained from orange peels. (Figure 153) Furthermore the bad recovery is a prominent issue. Sampling is a challenge, since very low concentrations of limonene (**83**) and carveol (**84**) are present.

One possibility is to take a sample of the aqueous solution and extract it with EtOAc. However due to the low concentration only a small amount of EtOAc can be taken and therefore the extraction might not be complete. The other possibility is to extract the whole solution with EtOAc. However the cells form with EtOAc a stable emulsion and only a small amount of EtOAc can be taken. Concentration of the organic layer is not possible, since limonene (**83**) is a very volatile compound. The work-up needs to be optimized in the future.

As it can be seen in Figure 153 there is a huge difference between the control values starting from limonene (**83**) and the orange peel derived results. Among the orange peel experiments approach I gave the highest carveol (**84**) yield, as expected from the high extraction yield. If orange peels and cell medium were mixed a small amount of limonene (**83**) and carveol (**84**) was obtained. Only slightly higher carveol (**84**) yields were observed with approach II. No explanation can be given for high limonene (**83**) value if a higher biomass loading was used without ionic liquid. The higher biomass loading was not beneficial for approach II. However the values presented suffer from a high standard deviation.

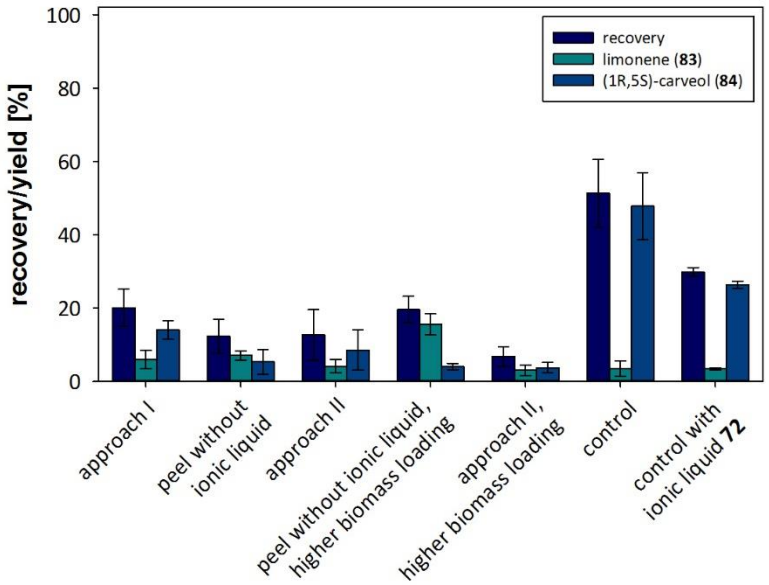


*Figure 153: Limonene (**83**) conversion to carveol (**84**) from orange peels using [C_2mim]OAc (**72**) (control uses limonene (**83**) as starting material), sampling after 12 h reaction time*

The values obtained were also related to the biomass used in those experiments. (Figure 154) Less than 0.5 wt% carveol (**84**) were obtained. However, standard deviation were as mentioned above very high.

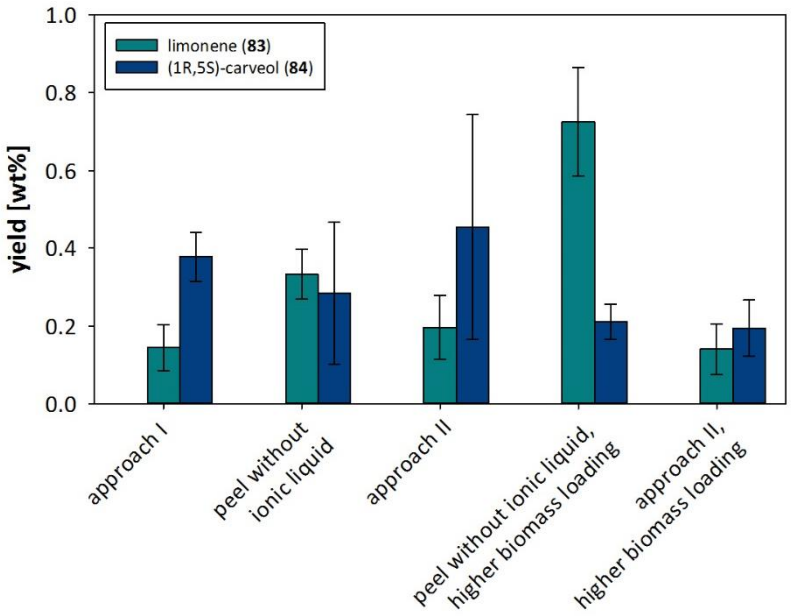


*Figure 154: Yields of limonene (**83**) and carveol (**84**) corresponding to used orange peels*

It was already shown that ionic liquids were suitable in biocatalytic systems when chosen carefully. In case of the multistep biocatalytic reaction of limonene (**83**) to lactone (**87**) ionic liquids were suitable additives in a low concentration of 50 mM. However for the direct conversion of extracted limonene (**83**) from orange peels to lactone (**87**), intensive investigations have to be performed.

4.6 Extraction of DNA from biomass[ix]

To expand the field of application of active ingredient isolation towards the isolation of biomolecules using ionic liquid technologies, a nucleic acid, DNA, was chosen as a target. This project was done in cooperation with the group of Dr. Brunner in IFA Tulln that is specialized in quantitative PCR for environmental, agricultural or food and feed research.

4.6.1 Biomolecule stabilization in ionic liquids

To evaluate the ability to apply ionic liquids for biomass extractions towards a fast isolation method for DNA from biomass and its analysis *via* quantitative real time PCR, a short review of recent literature confirmed that ionic liquids possess the ideal requirements for that task. Several aspects of the unique properties of ionic liquids should combined:

1 Ionic liquids can dissolve biomass.[33]

2 DNA is stabilized in hydrated ionic liquids.[315]

3 Ionic liquids have the ability to enhance PCR.[316]

Apart from the dissolution of biomass, hydrated ionic liquids provide a media for the stabilisation of biological compounds, such as proteins,[317] nucleic acid,[318] *e.g.* siRNA[319] and DNA.[315]

Using fluorescence measurements a thermodynamic stabilization of the protein monellin in the hydrophobic ionic liquid $[C_4mpy]NTf_2$ to 105 °C was observed compared to 40 °C in water.[165] The activity of proteins such as cytochrome c, microperoxidase-11 and Fe(III)protoporphyrin(IX) chloride (hemin) was significantly higher in hydrophobic ionic liquids such as $[C_4mim]N(Tf)_2$, $[C_4mim]PF_6$ and $[C_8mim]PF_6$.[320] Solubilisation without denaturation of cytochrome c was observed in $[C_2mim]NTf_2$.[321] Several publications showed the superior stabilisation of proteins in ionic liquid solutions compared to aqueous solutions:

A stabilisation effect of cytochrome c in the biocompatible choline dihydrogenphosphate [chol]dhp ionic liquid was observed compared to buffer solution. The protein stability was tremendously increased to at least 6 months, whereas denaturation in TRIS (Tris(hydroxymethyl)-aminomethan) buffer solution was observed after 2 weeks.[322] A set of ionic liquids was tested for cytochrome c stabilisation, resulting in following order for stabilization: [chol]dhp > $[C_4Mpy]$dhp > [chol]dbp > $[C_4mim]$OAc > $[C_4mim]$lactate > $[C_4mim]MeSO_4$. This order is based on the kosmotropicity and chaotropicity of the ions composing the ILs. Thermal denaturation of ribonuclease A around 60 °C occurred with increasing hydrophobicity of ILs.[323, 324]

A relation between protein denaturation and the Hofmeister Series was observed in a case study of aqueous solutions of Ribonuclease A (RNase A) using differential scanning calorimetry for the observation of thermal denaturation of RNase A around 60 °C. A destabilizing effect was observed with organic salts (except for $[C_{1,1,1,1}N]Cl$) which is contrary to many inorganic salts. With increasing hydrophobicity an increasing destabilisation effect was observed, resulting in decreasing Tm values and following destabilization series: $K^+ > Na^+ > [C_{1,1,1,1}N]^+ > Li^+ > [C_{2,2,2,2}N]^+ \approx [C_2mim]^+ >$ [bmpyrr]$^+$ >

[ix] In cooperation with the group of Dr. Brunner, IFA Tulln. While synthesis of ionic liquids and extraction experiments were performed by A.K. Ressmann, PCR analysis, agarose gels and calibration curves were done by E. Garcia Gonzalez.

$[C_4mim]^+ \approx [C_{3,3,3,3}N]^+ > [C_6mim]^+ \approx [C_{4,4,4,4}N]^+$. Having a $[C_2mim]^+$ cation, anions were also investigated and again the same influence of the hydrophobicity was observed: $[SO_4]^{2-} > [HPO_4]^{2-} > Cl^- > [EtOSO_3]^- > [BF_4]^- \approx Br^- > [MeOSO_3]^- > [TfO]^- > [SCN]^- \approx [N(CN)_2]^- > [Tf_2N]^-$.[323] A more detailed look at the stability of RNase A showed the enhanced thermal stability of the native state using [chol]dhp, whereas $[C_2mim]N(CN)_2$ acted as a strong denaturant.[324]

In 2012 the group of Weingärtner described that stabilisation of proteins against aggregation can be achieved with specific conditions using any ionic liquid, thus resulting in different stabilization efficiency. Hofmeister series is therefore not always an appropriate tool for protein stabilisation estimation, *e.g.* choline cation is classified as a denaturation agent according to the Hofmeister ranking at low concentrations though high concentrations of choline chloride are able to stabilize hen egg white lysozyme and α-lactalbumin.[325]

Recently, the group of Ohno published a study about the influence of water molecules to ion pairs of hydrated ionic liquids (ionic liquids that contain small amount of water) for biological activities. They found out that a minimum of hydration numbers of approximately seven is necessary for different biological activities, such as enzymatic acitvity. [326]

In contrast, the stability of DNA in ionic liquids remained unknown until the group of MacFarlane[315] focused on long-term stability of DNA in various choline based ionic liquid-water solutions. DNA analysis from salmon *via* circular dichroism spectra revealed that the double-helical structure of DNA dissolved in choline lactate ([chol]lac) was still present after 6 months of storage at room temperature, whereas the helical structure was lost after 1 month in water. Remarkably, DNA samples in the ionic liquid [chol]dhp (CDP/H_2O=50:50) could be stored for 1 year at room temperature without structural loss, whereas dissolution of DNA in conventional solvents such as methanol, DMSO or formamide resulted in rapid denaturation of DNA. The ionic liquid [chol]dhp was used for investigation towards base pair stability showing that in contrast to buffer systems A-T base pairs were more stable than G-C pairs in hydrated choline dihydrogen phosphate.[327] An elegant strategy of dissolution of DNA in ionic liquids was presented by Mukesh *et al*.[328] Two bio-based ionic liquids, choline-indole-3-acetate and choline-indole-3-butyrate were applied for the rapid dissolution of DNA followed by regeneration of DNA using isopropylalcohol. The ionic liquid was successfully recovered and further used for three consecutive DNA dissolution cycles.

Apart from this stabilization effect, ionic liquids were applied to DNA separation or purification. In 2007 Wang[329] extracted double-stranded (ds) DNA with $[C_4mim]PF_6$ from aqueous solution. The interactions between the cationic imidazolium groups and the phosphate groups in DNA were held responsible for the efficient extraction, and proteins and metal species did not interfere with the separation and purification process. In turn, Li[330] proposed a set of novel ionic liquids for *in situ* dispersive liquid-liquid microextraction of DNA with high extraction efficiency. An aqueous DNA sample was spiked with a complex matrix, *e.g.* proteins or metal ions, before addition of a hydrophilic ionic liquid. The addition of lithium bistriflimide $LiN(Tf)_2$ resulted in the formation of a biphasic system and the separation of DNA; best results were obtained with the ionic liquids 1-(1,2-dihydroxypropyl)-3-hexadecylimidazolium bromide and *N*,*N*-dodecyl-*N*-methyl-d-glucaminium bromide.

However, the role of ionic liquids is not only limited to dissolution and solubility, they are also suitable for analytical detection methods. In 2012 Shi *et al*. described the enhancement of PCR by ionic liquids such as the bicyclic 1-butyl-2,3-tetramethyleneimidazolium bromide, whereas commonly used enhancing reagents such as DMSO and betaine were found to be completely ineffective.[316] In 2014 a patent described a method for enhancing efficiency and sensitivity in nucleic acid amplification from biological materials such as mucus, blood, stool and tissue samples using the ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate.[331]

The combination of exceptional dissolution properties and stabilizing effects for DNA makes ionic liquids ideally suited for enhanced and efficient extraction of DNA from crude biomass. Since ionic liquids are also known to promote PCR amplification of DNA, they are predestined for challenging bioanalytical problems.

4.6.2 Quantitative real-time PCR

As an analytical method of choice quantitative real time polymerase chain reaction (qPCR) was chosen. This method relies on the conventional PCR (polymerase chain reaction) in combination with fluorescence measurements during or at the end of one PCR cycle. This enables therefore the quantification of synthesized or extracted DNA in real time. The fluorescence is direct proportional to the amount of PCR-products. Figure 155 shows a typical profile of a qPCR. The baseline represents the fluorescence signals that are beneath the detection limit. The threshold is defined as 10 times the standard deviation of the average signal of the baseline fluorescence signal. If the threshold signal is exceeded, the Ct (Cq) value can be evaluated. The lower the number, the more DNA was amplified, with the exception of zero. After a certain time the exponential amplification of DNA levels off and a plateau is reached. This phenomena is based on either inhibition of the polymerase reaction, reagent limitation due to accumulation of pyrophosphate molecules.[332]

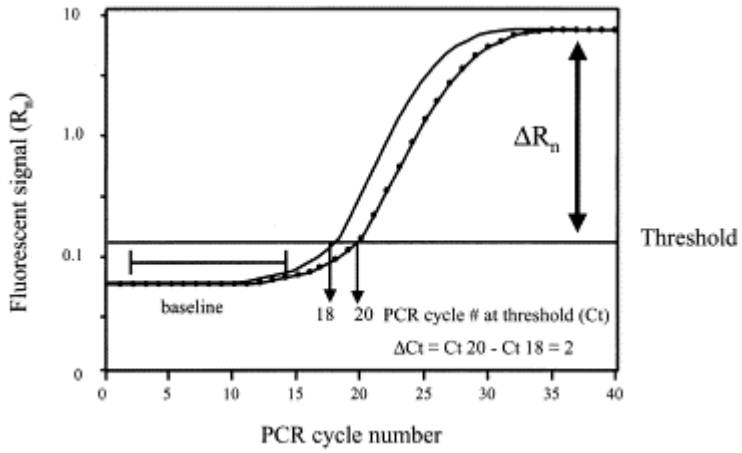


Figure 155: Explanation of qPCR[332]

Various detection methods can be applied: Early methods used the carcinogenic ethidium bromide with the drawback that only a total fluorescent signal was detected including nonspecific PCR products. Nowadays mostly 3 techniques are applied: 5’ nuclease assays including TaqMan probes, molecular beacons and SYBR Green I intercalating dyes. (Figure 156) While the first two methods are based on a fluorescent reporter probe method, the third method is based on double-stranded DNA-binding dyes as reporters.

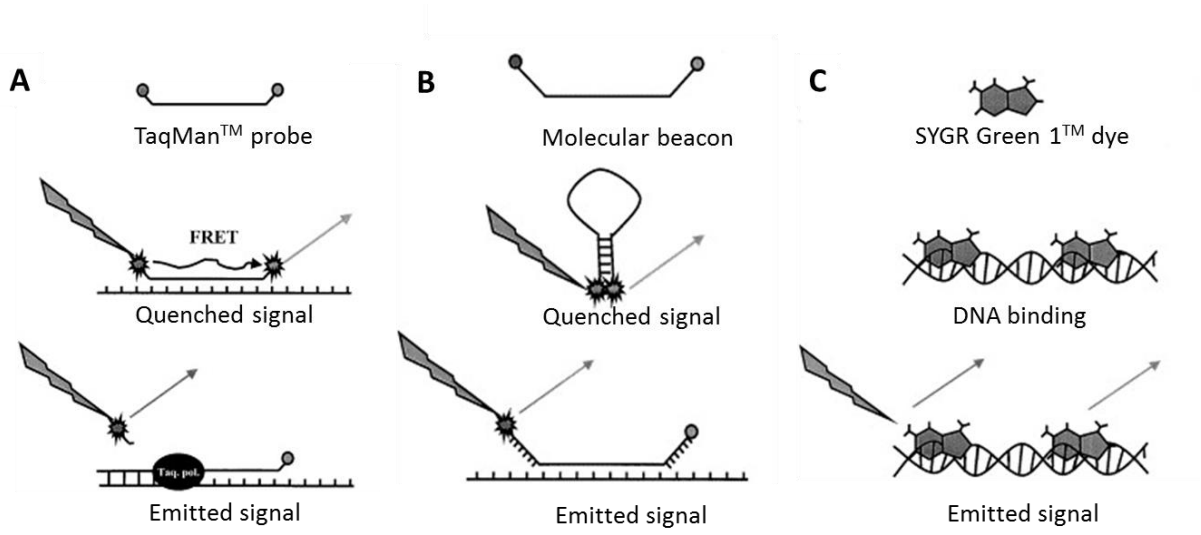


Figure 156: Methods for detection in qPCR adapted from[332]

In case of method A "TaqMan™" a probe consists of an oligonucleotide (primer) that is marked on one end with a reporter fluroescence dye and on the other end with a quencher. The reporter's fluorescence is suppresed by a quencher. The taq-polymerase that has additional to its polymerase activity a 5'-3'-exonuclease activity, releases the reporter. As the distance between reporter and quencher increases an increasing reporter fluorescence signal can be measured. After each elongation cycle the fluorescence is measured using the phenomene FRET (Förstener/fluorescence resonance energy transfer) that basically describes the mechanism of energy transfer between two light-sensitive molecules. A donor chromophore in electronic excited state can transfer energy to an acceptor.

Method B "molecular beacon" is similar to method A, as it also uses the principle of FRET and consists of a reporter and a quencher. Molecular beacons are oligonucleotides that are coupled with a reporter fluorophore and a quencher with a hairpin structure necessary to provide the quenching effect. In their un-hybridized state at room temperature molecular beacons fluorescence signals are prevented by the quencher. After hybridization the distance of the 5' dye molecule to the quencher increases and the signal is not quenched any more.

For method C "SYBR Green I" no third modified oligonucleotide has to be designed neither a hybridization probe. The dye binds to the minor groove of the DNA double strand and a signal is possible when the sample is excited by a light source. This method is cheaper but cannot discriminate between real template and artifact bands.

Figure 157 shows a qPCR plot obtained for DNA extracted with ionic liquids from maize using detection method A "TaqMan™".

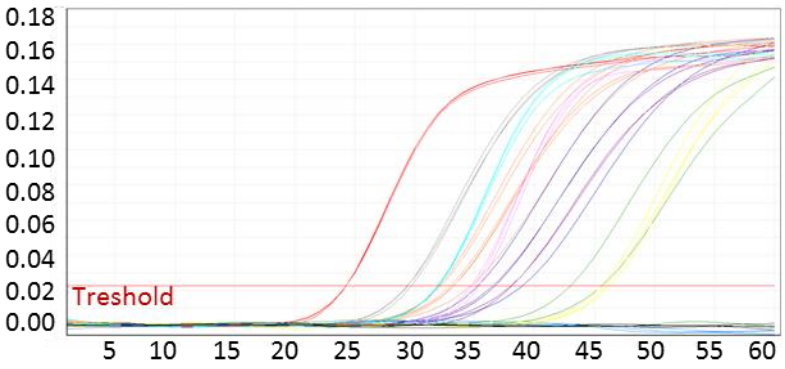


Figure 157: Quantitative real-time PCR from extracted DNA from maize

4.6.3 Extraction of DNA from maize

Ever since the production of the first genetically modified (GM) crop in 1983, the remarkable progress seen in biotechnology has taken farmers to introduce GM crops with characteristics of interest into their agricultural practices. The cultivation of these crops has been increasing at an annual rate of 6%, up to 170 million hectares of biotech crops in 2012.[x] At the same time, controversial discussions about the potential risks that genetically modified organisms (GMOs) might exert on the environment and the human health through foods have also intensified. In order to deal with growing public concern about GMOs, regulations have been implemented in numerous countries to enforce their management and labeling. The European Union adopted the regulation (EC) No 1829/2003 to set a limit of 0.9% for food containing GMOs; products exceeding this limit have to be labeled accordingly.[333] Usually, methods based on the polymerase chain reaction (PCR) are applied to quantify the GMO content of a sample and validated procedures have been developed for the most important crops, like maize, soy and canola. Distinct methods are available for the event-, construct-, or element-specific detection of GMOs. However, they are still time consuming and their procedures are complex. To overcome this drawback, only recently several approaches using simple isothermal amplification reactions have been published[334, 335], including the idea to perform on-site screening tests. However, they only focused on the development of simple amplification methods; none of them included a real simplification of the DNA extraction procedure. In this sense, common available methods are either based on the surfactant cetyltrimethylammonium bromide (CTAB) or on commercial kits, which are time consuming and tedious, as they include numerous pipetting and centrifugation steps which can limit on-site testing. All of these handling steps are prone to errors and furthermore, they bear a certain risk of cross contaminations. Additionally, the CTAB method uses organic solvents like chloroform and isoamylalcohol, which are environmentally problematic and might eventually become restricted. In addition, all currently used extraction protocols require specific laboratory instruments, such as centrifuges, which can limit its application for on-site testing.

4.6.3.1 Choice of ionic liquids

For the extraction of DNA from maize more than 40 ionic liquids were tested. A short look in literature gave already some hints: Based on experiences in the research group and on reviews from literature a set of mostly imidazolium based ionic liquids was chosen, since these ionic liquids are known to

[x] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. Official Journal of the European Union. L268

dissolve biomass. Especially the ionic liquid [C_2mim]DCA (**7**) that has the ability to dissolve starch was very promising for the dissolution of that biomass matrix. Classical imidazolium derivatives such as the acetate **72**, dimethylphosphate **4** and chloride ionic liquids **2**, **51**-**53** were chosen due to their powerful dissolution properties. (Figure 158)

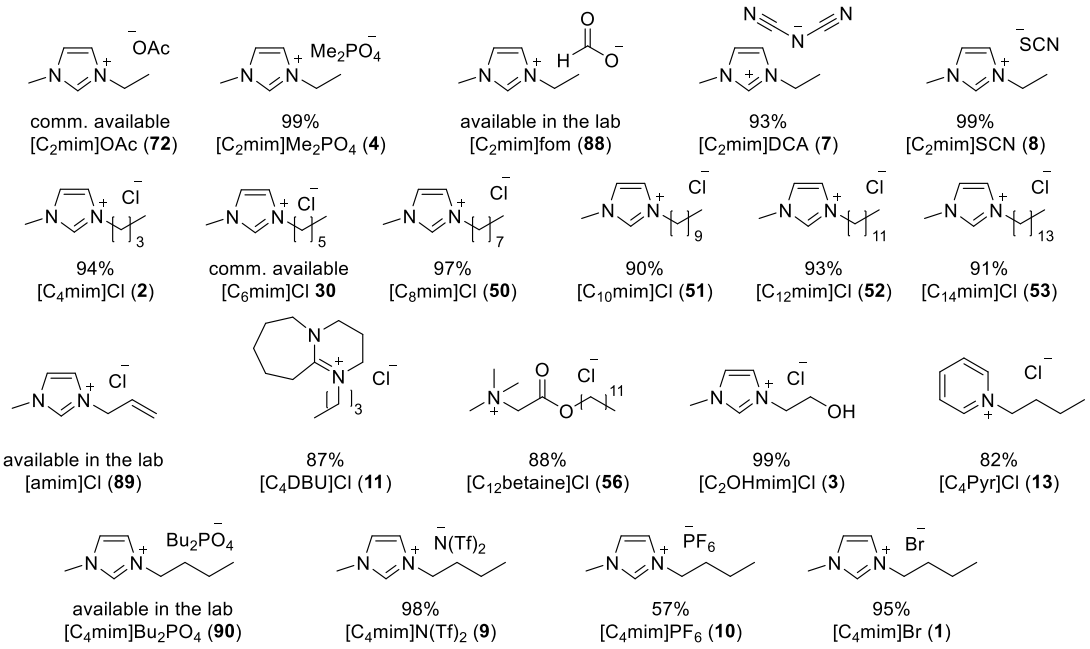


Figure 158: Ionic liquids chosen for the extraction of DNA from maize, part I

In the group of MacFarlane stabilization of DNA by choline ionic liquids was shown, making them particularly interesting for the extraction of DNA.[315] Another aspect was that ionic liquids should be easily available or synthesized with simple protocols. Therefore a group of protic ionic liquids was chosen, as they are usually obtained by just mixing a base and an acid. (Figure 159)

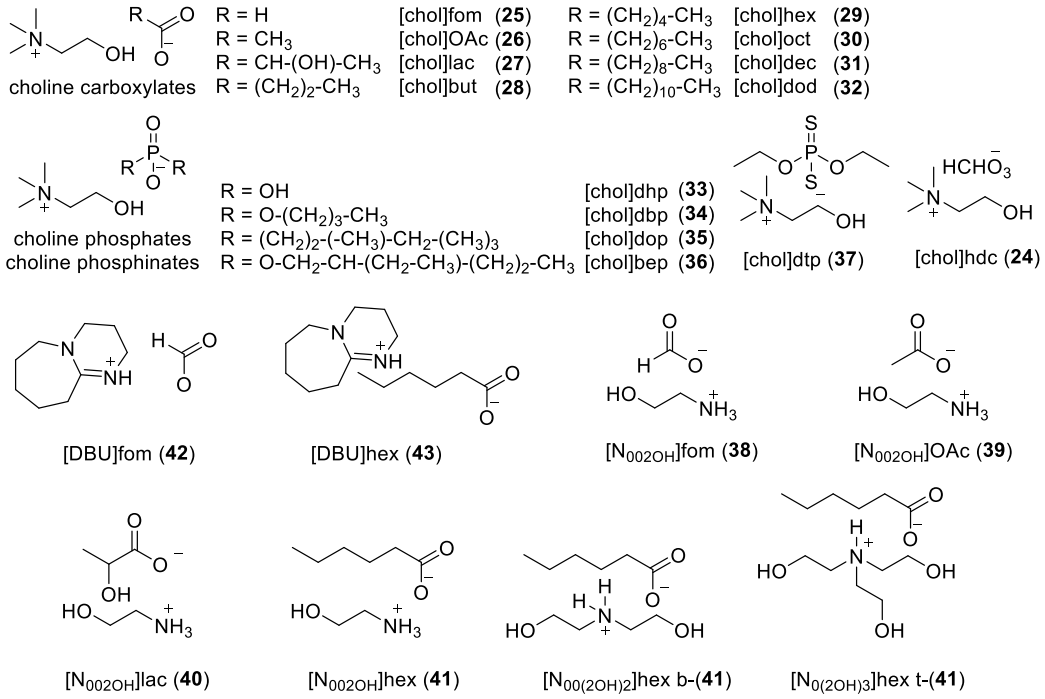


Figure 159: Ionic liquids chosen for the extraction of DNA from maize, part II (all yields > 99%)

Maize should be dissolved in pure ionic liquids or mixtures of ionic liquds/buffer solutions and the aqueous extract should then be analyzed with qPCR.

4.6.3.2 Maize extraction with ionic liquid-water mixtures

In order to develop a new method for the efficient extraction of genomic DNA from maize powder, the role of pure ionic liquids as extraction and dissolution media followed by a dilution with water was investigated. Initially, focus was put on ionic liquids that have been successfully applied for biomass dissolution,[101] e.g. [C_2mim]OAc (**72**), [C_4mim]Cl (**2**), [C_2mim]Me_2PO_4 (**4**) and the environmentally benign [chol]lac (**27**), which has already been shown to stabilise DNA.[315] A 10 wt. % solution of ground maize powder in ionic liquid was stirred for a certain time and temperature to dissolve biomass and extract DNA. After being diluted with water to 5 ml to precipitate biopolymers, the sample was denaturated at 95 °C, centrifuged and 400 µl from the supernatant was retrieved and later analyzed *via* quantitative real-time PCR. (Figure 160)

Figure 160: Flow scheme for the extraction of DNA from maize in ionic liquid/water systems

Among the 32 different ionic liquids tested, a clear trend towards hydrophilic ionic liquids was found – as to be expected from their biomass dissolving properties. (Table 74 in experimental part) Reasonable extraction yields were observed using [C_2mim]OAc (**72**) and [C_4mim]Cl (**2**), but also with protic ionic liquids, such as *N*-(2-hydroxyethyl) ammonium lactate (**40**), whereas only traces of DNA were extracted using [C_2mim]Me_2PO_4 (**4**). In contrast, hydrophobic ILs, such as [C_2mim]PF_6 (**10**) and [C_2mim]NTf_2 (**9**), but also long-chain imidazolium chlorides, failed to extract reasonable amounts of DNA. In comparison, the 2 step addition of LiN(Tf)$_2$ for the *in-situ* formation of the hydrophobic bistriflimide ionic liquid,[330] turned out to be successful and a good extraction performance was observed using [C_4DBU]Cl (**11**) in combination with LiN(Tf)$_2$. Choline derivatives performed particularly well and were therefore selected and chosen for focused investigations.

In order to investigate the influence of the carboxylate anion of choline ionic liquids, the carboxylate chain length was elongated from formate to acetate, lactate, butyrate (C_4COO^-), C_6COO^-,…*etc*. to $C_{12}COO^-$. Best results were obtained using choline hexanoate (**29**) and various conditions such as temperature (25 °C, 80 °C) and stirring time (15 min, 1 h) were optimized. (Figure 161)

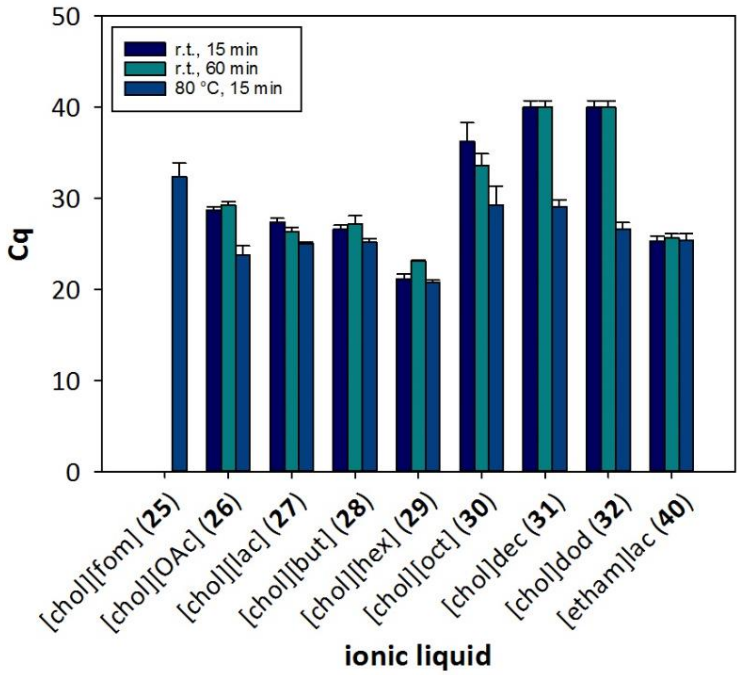


Figure 161: Amplification obtained using pure ionic liquids

However, despite these promising results, problems of reproducibility were observed, especially when choline derivatives were involved. This might be related due to variation in pH, since even traces of remaining acid from the preparation of ionic liquid could depurinate the DNA. In order to avoid the presence of free acid and investigate the influence of pH, the synthesis of [chol]hex (**29**) was performed with different molar ratios of choline hydrogen carbonate to acid (1:0.99, 1:0.95, 1:0.90, 1:0.80). A clear pH dependency of Cq values was seen (Figure 162). Changing the pH from 8.0 to 9.0 resulted in a substantial improvement of 1 cycle– corresponding to the double amount of DNA extracted. However, when the pH value was further changed to 9.5, the amount of extracted DNA could not be further increased.

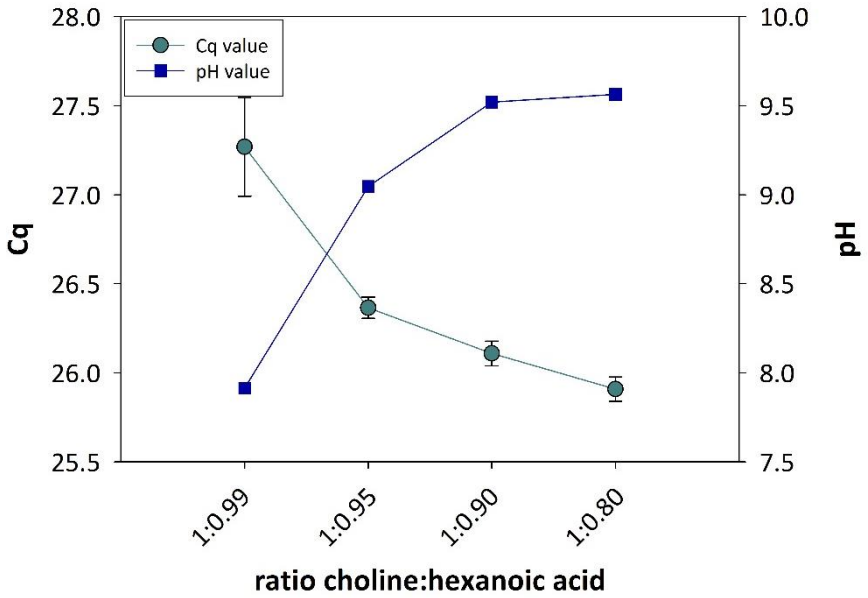


Figure 162: pH dependency of the extraction of DNA from maize

Due to this strong pH dependency, a switch to the combination of an aqueous ionic liquid/buffer system for pH regulation and to scavenge traces of free acid was chosen. As seen in previous experiments the ideal pH value is in the range of 8.5 to 9.0. Apart from the benefits of pH control and better reproducibility in aqueous ionic liquid /buffer system, it was seen that a lower concentration of the ionic liquid in the system was advantageous for the extraction process, as a higher concentration inhibits the polymerase during the PCR (Table 48, entries 1-3). Additionally, the phosphate buffer (Na_2HPO_4/NaH_2PO_4 buffer, 50mM, pH = 8.5) enhanced the extraction efficiency and therefore a combination of ionic liquids with this buffer was a suitable system for the extraction of DNA from maize powder.

*Table 48: Dependency of the Cq value and the concentration of choline hexanoate (**29**)*

Entry	Sample	IL [mg]	Water [mg]	Maize [mg]	Dilution [ml]	Conditions	Cq *ADH1*
1	[chol]hex (**29**)	892.1	-	100.03	5	RT, 15 min	34.3 ± 2.4
2		810.4	90	99.80			30.3 ± 0.4
3		448.4	450	100.30			29.5 ± 0.4

All experiments were carried out in triplicates.

4.6.3.3 Definition of best candidates for extraction with an ionic liquid/phosphate buffer system

Since a lower concentration of ionic liquid seemed to be beneficial for the amplification of DNA (Table 48) focus was set on 10% solution of ionic liquid in aqueous media and a decrease of volume from 5 ml to 1 ml of ionic liquid in phosphate buffer based on economic reasons.This mixture was stirred for 10 min at room temperatureand and denaturated at 95 °C for 10 min to deactivate any possible DNases that might be coextracted and that can later hinder the TaqMan polymerase during the amplification. Afterwards, they were centrifuged and the supernatant was analyzed *via* real-time PCR. (Figure 163)

Figure 163: Flow scheme for the extraction of DNA from maize in ionic liquid/aqueous buffer systems

A set of different ionic liquids was tested, including derivatives of choline, 1-ethyl-3-methylimidazolium, 1-allyl-3-methylimidazolium cations as well as aprotic ionic liquids containing an ethanolammonium or protonated DBU cation (Figure 164). Best results were obtained with [C_2mim]fom (**88**), [amim]Cl (**89**), [C_2mim]Me_2PO_4 (**4**) and [chol]fom (**25**); the best anion evaluated was the formate. In case of choline carboxylates, synthesis was performed with a slight excess of choline hydrogen carbonate to avoid the presence of free acid.

Results showed that when increasing the chain length of the carboxylate anion, the amount of extracted DNA decreased tremendously (from [chol]fom (**25**) with a yield of 1094.5 ± 65.2 µg DNA/g sample to [chol]lac (**27**) with 594 ± 80.2 µg DNA/g sample to [chol]dop (**32**) with 150.96 ± 47.4 µg DNA/g sample). The previously best performing [chol]hex (**29**) gave moderate extraction yield (662 ± 44.6 µg DNA/g sample), whereas only traces of DNA were observed when extracting with [chol]oct (**30**)

(193 ± 48.7 µg DNA/g sample). A further increase of the chain length resulted in either no extraction of DNA or no PCR amplification due to a polymerase inhibition caused by the ionic liquid. In this sense, a more hydrophobic and larger carboxylate resulted in a less efficient extraction effect of the ionic liquid. Among the choline derivatives, the best performing ionic liquid was the [chol]fom (**25**), which was further investigated.

In addition, a comparison between the anions from $[C_4mim]Bu_2PO_4$ (**90**) and $[C_2mim]Me_2PO_4$ (**4**) showed a difference of 10 Cq cycles, indicating that a longer chain length in anion and cation results in higher Cq values. The protic [DBU]fom (**42**) performed as well as [chol]fom (**25**).

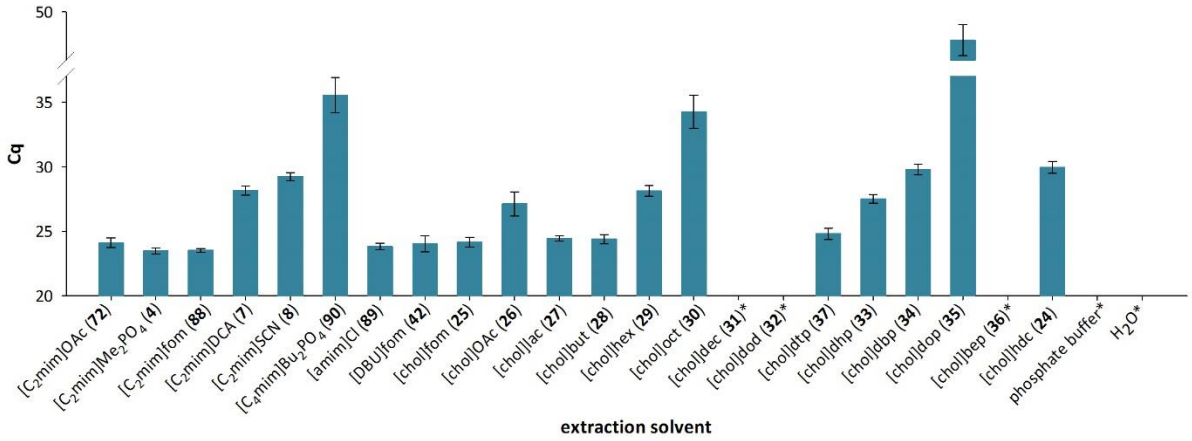


*Figure 164: Influence of different ionic liquids on the extraction of DNA from maize: 1 g of a 10 wt% solution of ionic liquid in buffer (1 ml phosphate buffer) and 100 mg maize powder were stirred for 10 min, denaturated at 95 °C, centrifuged and the supernatant analyzed via real-time PCR. The given Cq values are for the ADH1 gene of maize. *:No amplification observed. All experiments were carried out in triplicates.*

Among the best performing ionic liquids, two lead candidates for further proceeding were selected: [chol]fom (**25**), as it is considered to be environmentally friendly and $[C_2mim]Me_2PO_4$ (**4**), based on its previously reported biomass dissolving properties. Their usage confirms their potential in gDNA isolation from complex matrices, making them ideal for the development of rapid methods for DNA extraction with on-site applications.

4.6.3.4 Optimization of extraction parameters

With [chol]fom (**25**) as a promising candidate further investigations of the influence of different extraction conditions were made. To initially define the optimal concentration of ionic liquid **25** in phosphate buffer, experiments were carried out at room temperature and 80 °C with 10 min stirring. Moreover, the influence of the addition of prot K (5 µl, 20 mg/ml) was evaluated to see whether the digestion of proteins and enzymes present in the maize sample could improve the extraction and later the amplification of DNA (Figure 165). At low concentrations of ionic liquid **25** in buffer (1 wt%, 5 wt%) and the pure buffer (as a blank), an influence of prot K and temperature on the amplification was observed. Using a 1 wt% solution of **25** in buffer without the addition of prot K gave no amplification at room temperature, whereas low amounts of DNA were extracted when performing the reaction at 80 °C (209.2 ± 37.9 µg DNA/g sample). When increasing the temperature to 80 °C, a significant improvement of 10 cycles of the Cq value was obtained with the addition of prot K. With a higher concentration of ionic liquid **25** (5 wt%), amplification for all conditions was observed. However, with higher temperature and the presence of prot K the amount of extracted DNA increased

(1216 ± 60.8 µg DNA/g sample) compared to room temperature experiments (968 ± 76.6 µg DNA/g sample) and extractions without prot K (592 ± 56.7 µg DNA/g sample). A further increase of the concentration to a 10% solution of **25** in buffer resulted in the improvement of the Cq values obtained. At room temperature no significant influence of prot K was observed, while at 80 °C the presence of prot K approximately doubled the amount of DNA extracted (1176 ± 52.7 µg DNA/g sample) compared to the absence of prot K (660 ± 88.0 µg DNA/g sample). Higher temperature favoured the extraction. In contrast, when using even higher concentrations of ionic liquid in buffer like 50 wt% or 75 wt%, no amplification was obtained with or without the addition of prot K at both temperatures studied, which might be caused by a salting out process induced by the ionic liquid. Although the amount of DNA extracted using 80 °C was increased, the work at room temperature was chosen for potential on-site applications of the method.

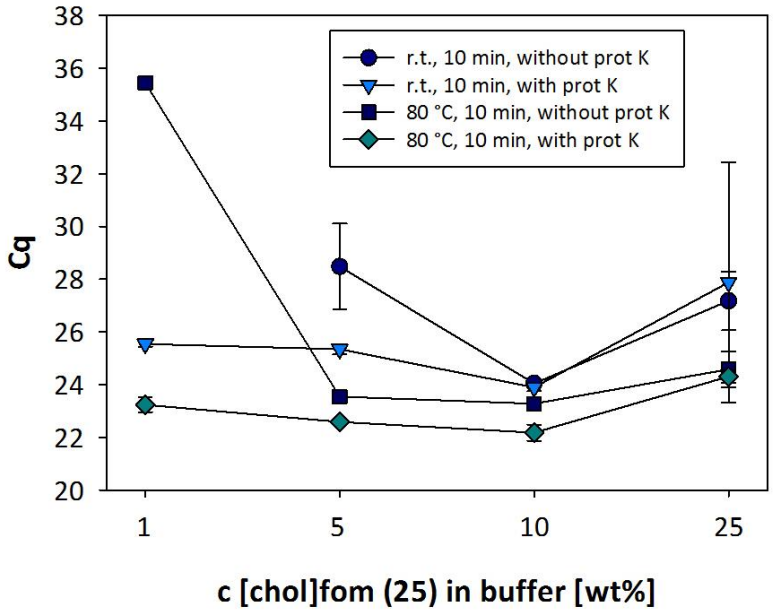


Figure 165: Optimization of the concentration of ionic liquid to use in the system ionic liquid-buffer.Analyses were carried out in the presence or absence of prot K and at room temperature and 80 °C. 1 g of a defined concentration of ionic liquid in buffer and 100 mg maize powder were stirred for 10 min, denaturated at 95 °C, centrifuged and analyzed via real-time PCR. The given Cq values are for the ADH1 gene of maize. All experiments were carried out in triplicates.

In addition, experiments were also carried out to know how $[C_2mim]Me_2PO_4$ (**4**) at a concentration of 10 wt% in phosphate buffer would perform when extracting at room temperature and 80 °C, with or without the addition of prot K (Figure 166). In this case it was seen that the Cq values were better when working without prot K at room temperature, whereas no significant difference was seen in extractions at 80 °C with or without prot K. A minor improvement in the amount of extracted DNA was obtained when working at 80 °C (980 ± 104 µg DNA/g sample at 80 °C and 744 ± 124 µg DNA/g sample at RT). It should be mentioned, that the Cq value obtained with pure buffer in Figure 166 represents only traces of DNA. However, having simple on-site applications in mind, the extractions were further performed at room temperature.

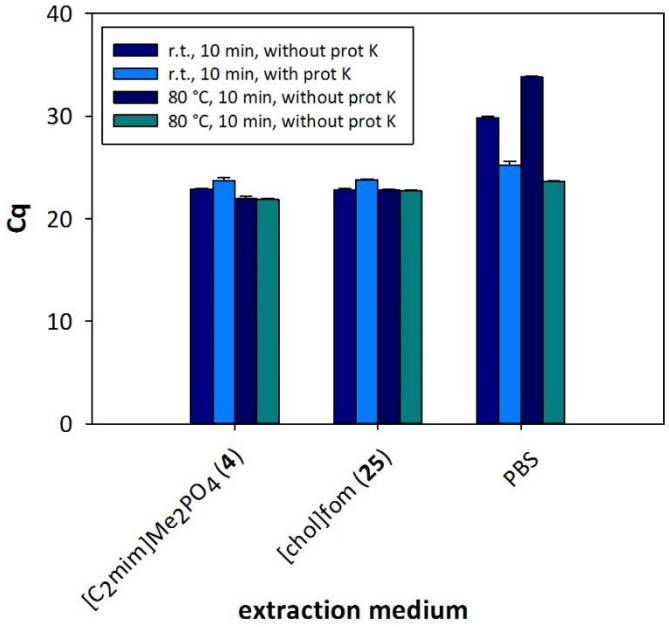


Figure 166: Comparison of different ionic liquid/buffer and buffer systems with or without the addition of prot K

When screening for the optimal incubation time, extractions were carried out for 5, 10, 15, 30 and 60 min. No dependency on time when extracting with both of the ionic liquids previously selected was found. Therefore, an extraction time of 5 min was chosen for keeping the protocol short and efficient.

4.6.3.5 Simplification of the extraction process

The extraction process was further optimized to see whether the final incubation step carried out at 95 °C for 10 min could be modified to facilitate the process. The extraction time was kept constant at 5 min, whereas the denaturation time was shortened from 10 min to 5 min. Alternatively, the extraction was performed without denaturation at all. As it can be seen in the results given in Table 49, a decrease of the denaturation time from 10 min to 5 min resulted in a slight increase of the Cq value, whereas a difference of 8 cycles was obtained when no denaturation was performed at all. In addition, when performing just a denaturation step for 10 min, only a minor increase of the Cq value was achieved. This suggests that the extraction step and denaturation of co-extracted enzymes can be combined while incubating at 95 °C for 10 min to further simplify the process.

*Table 49: Optimization of the denaturation step from the extraction protocol using 1 ml of a 10 wt% solution of [C₂mim]Me₂PO₄ (**4**) in phosphate buffer and 100 mg maize powder*

Extraction conditions	Cq *ADH1* ± STD
5 min stirring, 10 min denaturation	23.9 ± 0.2
0 min, 10 min denaturation	24.7 ± 0.2
5 min stirring, 10 min denaturation, syringe filtration	24.9 ± 0.4
5 min, 5 min denaturation	25.4 ± 0.5
10 m stirring, 0 min denaturation	31.9 ± 1.1
buffer, 5 min stirring, 10 min denaturation	29.8 ± 0.6

All experiments were carried out in triplicates

Moreover, a further simplification can be achieved using a syringe filtration over a PTFE membrane (pore size = 0.2 µm) instead of centrifugation. Filtration (Cq = 24.9 ± 0.4) slightly decreased the amount of amplifiable DNA when compared to centrifugation (Cq = 23.9 ± 0.2). Due to its simplicity, the filtration process can be used for further experiments.

4.6.3.6 Influence of the particle size

The influence of particle size on the Cq values was investigated next with the non-GM maize variety RWA38. In this case, the smaller particle size results in a more efficient extraction as demonstrated by applying the *ADH1* assay to the isolated DNA. When using $[C_2mim]Me_2PO_4$ (**4**) and [chol]fom (**25**), the same pattern was observed. Better results were obtained with $[C_2mim]Me_2PO_4$ (**4**) compared to [chol]fom (**25**) (Table 50).

Table 50: Influence of particle size for the extraction performance

Ionic liquid	Cq value corrected	
	< Particle Size	> Particle Size
$[C_2mim]Me_2PO_4$ (**4**)	27.2 ± 0.4	29.4 ± 0.4
[chol]fom (**25**)	29.2 ± 0.6	30.9 ± 0.8

Performed with 10 wt% solution of ionic liquid in phosphate buffer, stirring for 5 min, 10 min denaturation at 95 °C. All experiments were carried out in triplicates.

4.6.3.7 Stability over time and comparison to conventional CTAB method

Stability over time of the DNA extracted with the candidate ionic liquids [chol]fom (**25**) and $[C_2mim]Me_2PO_4$ (**4**) while storing the samples at room temperature for 10 days was also investigated. Real time PCR measurements were done every 2 days. Results show that the DNA is stable in the ionic liquid **4** and **25** and that the Cq values are comparable to those obtained with the CTAB extraction. (Figure 167)

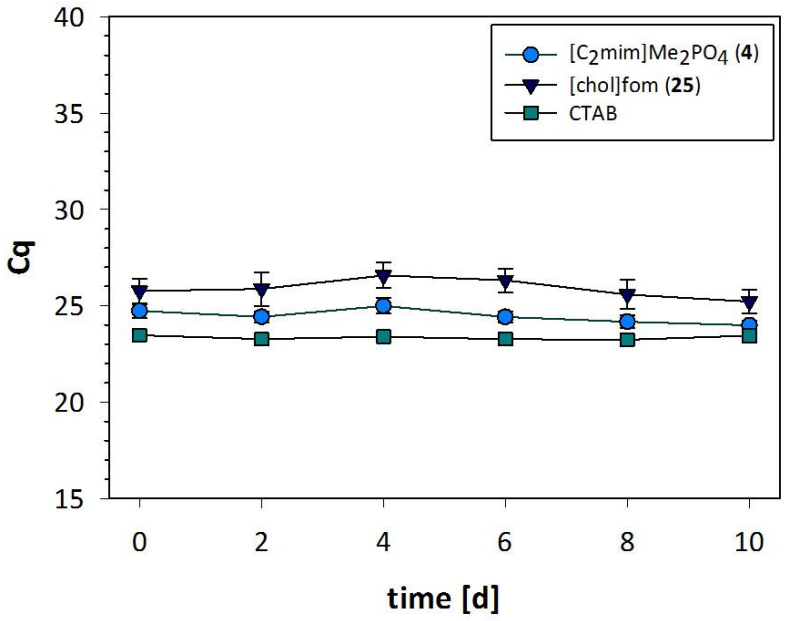


*Figure 167: Stability over time of DNA extracted with $[C_2mim]Me_2PO_4$ (**4**), [chol]fom (**25**) and the CTAB method and stored at room temperature. All experiments were carried out in triplicates.*

However the conventional used CTAB process is more time demanding and requires several centrifugation and heating steps, which made the whole process prone to contaminations. Figure 168 visualizes the steps necessary to extract DNA from maize samples: While for the CTAB method several heating steps, centrifugation steps and other work-ups are necessary, it is obvious that the developed ionic liquid-based method is more efficient and saves time. The developed method can even be shortened, since the dissolution process at the beginning is not necessary as (marked with a red cross. The estimated time of the CTAB method is around 5 hours, whereas in the developed process DNA was extracted within 20 minutes.

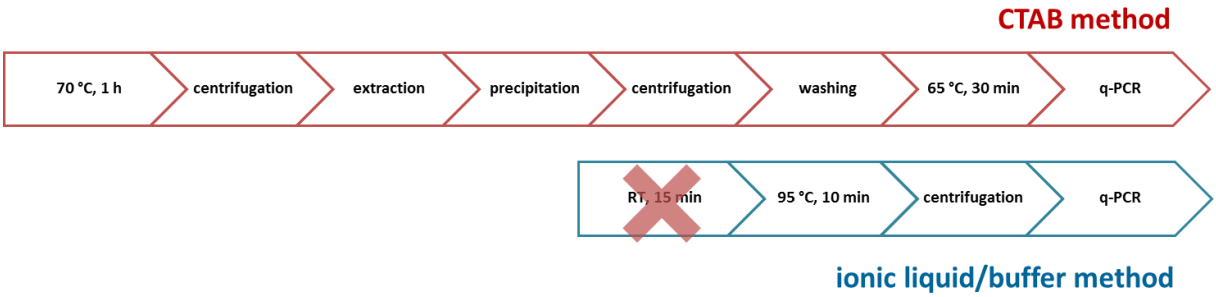


Figure 168: Comparison of conventional CTAB method and developed ionic liquid/buffer method

4.6.3.8 Scanning electron microscopy of maize treated with ionic liquids

To further elucidate the influence of ionic liquids on biomass, a scanning electron microscopy (SEM) image of recovered maize powder after extraction and denaturation was recorded. 100 mg maize powder were treated with 1000 mg of a 10 wt% solution of either $[C_2mim]Me_2PO_4$ (**4**) or [chol]fom (**25**) in phosphate buffer or 1000 mg water *via* stirring for 5 min, denaturation at 95 °C for 10 min and recovered *via* filtration. Despite the fact that ionic liquid-aqueous buffer solutions were applied rather than pure ionic liquid, some differences in starch morphology of the recovered maize powder were clearly found. This highlights the potential of ionic liquids for biomass dissolution and emphasizes their importance in the extraction process of DNA, as this break-up of starch structures could be held responsible for the improved extraction efficiency. (Figure 169)

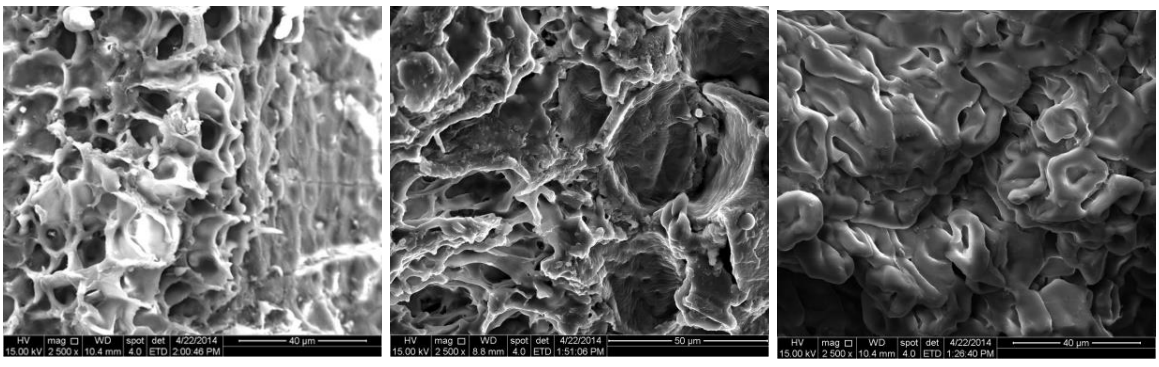


*Figure 169: SEM pictures of maize powder after extraction with water and ionic liquid-phosphate buffer solutions at 2500x magnification. Left: Water, middle: $[C_2mim]Me_2PO_4$ (**4**), right: [chol]fom (**25**).*

4.6.3.9 Detection of P35S promoter in Bt-11 maize

The suitability of the proposed extraction method for the detection of the *P35S* promoter from the *Cauliflower Mosaic Virus* inserted in the genome of the maize event variety Bt-11 was investigated by extracting DNA from Bt-11 certified reference material (Table 51). As it can be seen, the obtained Cq

values were comparable to those from the CTAB extraction. This fact makes the proposed extraction method suitable for the detection of this analytical target in various screening procedures. By a concentration of 0% of GM maize content, only traces were found as 50% of the replicates were negative for both methods. It could be expected that the results were not negative, as the reference materials are certified for Bt-11 event and not for the *P35S* promoter.

*Table 51: Detection of the P35S promoter in GM maize Bt-11 by the CTAB extraction method and when extracting with the [C₂mim]Me₂PO₄ (**4**). The given Cq values were obtained with the P35S assay. All experiments were carried out in triplicates.*

GM maize content (%)	CTAB Cq *P35S*	[C₂mim]Me₂PO₄ (4) Cq *P35S*
0.1	30.5 ± 0.8	30.9 ± 1.9
0.5	27.7 ± 0.3	28.6 ± 0.6
1	27.0 ± 0.3	27.8 ± 0.7

In addition, calibration curves were used to check for the presence of potential PCR inhibitors in the extracted gDNA for both maize varieties and when extracting with the CTAB method and the ionic liquids [C_2mim]Me_2PO_4 (**4**) and [chol]fom (Table 52). As it can be seen, all qPCR assays for the *ADH1* gene have high linearities (R^2=0.99) and efficiencies (>0.90). Furthermore, the efficiency and linearity of the extractions carried out with the method developed in this work are comparable to those obtained with the CTAB method. However, the efficiency and linearity in the case of the *P35S* promoter were lower than the ones from the *ADH1* gene.

*Table 52: Linearity and qPCR efficiencies of real-time PCR assays of extracted gDNA following the CTAB method and with the ionic liquids [C₂mim]Me₂PO₄ (**4**) and [chol]fom (**25**) for the non GM maize variety RWA38 and the GM maize Bt-11. DNA concentration of the extracts as well as the yield is also shown.*

Maize variety	Method of extraction	Target	c(ng/µl) ± STD	Yield (µg DNA/g sample) ± STD	Slope	Efficiency	R^2
RWA38	[C_2mim]Me_2PO_4 (**4**)	*ADH1*	180 ± 2	721.2 ± 8	-3.52	0.92	0.99
	[chol]fom (**25**)		274 ±2	1094.5 ± 6	-3.54	0.92	0.99
	CTAB		252 ± 1	252.3 ± 5	-3.22	1.04	0.99
Bt-11	[C_2mim]Me_2PO_4 (**4**)	*ADH1*	174 ± 4	696.7 ± 2	-3.51	0.93	0.99
	[chol]fom (**25**)		130 ± 2	519.3 ± 9	-3.33	0.99	0.99
	CTAB		256 ± 2	256.2 ± 9	-3.32	1.00	0.99
Bt-11	[C_2mim]Me_2PO_4 (**4**)	*P35S*	174 ± 4	696.7 ± 2	-3.77	0.84	0.98
	[chol]fom (**25**)		130 ± 2	519.3 ± 8	-4.05	0.77	0.99
	CTAB		256± 2	256.2 ± 9	-3.96	0.79	0.99

4.6.3.10 Quality of extracted genomic DNA

The quality of the extracted genomic DNA by using both $[C_2mim]Me_2PO_4$ (**4**) and the [chol]fom (**25**) was investigated by doing an agarose gel electrophoresis. The pattern of the separated DNA fragments was then compared to the CTAB method (Figure 170). As it can be seen, a smear of great intensity with fragments smaller than 500 bp was obtained in the sample extracted with the CTAB method. These short DNA molecules are typical for the fragmentation of the DNA during the extraction procedure. No visible sign of fragmentation was seen in the sample extracted with the ionic liquids.

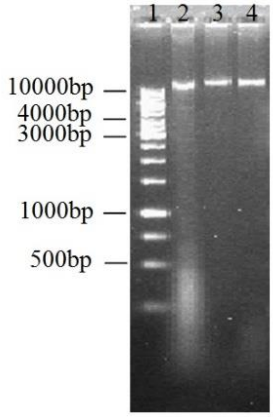


*Figure 170: Agarose gel electrophoresis for the genomic DNA extracted by using the CTAB method (well 2) and the ionic liquids $[C_2mim]Me_2PO_4$ (**4**) (well 3) and the [chol]fom **25** (well 4). The ladder was loaded into well 1.*

4.6.4 Extraction of DNA from meat

As the method presented in the previous chapter provided an extremely efficient and reliable strategy for the extraction and detection of DNA from maize samples, it should be expanded to a different food matrix and applied for the extraction of DNA from meat. Recently, the Horsegate or meat adulteration scandal in 2013 affected a number of European countries. In this sense, horse and pork meats were detected in beef, posing not only safety concerns, but also infringing religious faiths.[336] Additionally, manufacturing processes do not always comply with good manufacturing practices and the traceability of the origin of the ingredients can be poor. As a consequence, carryover meat or proportions of unexpected meats can be found in the final product.

To address these problems, laboratories must be able to determine the composition of meats in processed products. The first step in the screening process is the extraction of the DNA so as to later amplify the species-specific sequences. Current methods of isolation are highly time consuming and tedious as they involve several steps of filtration and centrifugation, which can deteriorate the quality of the DNA. Additionally, some of these methods require the use of aquatoxic surfactants such as SDS as well as of chloroform for extraction, thereby providing a serious environmental risk of exposure for on-field applications. Other methods are based on commercial kits, which can be expensive and can require special devices and handling. Hence it is important to develop a fast, inexpensive and simple extraction protocol for the isolation of DNA from meat.

For this purpose the ionic liquid/phoshphate buffer extraction should be applied for the extraction of DNA from meat.

4.6.4.1 Choice of ionic liquids

Based on the experience with maize similar ionic liquids were chosen. The two most promising were [chol]fom (**25**) and [C_2mim]Me_2PO_4 (**4**). Special focus was again put on the choline derivatives. Apart from that the influence of the chain length should not only be investigated using choline ionic liquids, but also guandine and methy imidazolium derivatives. (Figure 171)

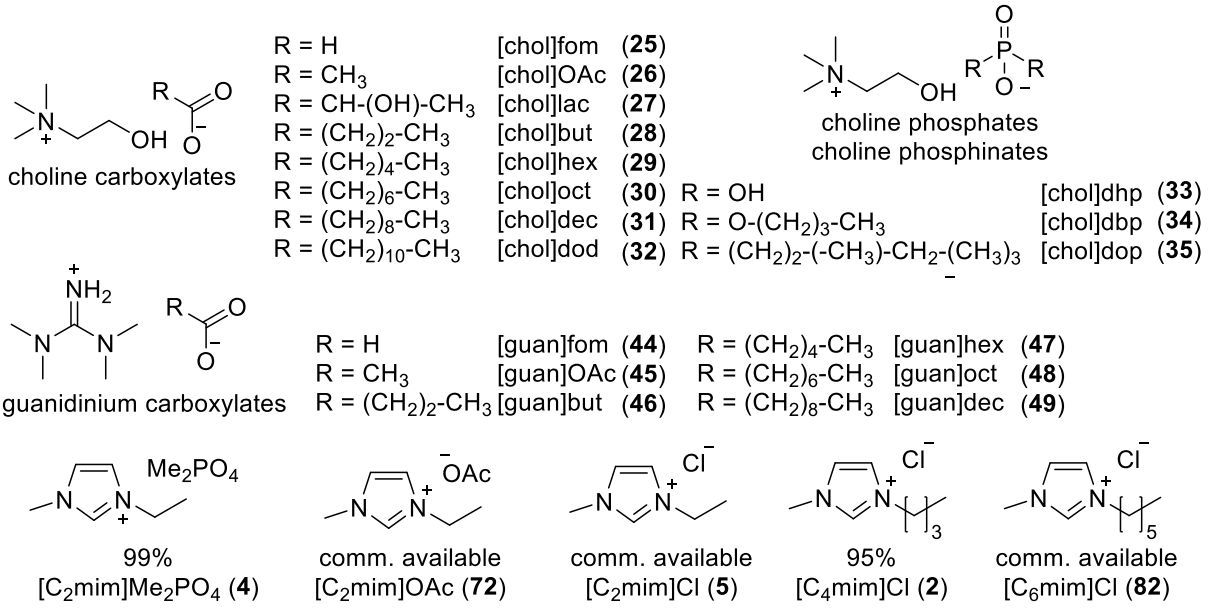


Figure 171: Ionic liquids chosen for the extraction of DNA from meat

The strategy for the extraction of DNA from meat was based on the developed process for maize. Meat should be pretreated with ionic liquid/buffer solutions and DNA should be extracted and stabilized in there. (Figure 172)

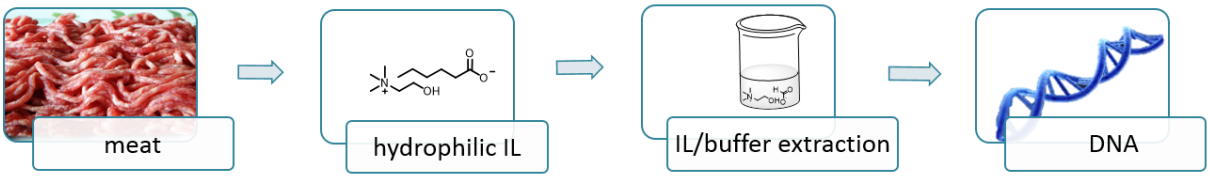


Figure 172: Strategy for the isolation of DNA from meat

4.6.4.2 Optimazition of conditions

The screening for the best extraction conditions was performed by using a modified procedure from the maize extraction[337] starting with the imidazolium derivative [C_2mim]Me_2PO_4 (**4**) and the choline derivative [chol]fom (**25**), since these two ionic liquids have shown superior performance in the previous work on DNA extraction from maize sample.[337] Three different buffer systems were investigated at 50 mM and pH 8.5 including sodium phosphate buffer, 2-amino-2-methyl-1-propanol buffer (AMP) and tris(hydroxymethyl)-aminomethan buffer (TRIS).

Figure 173: Flow scheme for the extraction of DNA from meat

Initially, 100 mg of ionic liquid were mixed with 900 µl of the corresponding buffer. (Figure 173) 200 mg of minced beef were chosen as starting material, which were added to the ionic liquid buffer mixture. The solution was stirred for 15 min at room temperature. Afterwards, a denaturation was performed at 95 °C for 10 min to deactivate any possible DNases that might be coextracted and that can later hinder the TaqMan polymerase during the amplification. The sample was centrifuged (13000 rpm, 5 min) and 400 µl of supernatant were taken and analyzed *via* quantitative PCR.

Table 53 shows that the phosphate buffer gave better results than the AMP and TRIS buffer in combination with the choline derivative. [chol]fom (**25**) performed significantly better than [C_2mim]Me_2PO_4 (**4**), which gave poor or no amplification (Table 54), although this ionic liquid was previously suitable for the extraction of DNA from maize.

Table 53: Influence of different buffers on the Cq values obtained when extracting DNA from minced beef with [chol]fom

Entry	Buffer	[chol]fom Cq (25) [a]	Pure buffer Cq [b]
1	phosphate	27.4 ± 0.7	33.9 ± 0.5
2	AMP	28.2 ± 1.3	43.4± -
3	TRIS	28.3± 0.7	35.1 ± 2.1

[a] Performed with 100 mg (± 10.0 mg) **25**, 900 µl corresponding buffer and 200 mg (± 15.0 mg) minced beef for 15 min at room temperature and denaturation for 10 min at 95 °C. [b] Performed with 1000 µl corresponding buffer and 200 mg (± 15.0 mg) minced beef for 15 min at room temperature and denaturation for 10 min at 95 °C. All experiments were carried out in triplicates.

For the removal of fat in meat, samples were optionally treated with *n*-heptane before extraction. However, no influence of this pretreatment on the Cq values using $[C_2mim]Me_2PO_4$ (**4**) was observed. (Table 54)

*Table 54: Influence of different buffers and pretreatment of meat in the Cq values obtained when extracting mtDNA from beef with $[C_2mim]Me_2PO_4$ (**4**)*

Entry	Buffer	$[C_2mim]Me_2PO_4$ (4) untreated meat[a] Cq[c]	buffer untreated meat[b] Cq	$[C_2mim]Me_2PO_4$ (4) treated meat[a] Cq	buffer treated meat[b] Cq
1	phosphate	-	33.9 ± 0.5	-	35.6 ± 0.9
2	AMP	-	43.4 ± 0.0	-	37.2 ± 0.2
3	TRIS	34.1 ± 0.6	35.1 ± 2.1	37.4 ± 0.6	33.6 ± 0.8

[a] Performed with 100 mg (± 10.0 mg) IL, 900 µl corresponding buffer and 200 mg (± 15.0 mg) minced beef for 15 min at room temperature and denaturation for 10 min at 95 °C. [b] Performed with 1000 µl corresponding buffer and 200 mg (± 15.0 mg) minced beef for 15 min at room temperature and denaturation for 10 min at 95 °C. [c] A difference in 1 Cq value corresponds to double the concentration of extracted DNA. The smaller the Cq value, the higher the concentration of extracted DNA. All experiments were carried out in triplicates.

Therefore all experiments were made without performing this pretreatment. Furthermore, it was seen that a denaturation step at 95 °C was sufficient (27.9 ± 0.9), as no influence in the Cq values was seen when comparing the experiments without stirring time to the experiments with an additional 15 min stirring time. (27.4 ± 0.7)

4.6.4.3 Influence of different ionic liquids on DNA extraction

After evaluation of the best conditions for the extraction, 20 other ionic liquids including choline derivatives, guanidine derivatives and imidazolium derivatives were tested. Particular interest was raised in choline derivatives, as they are generally classified as environmentally benign and biodegradable ionic liquids with low toxicities.[205,206] The toxicity of choline carboxylates [chol]but (**28**) and [chol]hex (**29**) was investigated towards *P. corylophilum*, showing a low growth inhibition and therefore low toxicity. Furthermore the biodegradation of [chol]hex (**29**) was evaluated by Garcia et al and the biodegradation of the anion was confirmed by NMR spectroscopy.[30] Additionally, choline hexanoate has already been used for cork dissolution and suberin isolation from cork,[30, 338] indicating that this ionic liquid is suitable for the dissolution of biomass.

An interesting relationship between the length of the carboxylate chain of the choline ionic liquids and the Cq values was observed. (Figure 174) While extending the carboxylate anion from formate to acetate an insignificant influence (< 1 cycle) of the Cq value was observed. Further increase to the butyrate anion decreased the Cq value significantly for more than two cycles. However best results were obtained using [chol]hex (**29**) (25.53 ± 0.58), with a further decrease of more than one cycle. With a longer chain length of 8 carbon atoms a slight increase in Cq value was observed. Further increase to [chol]dec (**31**) and [chol]dod (**32**) resulted in no amplification. Other choline derivatives such as [chol]dhp (**33**) and [chol]dop (**35**) gave no amplification, whereas [chol]dbp (**34**) exhibited a Cq value of 25.69 ± 0.29, which is comparable to the Cq value obtained with [chol]oct (**30**). (Table 55, entry 1-3)

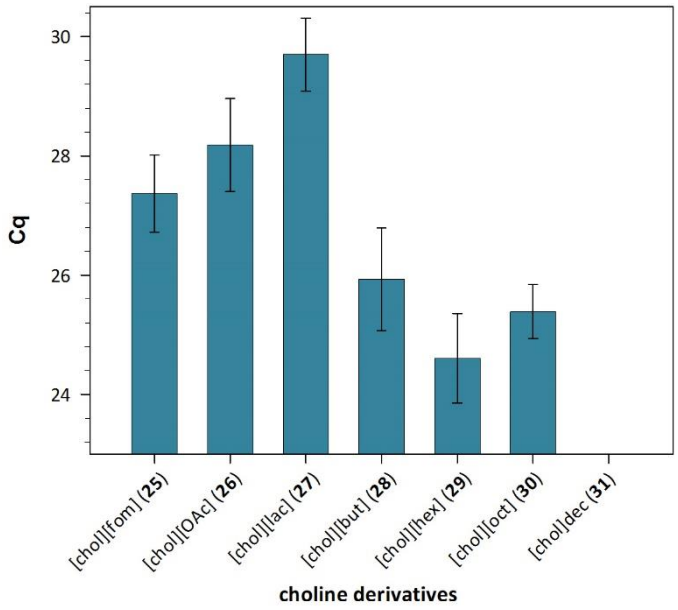


Figure 174: Screening of different choline derivatives performed with 100 mg (± 10.0 mg) ionic liquid, 900 µl phosphate buffer and 200 mg (± 15.0 mg) minced beef for 10 min at 95 °C.

The influence of the chain length of the carboxylate anion was also investigated using a *N,N,N,N*-tetramethylguanidinium cation. While [guan]fom (**44**), [guan]OAc (**45**) and [guan]but (**46**) did not lead to any amplification, [guan]hex (**47**) gave very promising Cq values (27.54 ± 0.47) and [guan]oct (**48**) performed tremendously better (25.25 ± 0.28). As seen before, a further increase in the chain length to 10 carbon atoms resulted in no PCR amplification. (Table 55, entry 9) The imidazolium based ionic liquids tested gave poor or no amplification. (Table 55, entry 10-14)

Table 55: Phosphate choline derivatives, guanidinium derivatives and imidazolium derivatives tested for the extraction of mtDNA from minced beef.

Entry	Ionic liquid[a]	Cq
1	[chol]dhp (**33**)	-
2	[chol]dbp (**34**)	25.7 ± 0.3
3	[chol]dop (**35**)	-
4	[guan]fom (**44**)	-
5	[guan]OAc (**45**)	-
6	[guan]but (**46**)	-
7	[guan]hex (**47**)	27.5 ± 0.5
8	[guan]oct (**48**)	25.3 ± 0.4
9	[guan]dec (**49**)	-
10	[C_2mim]Me_2PO_4 (**4**)	-
11	[C_2mim]OAc (**72**)	-
12	[C_2mim]Cl (**5**)	33.3 ± 1.6
13	[C_4mim]Cl (**2**)	41.7 ± 3.3
14	[C_6mim]Cl (**82**)	-

[a] Performed with 100 mg (± 10.0 mg) ionic liquid, 900 µl phosphate buffer and 200 mg (± 15.0 mg) minced beef for 10 min at 95 °C. All experiments were carried out in triplicates.

An agarose gel electrophoresis of the amplicons obtained from the beef extracts carried out with the choline derivatives was also performed (Figure 175). As it can be seen, by-products when extracting with [chol]OAc (**26**), [chol]lac (**27**) and [chol]but (**28**) were obtained, probably due to unspecific amplification during the real-time PCR, not so in the extracts with [chol]hex (**29**) and [chol]oct (**30**). This contributes to confirm [chol]hex (**29**) as best candidate for extraction.

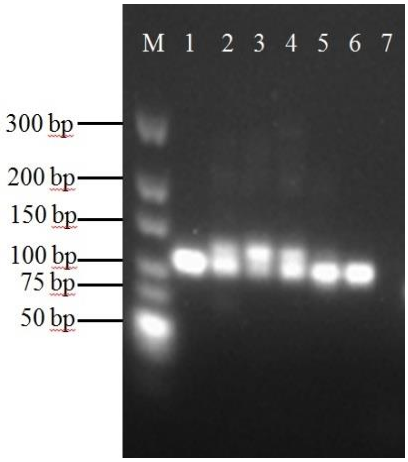


Figure 175: Agarose gel electrophoresis of the amplicons obtained from the beef extracts with choline derivatives. Amplicon size: 96 bp. M: Ultra Low Range Ladder (50 bp – 300 bp), 1: [chol]fom (yield = 488.7 µg DNA/g sample), 2: [chol]OAc (yield = 281.3 µg DNA/g sample), 3: [chol]lac (yield = 493.2 µg DNA/g sample), 4: [chol]but (yield = 323.7 µg DNA/g sample), 5: [chol]hex (yield = 487.7 µg DNA/g sample), 6: [chol]oct (yield = 376 µg DNA/g sample), 7: [chol]dec (yield = 413.7 µg DNA/g sample).

Furthermore, minced meat was frozen with liquid nitrogen and triturated in a mortar. However, this procedure did not improve the amount of extracted DNA using [chol]hex (**29**). (26.4 ± 0.32) As a sample pre-treatment by grinding the samples can be avoided, the prominence of ionic liquids is therefore highlighted, since they can effectively extract DNA.

4.6.4.4 Influence of the concentration of ionic liquid in the extraction buffer

The role of the concentration of the ionic liquid **29** in the buffer system was investigated. A low concentration of 1 wt% of ionic liquid **29** in buffer was not sufficient to exert a greater extraction effect on the matrix. (Table 56, entry 2) However, it can be seen that such a low concentration can improve the Cq value for one cycle when compared to pure phosphate buffer. A further increase to 5 and 10 wt% **29** in buffer, (Table 56, entry 3-4) enhanced the Cq value in 5 to 6 cycles respectively. However a higher concentration of ionic liquid **29** in buffer to 25 wt% gave a higher Cq value, whereas a 50 wt% solution resulted in no amplification probably due to an inhibitory effect on the PCR as discussed later in detail.

Table 56: Influence of the concentration of ionic liquid in buffer system.

Entry	C [chol]hex (29)/buffer [wt%][a]	Cq
1	0	33.9 ± 0.5
2	1	32.5± 0.7
3	5	27.2 ± 0.5
4	10	26.6 ± 0.5
5	25	29.9 ± 0.6
6	50	-

[a] Performed with x mg ionic liquid and y mg phosphate buffer (x + y =1000) and 200 mg (± 15.0 mg) minced beef 10 min at 95 °C. All experiments were carried out in triplicates.

4.6.4.5 Application on different types of meats

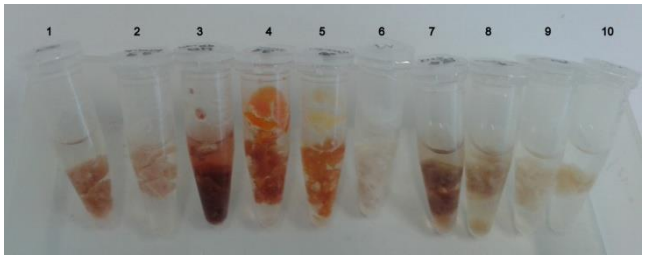


Figure 176: Extracts of different meats and meat derived products. 1: Leberkäse. 2: Knackwurst. 3: Bloodsausage. 4: Garlic sausage. 5: Gouchos. 6: Pork. 7: Horsemeat. 8: Beef. 9: Pork treated with buffer. 10: Chicken.

To expand the developed method other meats, such as pork, horse meat, chicken were tested. Figure 176 shows different meat samples after treatement with [chol]hex (**29**)/buffer mixture. The different types were tested using choline hexanoate. As it can be seen in Figure 177 DNA was successfully extracted from meat derived from different animals. In all studied cases the ionic liquid/buffer system performed significantly better than the pure phosphate buffer. However, a substantial difference between minced beef and beef meat was observed, giving the pretreatment of meat an important role in the extraction of DNA using such a great extent when compared to the ionic liquid/buffer systems.

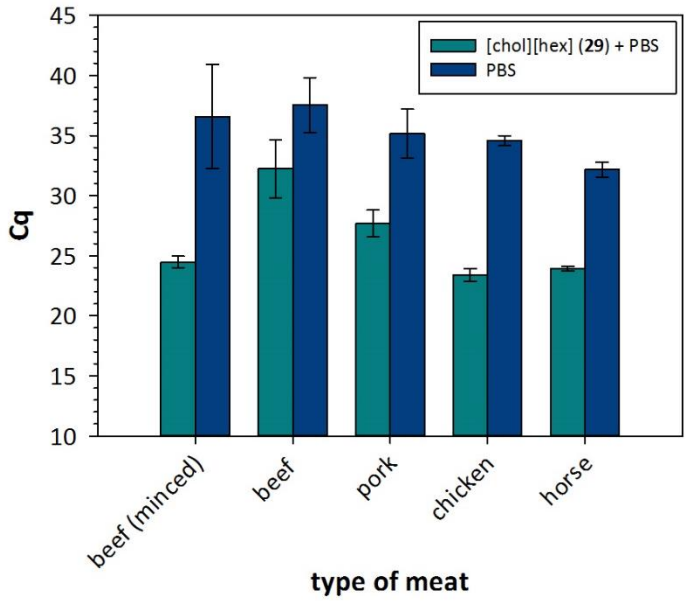


*Figure 177: Screening of different types of meat performed with 100 mg (± 10.0 mg) [chol]hex (**29**), 900 µl phosphate buffer and 200 mg (± 15.0 mg) meat for 10 min at 95 °C.*

In order to check the suitability of the proposed method in processed products containing horse meat, different meat derived products were also extracted. (Figure 178) In comparison, the Cq values for the sausages were significantly lower (approximately 8 cycles) than the horsemeat sample previously analysed, probably due to the manufacturing process of the samples, which seemed to favour the DNA extraction, therefore increasing the amount of DNA in the extracts. In the case of blood sausage, the Cq value was higher than the other ones obtained, which might be caused by the fact that blood sausages consist mainly of clotted blood with a content higher than 90% of erythrocytes (do not contain nuclei or mitochondria). In this case, extracted DNA comes from leukocytes and thrombocytes which represent a much lower percentage of the blood composition.[339, 340]

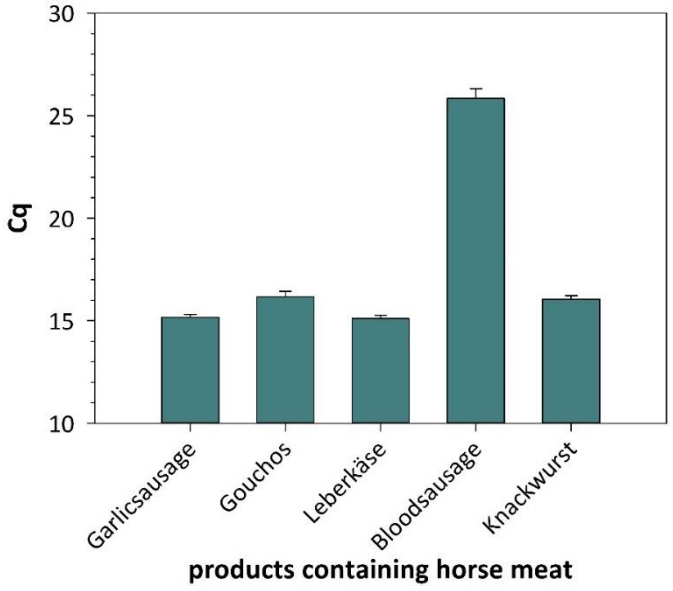


*Figure 178: Different products containing horse meat tested with [chol]hex (**29**)/buffer*

Sausages with different concentrations of horse meat were also investigated using the [chol]hex (**29**)/buffer system. According to Table 57, a low amount of 0.1% of horse meat could be detected with

a low Cq value of 22.19 ± 0.35. This confirms that the developed method can be successfully used for detection of adulteration of highly processed food products with horsemeat contents as low as 0.1%.

Table 57: Extraction of DNA from sausages containing different concentrations of horse meat

Entry	Horse meat content [%][a]	Cq[c]
1	0	-
2	0.1	22.2 ± 0.4
3	0.5	20.7 ± 0.1
4	1	19.6 ± 0.1
5	5	17.5 ± 0.1

[a] Performed with 100 mg (± 10.0 mg) [chol]hex **29**, 900 µl phosphate buffer and 200 mg (± 15.0 mg) horse sausages 10 min at 95 °C. [c] A difference in 1 Cq value corresponds to double the concentration of extracted DNA. The smaller the Cq value, the higher the concentration of extracted DNA. All experiments were carried out in triplicates.

4.6.4.6 Comparison of the ionci liquid method to other methods

The method proposed in this work was compared with other commercially available methods, such as the Wizard® Genomic DNA Purification Kit (Promega Corporation) and the SureFood® PREP Animal (r-Biopharm), as well as the method by *Amani et al.*[341] (Figure 179). Extractions were carried out with beef, chicken, pork and horsemeat. As it can be seen, results obtained when extracting with the method proposed in this work do not differ to a greater extent when compared with the other analysed methods and when extracting DNA from beef, chicken and pork. A greater difference is seen though in horsemeat, where lower Cq values were obtained when extracting with the other methods. However, the Cq value for horsemeat is comparable to those from beef, chicken and pork when extracting with [chol]hex (**29**) while following the protocol developed in this work.

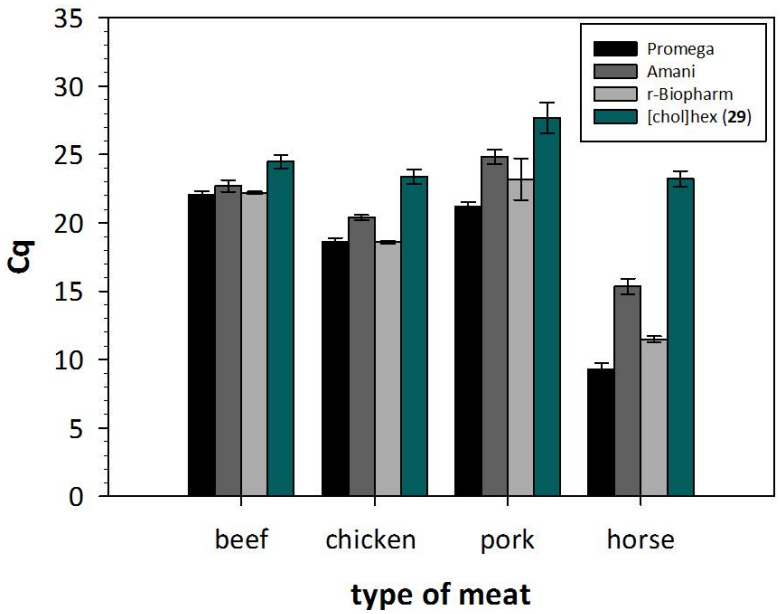


Figure 179: Comparison of different conventional methods and kits and the ionic liquid-based method using different types of meats

In spite of this, the extraction method proposed in this work stands out for its simplicity, it saves time as it is five or more times faster. Additionally, tedious centrifugation and heating/cooling steps (Figure

180) that are required in the other conventional methods could be reduced, thereby adding to the energy efficiency of the process. As a result, this method is considered to be suitable for rapid extractions while obtaining reliable results.

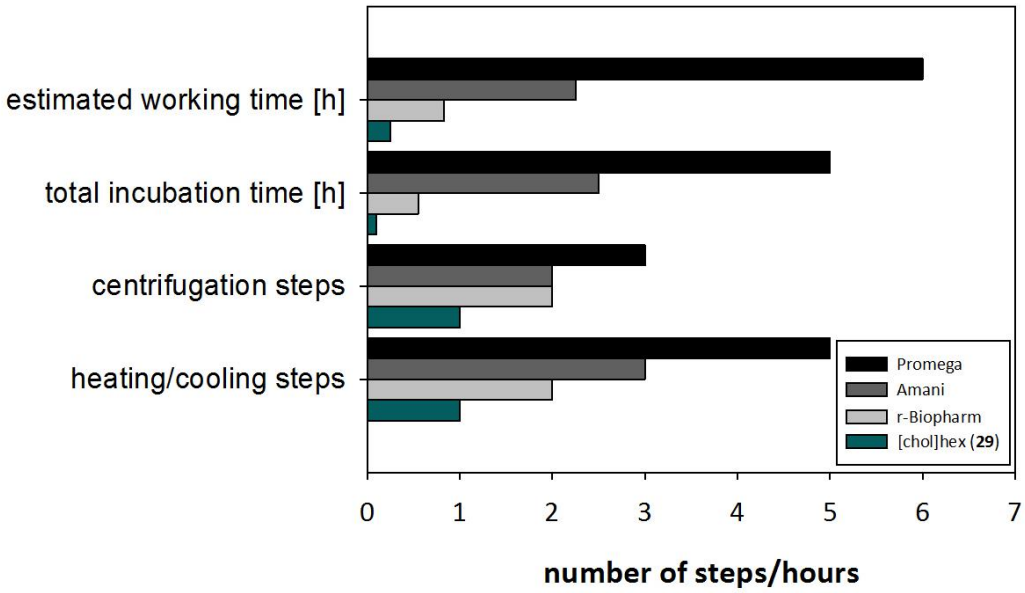


Figure 180: Comparison between conventional methods and kits and the developed method

Furthermore, calibration curves were made to check for the presence of potential PCR inhibitors in the extracted DNA from beef, chicken, pork and horsemeat when extracting with the method proposed in this work and the one from Amani *et al*., 2011, as well as the r-Biopharm and the Promega kits. As it can be seen (Table 58), all qPCR have high linearities (R^2 = 0.99) and high efficiencies (>0.90). Values obtained with the method proposed in this work are comparable to those from the other methods.

Table 58: Photometric data of correlation coefficients and qPCR efficiencies of real-time PCR assays of extracted mtDNA (2 ml template DNA) from beef, chicken, pork and horsemeat while following the protocol proposed by the Wizard® Genomic DNA Purification Kit (Promega Corporation), Amani et al., 2011, SureFood® PREP Animal (r-Biopharm) and the method developed with [chol]hex in this work.

Types of meat	c (ng/µl) ± STD	Yield (µg DNA/g sample) ± STD	Slope	Efficiency	R^2
Wizard® Genomic DNA Purification Kit (Promega Corporation)					
Beef	104.2 ± 9.1	520.8 ± 45.4	-3.45	0.95	0.9996
Chicken	270.7± 0.8	1353.3 ± 3.8	-3.53	0.92	0.9992
Pork	56 ± 0.5	280 ± 2.5	-3.31	1.00	0.9974
Horse	133.5 ± 12.8	667.5 ± 63.8	-3.36	0.98	0.9990
Amani *et al.*, 2011[341]					
Beef	123.5 ± 7.1	123.5 ± 7.0	-3.47	0.94	0.9979
Chicken	161.2 ± 1.6	161.2 ± 1.6	-3.56	0.91	0.9995
Pork	113.5 ± 3.8	113.5 ± 3.8	-3.43	0.96	0.9990
Horse	107.8 ± 2.2	107.8 ± 2.2	-3.44	0.95	0.9994
SureFood® PREP Animal (r-Biopharm)					
Beef	140.5 ± 3.1	281 ± 6.2	-3.48	0.94	0.9961
Chicken	450.3 ± 10.5	900.7 ± 21	-3.22	1.05	0.9982
Pork	96.2 ± 1.3	192.3 ± 2.5	-3.56	0.91	0.9999
Horse	197.3 ± 3.8	394.7 ± 7.6	-3.36	0.98	0.9998
Method developed with [chol][hex] (**29**)					
Beef	329.3 ± 5.0	658.7 ± 10.1	-3.18	1.06	0.9996
Chicken	285.7 ± 5.5	571.3 ± 11.1	-3.30	1.01	0.9929
Pork	316.8 ± 6.3	633.7 ± 12.7	-3.40	0.97	0.9862
Horse	265 ± 19.4	530 ± 38.7	-3.42	0.96	0.9976
Horse meat in sausage (5 %)	209.3 ± 4.2	418.7 ± 8.3	-3.59	0.90	0.9994

4.6.4.7 Influence of ionic liquids on DNA stabilization

In order to get more information on the role of various ionic liquids in the extraction, stability over time of the DNA extracted from beef with the choline derivatives [chol]fom (**25**), [chol]OAc (**26**), [chol]lac (**27**), [chol]but (**28**), [chol]hex (**29**) and [chol]oct (**30**), as well as the method proposed by Amani *et al.*[341], and the kits from r-Biopharm and Promega was investigated. (Figure 181) Samples were stored at room temperature for 20 days and real time PCR measurements were done every two days. Results show that at the beginning of the study, the lower the number of carbon atoms in the side chains of the ionic liquids (from 1 to 4 carbon atoms), the greater the instability of the Cq values, tending to stabilization by the end of the study (after 11 days). Cq values of the extractions made with [chol]hex (**29**) were kept approximately constant throughout this time and were comparable to those obtained when extracting with the protocol from Amani and the commercial kits from r-Biopharm and Promega.

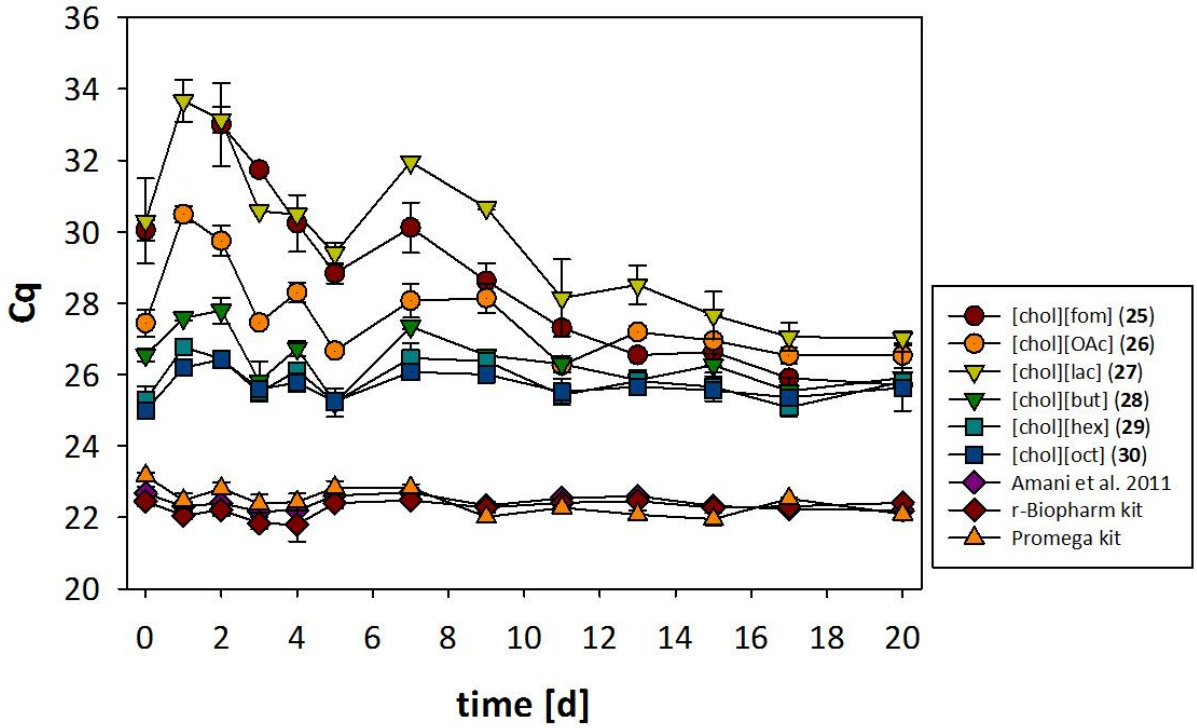


Figure 181: Stability of extracted DNA from minced beef using choline derivatives and conventional methods and kits.

This observed stabilisation of DNA in ionci liquid–buffer mixtures might be related to the unique interactions between cations and DNA strands: according to a study published by Vijayaraghavan *et al*.,[315]the native double helical structure of DNA is retained in choline derivative solutions, which might be associated with the ability of the ionic liquid ions to engage in hydrogen bonding with the exterior of the DNA helix. In this sense, the most likely H-bonding would be with the choline cation hydroxyl group. A molecular dynamics study on DNA solvation in pure ionic liquids reported by Cardoso *et al*. also suggested that cations are able to establish edge-to-face NH–π interactions with the DNA bases.[342]

4.6.4.8 Influence of ionic liquids on DNA amplification

To further investigate the influence of the ionic liquids on the DNA amplification during real-time qPCR, DNA extracts from beef, chicken, pork and horsemeat performed with the Wizard® Genomic DNA Purification Kit were diluted with [chol]fom (**25**), [chol]OAc (**26**), [chol]lac (**27**), [chol]but (**28**), [chol]hex (**29**), [chol]oct (**30**), [chol]dec (**31**), [chol]dod (**32**), sodium phosphate buffer (pH=8.5) and water. Final concentrations of the ionic liquids in the qPCR reactions were 33 ng/µl (7.5%), 11 ng/µl (2.5%, concentration present at DNA extraction experiments performed with 10 wt% solution of ionic liquid in buffer), 5.5 ng/µl (1.25%) and 2.2 ng/µl (0.5%).

When working at the highest concentration (33 ng/µl, 7.5%), no amplification of DNA was achieved when mixing the extracted DNA with choline derivatives with a chain length greater than 4 carbon atoms (Figure 182, left). This is in accordance with our previous observations, where no DNA was detectable during the PCR using high concentrations of 50 wt% of the ionic liquid [chol]hex (**29**) in the extraction experiments. (Table 56, entry 6)

In all cases, the lowest Cq value was obtained when diluting just with water, suggesting a possible inhibition of the amplification process when diluting not only with all of the ionic liqiuds, but also with

sodium phosphate buffer. In all types of meat, the lowest Cq value was obtained with the [chol]lac (**27**).

A further decrease of the ionic liquid concentration to 11 ng/µl (2.5%, corresponding to a 10 wt%. solution of ionic liquid in buffer in the extraction experiment) stabilized and lowered the Cq values obtained for all the meat sorts and when diluting the extracts with the choline derivatives with chain lengths varying from one to six carbon atoms (Figure 182, right). No amplification was achieved with a dilution with [chol]dec (**31**) and [chol]dod (**32**) for all meats, while a successful amplification was accomplished with [chol]oct in beef, pork and horsemeat, not so in chicken (in comparison to [chol]hex (**29**), Cq values were lower in approximately 9 cycles). This influence of ionic liquid chain length on DNA amplification might be related to the surface-activity of ionic liquids with longer alkyl chains, as is known that detergents inhibit the PCR[343, 344] due to the degradation of the DNA polymerase.

In contrast a concentration of 7.5% ionic liquid already resulted in an increase of the Cq values for all short chain choline derivatives ([chol]fom (**25**), [chol]OAc (**26**), [chol]lac (**27**) and [chol]but (**28**))and no amplification was observed in the case of [chol]hex (**29**) and [chol]oct (**30**). This is in accordance with the extraction experiments shown in Table 56, where an ionic liquid concentration of 25 wt% in buffer resulted in a higher Cq value and a concentration of 50 wt% resulted in no amplification caused by inhibition of qPCR.

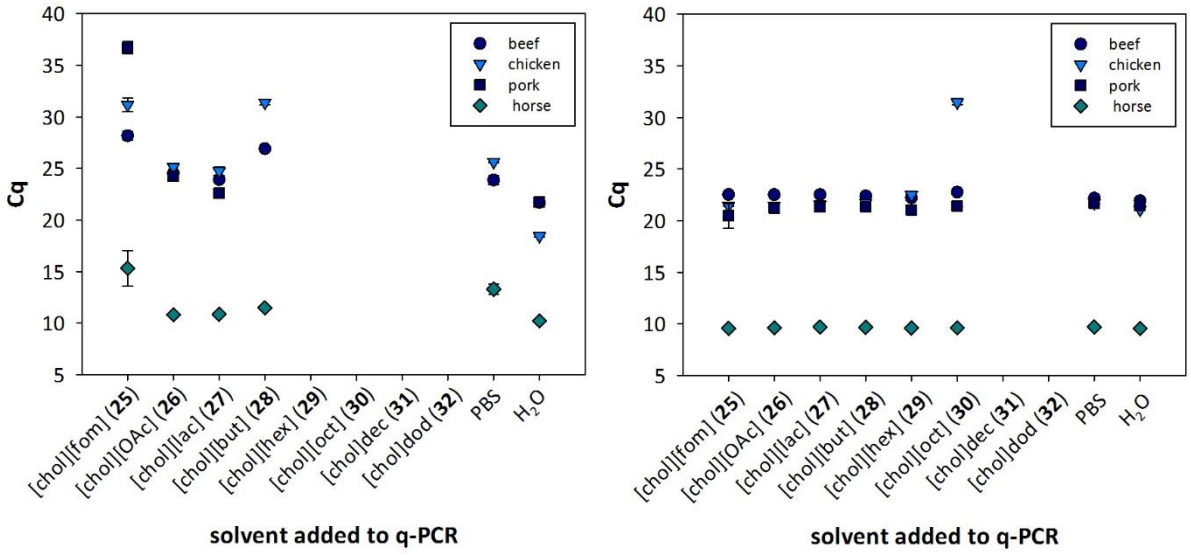


Figure 182: Ampflification of DNA in the presence of 7.5% solvents (left) and 2.5% (right)

Figure 183 and Table 59 outline the influence of the long chain choline derivatives on the amplification of DNA.

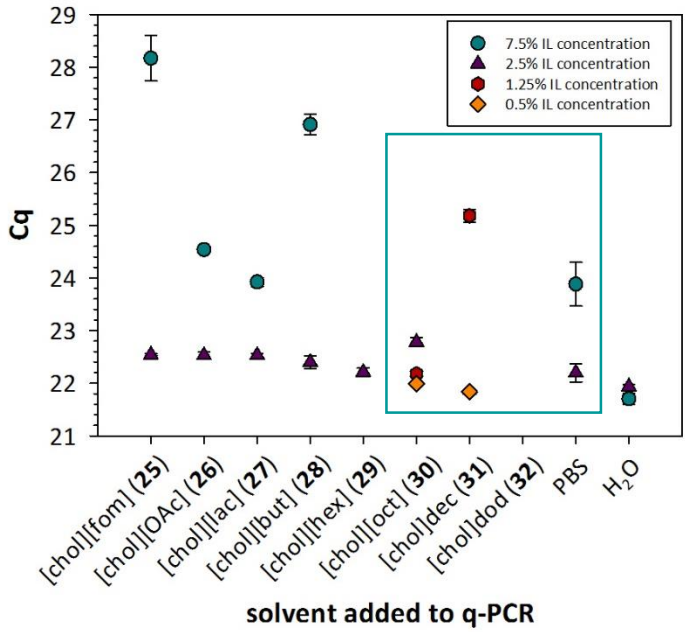


*Figure 183: Influence of choline ionic liquids on the amplification process during real time PCR. Concnetrations of 1.25% and 0.5% were only tested for [chol]oct (**30**), [chol]dec (**31**) and [chol]dod (**32**) (blue frame)*

To continue investigating how [chol]oct (**30**), [chol]dec (**31**) and [chol]dod (**32**) can influence the DNA amplification during the qPCR, the concentration of the these ionic liquids was lowered to 5.5 ng/µl (1.25%). At this concentration, it was then possible to amplify the DNA when diluting the samples with [chol]oct (**30**) and to a lesser extent with [chol]dec (**31**). No amplification was possible with [chol]dod (**32**). A decrease in concentration of these ionic liquids in the qPCR to 2.2 ng/µl (0.5%), made it possible for the [chol]dec (**31**) to have a similar Cq value as the [chol]oct (**30**). However, no amplification was obtained for extracts from meats diluted with [chol]dod, except for chicken. In the case of the beef samples extracted with [chol]dec following the protocol proposed in this work, amplification was only possible after diluting 16 times (Cq = 28.1 ± 0.1), while a dilution of 64 times was necessary to be able to amplify with [chol]dod (**32**) (Cq = 25.1± 0.06) (data not shown).

In case of meat obtained from chicken, the influence of the concentration of choline ionic liquids with different chain lengths on the amplification process during real time PCR indicates that a longer alkyl chain length of the carboxylate anion results increased inhibition.

Table 59: The amplification process during real time PCR, c ionic liquid=5.5 ng/µl (1.25%) and c ionic liquid=2.2 ng/µl (0.5%)

Entry	Meat	c ionic liquid	[chol]oct (30) Cq	[chol]dec (31) Cq	[chol]dod (32) Cq
1	beef	5.5 ng/µl	22.2 ± 0.1	25.2 ± 0.1	-
2	chicken	5.5 ng/µl	19.6 ± 0.1	29.1 ± 0.6	-
3	pork	5.5 ng/µl	21.9 ± 0.1	30.5 ± 1.5	-
4	horse	5.5 ng/µl	8.9 ± 0.1	9.1 ± 0.1	-
5	beef	2.2 ng/µl	22.0 ± 0.0	21.8 ± 0.1	-
6	chicken	2.2 ng/µl	18.9 ± 0.1	19.2 ± 0.1	28.7 ± 0.9
7	pork	2.2 ng/µl	22.2 ± 0.1	21.8 ± 0.0	-
8	horse	2.2 ng/µl	8.9 ± 0.1	8.8 ± 0.0	-

Those results obtained suggest that PCR amplification of DNA extracted with choline derivatives is highly dependent on the concentration of ionic liquid present in the sample and that this effect is greater with a higher amount of carbon atoms in the side chain of the choline structure. For DNA amplification and suitability for extraction experiments it can be stated that with increasing chain length the extraction efficiency increases. However, the inhibition of the qPCR also increases.Figure 184 outlines the relation of the phenomena described above. To compromise between extraction efficiency and inhibition of PCR for the fatty meat matrix a choline derivative with a chain length of 6 carbon atoms was the optimum candidate.

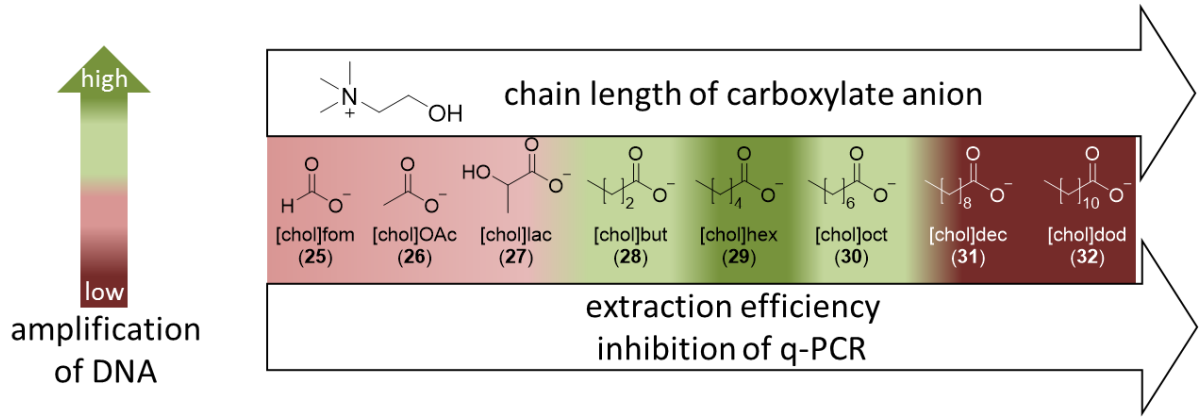


Figure 184: Graphical representation of the influence of the carboxylate chain length of choline derivatives on the extraction efficiency and the inhibition of qPCR.

In the last part of this thesis ionic liquid buffer solutions were successfully applied for the extraction of DNA from biomass. It was demonstrated that this approach is very versatile and its application might be expanded to other food matrices or e.g. for the isolation of human DNA for forensic analysis. Apart from that this method could also be expanded to e.g. mycotoxines, since they are known to be barely soluble.

5 Outlook

To aim for iseugenol (**64**) or vanillin from the crude extracts of cloves two new approaches are suggested (Figure 185): The first one uses functionalized ionic liquid, e.g. the basic phosphonium ionic liquid [P_{4444}]OH (**76**) that showed already good dissolution properties of wood. It might be possible that this basic ionic liquid or other functionalized ionic liquids can isomerize eugenol (**63**) to isoeugenol (**64**), as the isomerization ash also been described under highly basic conditions. The second approach would be similar to the limonene project presented in this thesis. Since the biotechnological tranformation of eugenol to vanillin has been reported in literature[345, 346] the combination of extraction and biocatalysis can be combined using ionic liquids. However it was already seen that bacterial systems are very sensitive towards the addition of ionic liquids therefore accurate investiagations are needed.

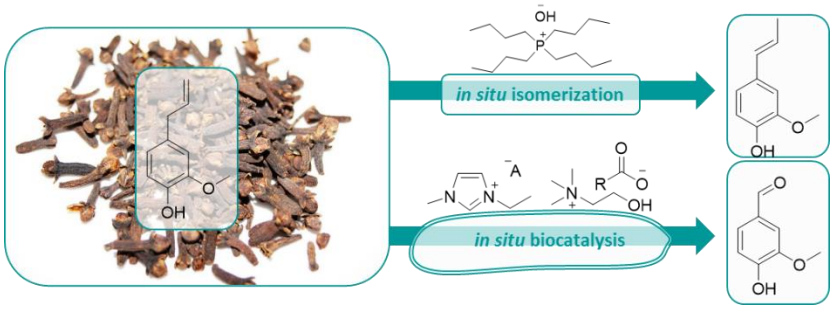


*Figure 185: Possible in situ extraction and conversion from eugenol (**63**) to isoeugenol (**64**) or vanillin*

5.1 Extraction of 10-deacetyl-baccatin III (91) from yew needles

An unfinished part of this PhD thesis deals with the dissolution of European yew needles in ionic liquids for the improved isolation of 10-deacetyl-baccatin III (**91**). (Figure 186)

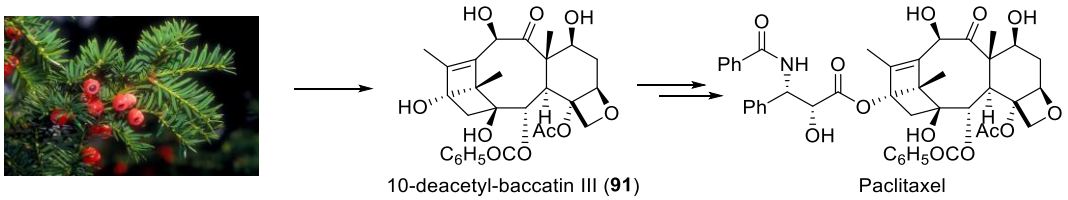


*Figure 186: The european yew plant, 10-deacetly-baccatin III (**91**) and Paclitaxel*

Paclitaxel is a complex diterpene amide derived from the bark of Pacific yew tree (*Taxus brevifolia*), and is currently the best known drug approved for use in the treatment of breast, ovarian, and nonsmall cell lung cancer, and AIDS-related Kaposi's sarcoma.[6] The reported isolation for Paclitaxel varies between 0.004–0.01% dry from yew bark, and thus Paclitaxel is produced from 10-deacetylbaccatin III (**91**) isolated from European yew (*Taxus baccata*), since its needles supply most of the 10-deacetylbaccatin III (**91**) needed for this synthesis.[347] However, the European yew contains approximately 0.2% in the needles, thereby making it a more attractive and a sustainable source for the active ingredient.[348]

The strategy for the enhanced extraction yields of 10-deacetylbaccatin III (**91**) is based on the powerful dissolution properties of ionic liquids, in combination with consecutive supercritical fluid extraction (SFE) of the valuebale ingredient. Supercritical fluid extraction gained attention due to its

nondestructive extraction of sensitive natural compound. Especially the use of supercritical carbon dioxide ($scCO_2$) was promising for the isolation of pharmaceutical products due to its low toxicity.[21] The combination of highly volatile $scCO_2$ and non-volatile polar ionic liquids might be useful for the extraction of active ingredients. Due to their different miscibilities the mix of $scCO_2$ and ionic liquids leads to the formation of a biphasic system, in which $scCO_2$ is soluble in the ionic liquid due to the pressure, but not *vice versa*. Furthermore the viscosity is remarkably reduced by the dissolved $scCO_2$ in the ionic liquid. The combination of $scCO_2$ with ionic liquids enables a variety of application such as the extraction of naphtalene from $[C_4mim]PF_6$ (**10**), metal catalyzed reactions, etc… [349]

Especially for 10-deacetylbaccatin III (**91**) since this natural product was reported to be soluble in $scCO_2$. Enhanced yields with low extraction times are expected for the isolation of 10-deacetylbaccatin III (**91**).

5.1.1 Design and synthesis of ionic liquids

Before addressing the key step of supercritical fluid extraction, the dissolution of yew needles and the extraction of **91** should be separately investigated. Based on previous experience, a set of ionic liquids based on imidazolium, ammonium or phosphnoum, cholinium based ionic liquids was selected. (Figure 187)

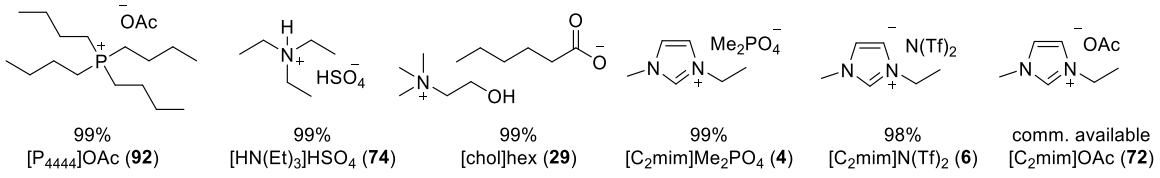


*Figure 187: Ionic liquids selected for the extraction of 10-deacetyl baccatin III (**91**) from yew needles*

Hydrophilic and hydrophobic ionic liquids were both investigated. In case of hydrophilic ionic liquids aqueous solutions should be tested as well.

5.1.2 Ionic liquid-based extraction process

After setting an HPLC strategy including a standard calibration, optimum conditions were identified as stirring time of 1 h at room temperature and a 40 wt% biomass loading. The influence of different ionic liquids including imidazolium ionic liquids, choline ionic liquids, phosphonium ionic liquids and ammonium ionic liquids was investigated. The imidazolium ionic liquids $[C_2mim]Me_2PO_4$ (**4**) and $[C_2mim]OAc$ (**72**) performed equally well and gave good extraction yields of 0.05 wt%. In comparison, lower yield of 0.03 wt% were observed when using the hydrophobic imidazolium ionic liquid $[C_2mim]NTf_2$ (**6**). The choline based [chol]hex (**29**) gave low yield of 0.03 wt% as well, whereas no yield was obtained using $[P_{4444}]OAc$ (**92**) probably due to mixing and viscosity issues at 80 °C extraction temperature.

Conventional solvents were also tested as extraction media and compared to the ionic liquids: The toxic solvent methanol gave slightly higher yields than $[C_2mim]OAc$ (**72**), whereas the volatile and toxic organic solvents such as chloroform, dichloromethane, toluene, gave lower yields, as well as heptane, EtOAc and water.

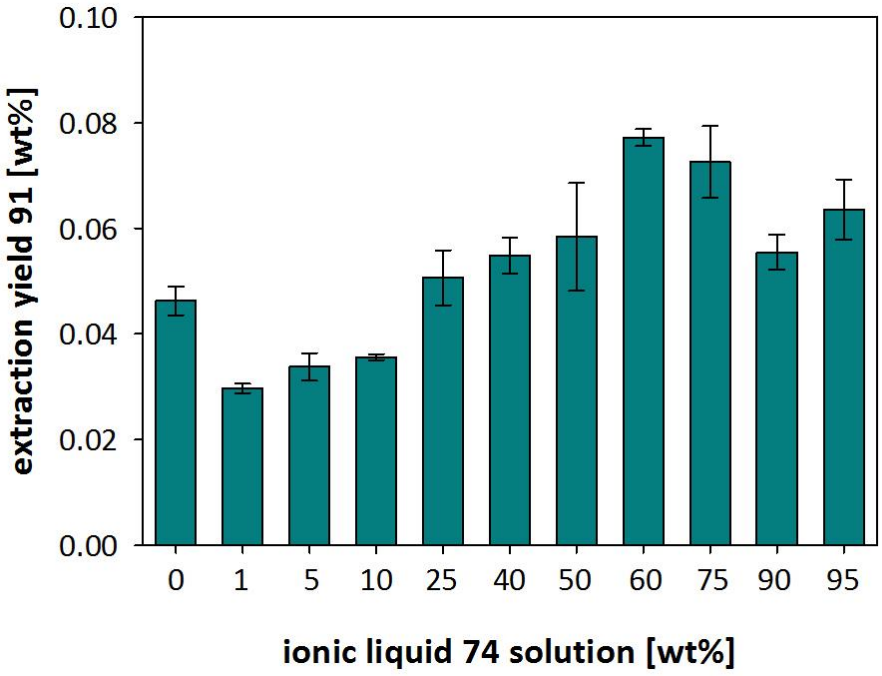


*Figure 188: 10-deacetyl-baccatin III (**91**) extraction using aqueous solutions of [NHEt$_3$]HSO$_4$ (**74**)*

To compromise between solubility properties and the high price of ionic liquids, aqueous ionic liquid solutions were investigated: If the concentration of ionic liquid in water was increased from 1% to 50%, a tremendous increase in extraction efficiency was observed, whereas at higher ionic liquid concentrations the extraction efficiency decreases again. In case of [C$_2$mim]Me$_2$PO$_4$ (**4**), [C$_2$mim]OAc (**72**) and [P$_{4444}$]OAc (**92**) up to 0.05 wt% 10-deacetyl-baccatin III (**91**) was extracted. The best results were obtained using [chol]hex with a maximum of 0.06 wt% using a 50% solution and the cheap protic ionic liquid [NHEt$_3$]HSO$_4$ (**74**) gave extraction yields up to 0.08 wt% using a 60% solution of ionic liquid in water. (Figure 188)

The cheap ionic liquid [NHEt$_3$]HSO$_4$ (**74**) could already successfully applied as aqueous solution for the extraction of baccatine from yew needles. To date, the extraction efficiency is still below 0.1 wt% and this process needs to be optimised. However, a first step for a future process combining ionic liquids and using supercritical CO_2 was made, and research efforts in this regard will be intensified in future.

6 Conclusion

In the first part of this thesis a set of more than 50 ionic liquid s was successfully synthesized in very good yields over typically 1-3 steps. A variety of cations (Figure 189) was obtained including anions such as different halogens, carboxylates, phosphates and e.g. bistriflimides.

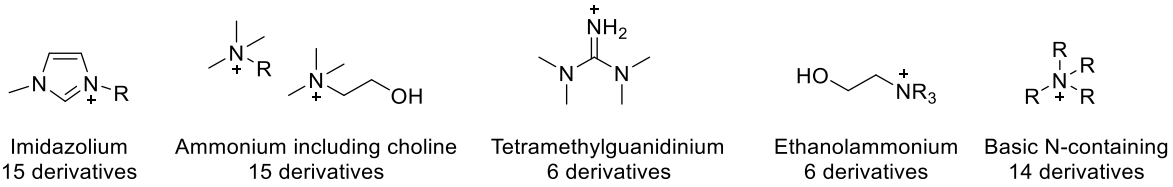


Figure 189: Cationic backbones of synthesized ionic liquids

The tuneability of the ionic liquids made it possible to synthesize the ideal candidate for each isolation problem. For the extraction of piperine and eugenol surface-active ionic liquids were successfully applied. In contrast, a hydrophilic phosphonium based ionic liquid was most suitable for the extraction of betulin, while for the isolation of limonene (**83**) a hydrophilic imidazolium based ionic liquid was a promising extraction solvent. For the extraction of DNA ionic liquids dissolvable in buffer solution were applied and imidazolium derivatives and choline ionic liquids with different carboxylate chain length were eventually selected.

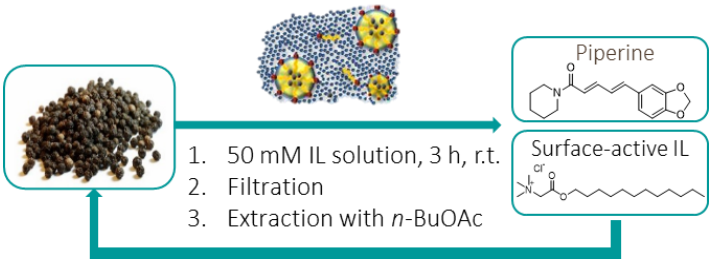


Figure 190: Summary of the isolation of piperine from black pepper

Based on the isolation of piperine from black pepper it was demonstrated that the extraction efficiency of various aqueous-ionic liquid micellar solutions strongly depends on the CMC of the respective ionic liquid. Using a biodegradable betaine derivative, a simple and scalable isolation procedure was developed, and it allowed separation of piperine (**58**) from the extraction media and the recycling of the aqueous solution for four times without loss in the extraction efficiency. The micellar extract could be directly used for a subsequent reaction. (Figure 190)

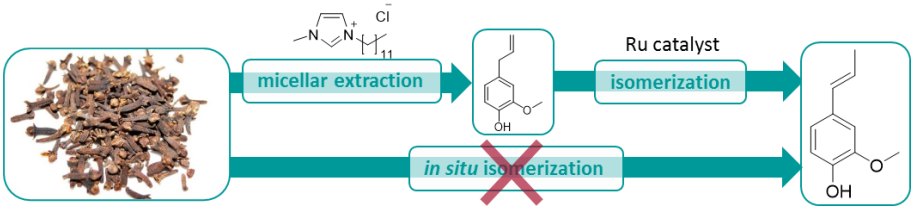


*Figure 191: Approach for the isolation of eugenol (**63**) and derivatization*

The use of those micellar systems was expanded to the extraction of eugenol from cloves. (Figure 191) Again, micellar solutions showed a powerful extraction efficiency compared to pure water, in a very short extraction times. The use of these ionic liquids for a ruthenium catalyzed reactions gave

promising results, however intensive screening is needed for the optimization of the isomerization. Different catalyst have to be investigated as well as different ionic liquids, e.g. the 1,3 alkylated imidazolium derivatives and 1,2,3 alkylated. The combination of extraction and catalysis could not be performed so far, as the concentration of ionic liquid and starting material behaved contrary to each other. Therefore a catalyst that can convert quite low concentrated amounts of eugenol (**63**) has to be identified in future.

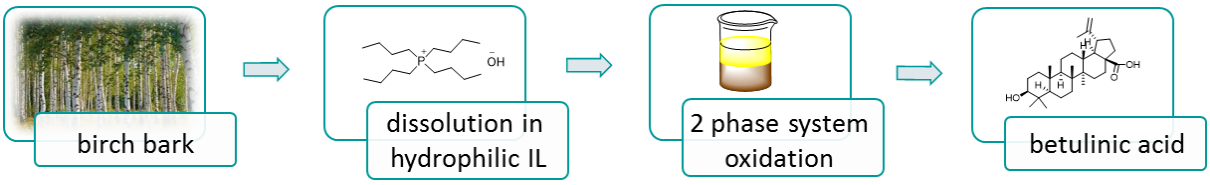


*Figure 192: Appoach for the isolation of betulin (**69**) and betulinic acid (**70**)*

Wood, in particular birch bark, was partially dissolved in the phosphonium based ionic liquid [P_{4444}]OH (**76**) allowing the isolation of betulin (**69**) in high yields in a very short time at room temperature. As organic extration medium BuOAc was used. (Figure 192) This environmentally benign method enables not only the extraction of betulin (**69**) in enhanced yields, but also the possibility for the direct conversion of the extracted betulin (**69**) towards biological interesting and value-added derivatives. This was demonstrated with the combined extraction of betulin (**69**) and its direct oxidation reaction towards betulinic acid (**70**). High yields of betulinic acid (**70**) were obtained in an energy-efficient process that outperforms current strategies for the production of betulinic acid **70**.

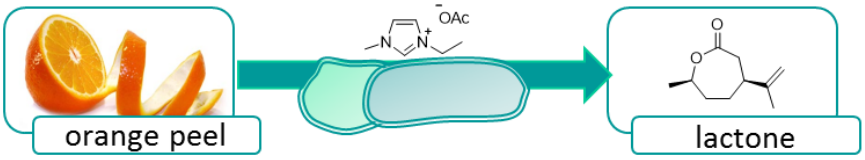


*Figure 193: Appoach for the isolation of lactone (**87**) from orange peels*

Ionic liquids were also investigated for the *in situ* extraction and biocatalytic transformation of limonene (**83**) from orange peels towards a valuable chiral polymer building block. Some ionic liquids were compatible with two different bacterial strains. However the direct extraction and conversion is still under investigations.

In the last part of this project DNA was extracted from different food matrices. (Figure 194)

Figure 194: Work flow for the extraction of maize and meat using an ionic liquid-based strategy

A simple, fast and efficient strategy using an ionic liquid/buffer mixture was developed for the extraction of DNA directly from a complex natural matrix. While the use of pure ionic liquids for genomic DNA extraction was not suitable as reproducibility problems arose, it was found that combined IL-aqueous buffer systems could notably increase the amount of extracted DNA compared to conventional buffer systems. Significant changes in starch morphology were observed after

incubation of maize with ionic liquid-aqueous buffer systems, thereby emphasizing the influence of ionic liquids in biomass treatment – even when used in aqueous solutions. Among all systems tested, the ionic liquid $[C_2mim]Me_2PO_4$ (**4**)in combination with sodium phosphate buffer (50 mM, pH=8.5) was found as the most efficient extraction system. DNA extracted with $[C_2mim]Me_2PO_4$ (**4**)had the highest quality and was stable when stored at room temperature for 10 days. Optimization of extraction parameters resulted in a simplified and time-efficient procedure compared to a conventional CTAB method for the direct extraction of genomic DNA from maize powder, as a save of time of approximately three hours was achieved when extracting 10 samples in parallel with both methods.

The developed extraction method for DNA was expanded to another food matrix. DNA was extracted from different types of meats and meat derived products using a mixture of the ionic liquid choline hexanoate (**29**) and a phosphate buffer. Among all systems tested, choline hexanoate (**29**) at a concentration of 10 wt% in sodium phosphate buffer (50 mM, pH = 8.5) was found to be most efficient. DNA extracted with this ionic liquid showed high specificity as no by-products were detected when performing an agarose gel electrophoresis, had a high yield and was stable when stored at room temperature for 20 days. Further investigations showed a strong influence of the alkyl chain length and the concentration of ionic liquids on the extraction of DNA from meat and on the amplification process. When extracting with choline carboxylates with chain lengths containing less than 8 carbon atoms concentrations of the ionic liquids lower than 11 ng/ml did not affect the amplification of DNA in the qPCR reaction. These results were in accordance with the observed concentration dependency in the extraction experiments, where a higher concentration of ionic liquid in buffer influenced DNA amplification and resulted in higher Cq values. The optimised extraction procedure based on the biodegradable ionic liquid choline hexanoate (**29**) in aqueous buffer systems provides several advantages, as it avoids not only the use of toxic and volatile organic solvents, but provides a time and energy saving process. The easily applicable protocol avoids washing and filtration steps and reduced therefore accumulation of waste. When compared to conventional methods such as the extraction kits from Promega, r-Biopharm and Amani *et al.*, 2011, the here proposed protocol resulted in a simple, inexpensive and five times or more faster extraction procedure, while having reliable results and a low environmental footprint.

7 Experimental part

7.1 Materials and methods

Chemicals and reagents:

All reagents were purchased from commercial suppliers and used without further purification unless noted otherwise. CH_2Cl_2, Et_2O, MeOH, THF and toluene intended for water-free reactions were pre-distilled and desiccated on Al_2O_3 columns (PURESOLV, Innovative Technology). Anhydrous triethylamine was stored over KOH and distilled from calcium hydride prior to use.

All ionic liquids were dried for at least 24-48 h at room temperature or 50 °C and 0.01 mbar before use and were stored under argon.

Chromatography solvents were distilled prior to use. Column chromatography was performed on a Büchi Sepacore Flash System (2 x Büchi Pump Module C-605, Büchi Pump Manager C-615, Büchi UV Photometer C-635, Büchi Fraction Collector C-660) or standard manual glass columns using silica gel from Merck (40-63 µm) with PE/EtOAc or MeOH/CH_2Cl_2 mixtures as eluates.

Preparative HPLC:

Preparative HPLC was performed on a Shimadzu preparative HPLC equipped with SDP20A PDA detector using a C18(2) column (250 cm x 21.20 cm ID) and MeOH/H_2O as eluent at a flowrate of 20 ml/min.

TLC-analysis:

TLC-analysis was carried out using precoated aluminum-backed plates purchased from Merck (silica gel 60 F_{254}). UV active compounds were detected at 254 nm.

Table 60: TLC staining solutions

TLC staining solution 1 (general purpose)		**TLC staining solution 2 (general purpose)**	
2 g	$KMnO_4$	4.5 g	phosphomolybdic acid hydrate
1 g	NaOH	0.1 g	cerium ammonium nitrate
40 g	K_2CO_3	100 ml	H_2SO_4 (10%)
320 mL	deion. H_2O	300 mL	EtOH

NMR analysis:

^{1}H, ^{13}C and ^{30}P NMR spectra were recorded from $CDCl_3$, CD_2Cl_2, d_4-MeOD or d_6-DMSO solutions on a Bruker AC 200 (200 MHz) or Bruker Advance UltraShield 400 (400 MHz) spectrometer and chemical shifts (δ) are reported in ppm using tetramethylsilane as internal standard coupling constants (J) are in Hertz (Hz). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, sext = sextet, m = multiplet, brs = broad.

TGA measurements:

Thermal stabilities were determined on a Netzsch TGA in a range of 25 to 500 °C with a heating rate of 10 °C/min. Decomposition temperatures ($T_{5\%onset}$) were reported from onset to 5 wt% mass loss.

Melting points:

Melting points above room temperature were measured on a Kofler hot-stage microscope or on an automated melting point system OPTI MELT of Stanford ResearchSystems and are uncorrected.

Infrared spectra:

Infrared spectra were recorded on a Perkin-Elmer Spectrum 65 FT IR spectrometer equipped with a specac MK II Golden Gate Single Reflection ATR unit.

Elemental analysis:

Elemental analysis was performed at Vienna University, Department of Physicochemistry- Laboratory for Microanalysis, Währingerstraße 42, A-1090 Vienna.

HR-MS:

A Thermo Scientific LTQ Orbitrap XL hybrid FTMS (Fourier Transform Mass Spectrometer) equipped with Thermo Scientific MALDI Interface or Thermo Fischer Exactive Plus Orbitrap (LC-ESI+) and a Shimadzu IT-TOF Mass Spectrometer were used for high resolution mass spectrometry.

Microwave synthesis:

Microwave reactions were performed on a BIOTAGE Initiator™ sixty microwave unit. The reported times are hold times. For this purpose the reaction mixtures were transferred into a glass pressure microwave tube equipped with a magnetic stirring bar, sealed with a Teflon septum and exposed to microwave irradiation at the required temperature. The absorption mode was set to *„Very high“*.

LC-MS:

Measurements were performed on an *Esquire/HCT* ion trap mass spectrometer from *Bruker Daltonics*, coupled to an *Agilent 1100 series* LC system *via* an electrospray interface. The separations were conducted at 25 °C on an *Agilent ZORBAX Eclipse* XDB-C18 (150 x 4.6 mm, particle size 5 µm) LC column with a flow rate of 0.8 ml/min. The mobile phase consisted of 87% MeOH in water with 0.1% HOAc added.

Alternatively a Nexera LC-30AD UPLC systems equipped with a Shimadzu LC-MS 2020 detector was used with an *Agilent ZORBAX Eclipse* XDB-C18 (150 x 4.6 mm, particle size 5 µm) LC column with a flow rate of 1 ml/min. The mobile phase consisted of 90% MeOH in water with 5 mM formic acid added.

HPLC methods:

HPLC analysis for piperine and eugenol was performed on a Thermo Finnigan Surveyor chromatograph equipped with a PDA plus detector.

Method A: Method for the detection of piperine

For the determination of piperine a Phenomenex Luna 10 µm C18 100A column (250 × 4.60 mm) was used with CH_3CN/H_2O 55/45 as solvent and a flow of 1 ml/min; detection was done at 271 nm, at 30 °C column oven temperature, 4 °C tray temperature. Retention times were 4.1 min for phenol and 9.2 min for piperine. Standard calibrations were performed for aqueous and organic extraction.

*Method B: Method for the detection of eugenol (**63**)*

For the determination of eugenol (**63**) a Phenomenex Luna 10 µm C18 100A column (250 × 4.60 mm) was used with CH_3OH/H_2O (0.1% TFA) 50/50 as solvent and a flow of 1 ml/min; detection was done at 270 nm, at 40 °C column oven temperature, 25 °C tray temperature. Retention times were 5.9 min for phenol and 19.5 min for eugenol (**63**).

*Method C: Method for the detection of 10-deacetylbaccatin III (**91**)*

For the determination of 10-deacetylbaccatin III (**91**) a Phenomenex Luna 10 µm C18 100A column (250 × 4.60 mm) was used with MeOH:H_2O (0.1% TFA) = 48:52 as solvent and a flow of 1 ml/min; detection was done at 270 nm, at 40 °C column oven temperature, 4 °C tray temperature. Retention times were 11.8 min for **91** and 18.7 min for methyl benzoate.

HPLC analysis for betulin (**69**), its derivatives and limonene (**83**) was performed on a Jasco HPLC unit equipped with a PDA detector.

Method D: Method for the simultaneous detection of betulin (**69**)*, betulonic acid* (**80**) *and betulonic aldehyde* (**79**) *(TPAP/NMO oxidation)*

For the determination of betulin (**69**), betulonic acid (**80**) and betulonic aldehyde (**79**) a Maisch ReproSil 100 C18 250x4.6, 5 µm was used with MeOH:H_2O (0.1% TFA) = 85:15 as solvent and a flow of 1 ml/min; detection was done at 210 nm, at 30 °C column oven temperature, 25 °C tray temperature. Retention times were 7.2 min for the internal standard, 13.3 min for betulin (**69**), 17.4 min for betulonic acid (**80**) and 29.0 min for betulonic aldehyde (**79**).

*Method E: Method for the detection of betulinic acid (**70**) in the extraction experiments*

For the determination of betulinic acid (**70**) a Maisch ReproSil 100 C18 250 x 4.6, 5 µm was used with MeOH:H_2O (0.1% TFA) = 85:15 as solvent and a flow of 1 ml/min; detection was done at 210 nm, at 30 °C column oven temperature, 25 °C tray temperature. Retention times were 7.1 min for the internal standard and 15.0 min for betulinic acid (**70**).

*Method F: Method for the detection of betulin (**69**) in the extraction experiments*

For the determination of betulin (**69**) a Maisch ReproSil 100 C18 250 x 4.6, 5 µm was used with MeOH:H_2O (0.1% TFA) = 87:13 as solvent and a flow of 1 mlL/min; detection was done at 210 nm, at 30 °C column oven temperature, 25 °C tray temperature. Retention times were 7.3 min for the internal standard and 15.7 min for betulin (**69**).

*Method G: Method for the simultaneous detection of betulin (**69**), betulinic acid (**70**), betulinic aldehyde (**78**) (BAIB oxidation)*

For the determination of betulin (**69**), betulinic acid (**70**) and betulinic aldehyde (**78**) a Maisch ReproSil 100 C18 250 x 4.6, 5 µm was used with MeOH:H_2O (0.1% TFA) = 87:13 as solvent and a flow of 1 ml/min; detection was done at 210 nm, at 30 °C column oven temperature, 25 °C tray temperature. Retention times were 7.3 min for the internal standard, 15.73 min for betulin (**69**), 16.5 min for betulinic acid (**70**) and 28.0 min for betulinic aldehyde (**78**).

*Method H: Method for the simultaneous detection of betulinic acid (**70**), bevirimat (**71**) and regioisomer iso-(**71**)*

For the determination of betulinic acid (**70**), bevirimat (**71**) and regioisomer iso-(**71**) a Maisch ReproSil 100 C18 250 x 4.6, 5 µm was used with MeOH:H_2O (0.1% TFA) = 88:15 as solvent and a flow of 1 ml/min; detection was done at 210 nm, at 30 °C column oven temperature, 25 °C tray temperature. Retention times were 7.1 min for the internal standard, 15.0 min for betulinic acid (**70**), 23.4 min for regioisomer iso-(**71**) and 24.3 min for bevirimat (**71**).

*Method I: Method for the detection of limonene (**83**)*

For the determination of limonene a Maisch ReproSil 100 C18 250 x 4.6, 5 µm was used with MeOH:H_2O(0.1% TFA) = 87:13 as solvent and a flow of 1 mL/min; detection was done at 210 nm, at 30 °C column oven temperature, 25 °C tray temperature. Retention times were 7.0 min for the internal standard and 9.5 min for limonene (**83**).

GC-methods:

GC analysis for eugenol (**63**), isoeugenol (**64**) and limonene (**83**) was performed on a Thermo Finnigan Trace 1310 equipped with a Thermo Scientific TR-5MS (15 m x 0.1 µm ID).

*Method J: Method for the simultaneous detection of eugenol (**63**) and isoeugenol (**64**) (Z and E)*

80 °C 0.8 min – 100r → 115 °C – 80r → 175 °C – 50r → 190 °C – 20r → 210 °C – 50r → 300 °C, duration 5.0 min.

Retention times: Methylbenzoat 1.51 min, eugenol 2.07 min (**63**), *Z*-isoeugenol (*Z*)-(**64**) 2.18 min, *E*-isoeugenol (*E*)-(**64**) 2.26 min

*Method K: Method for the detection of limonene (**83**) in extraction experiments*

60 °C 1 min – 80r → ramp to 200 °C, 2.75 min duration

Retention times: limonene (**83**) 1.87 min, methylbenzoat 2.07 min

GC analysis for biocatalytic reactions was conducted with a Thermo Finnigan Focus GC / DSQ II equipped with a FID detector using a standard capillary column BGB5 (30m x 0.32 mm ID) at a flow rate of 2.0 ml/min and a split ratio of 20 with Helium as carrier gas.

*Method L: Method for the simultaneous detection of limonene (**83**), carveol (**84**), carvone (**85**), dihydrocarvone (**86**) and lactone (**87**)*

80 °C – 50r → 140 °C – 20 r → 200 °C – 50r → 280 °C, duration 7 min

Retention times: 3.3 min limonene (**83**), 3.6 min ISTD (methyl benzoate), 4.2 min dihydrocarvone (**86**), 4.2 min carveol (**84**), 4.4 min carvone (**85**), 5.3 min lactone (**87**)

SEM pictures

Scanning electron microscopy (SEM) pictures were taken with a FEI Inspect F50 at 15 kV. All samples were coated with a 3.5 nm thick gold-layer using a Leica Cool Sputter Coater EM SCD005.

Pepper samples

Ground black pepper (*Piper nigrum*) was bought in a local supermarket and used as obtained.

Cloves samples

Ground cloves (*Caryophyllaceae*) were bought in a local market and used as obtained.

Preparation of plane bark

Plane bark (P*latanus acerifola*) was gratefully provided by the Botanic Institute in Vienna, frozen with liquid nitrogen and milled to a particle size of 1 µm.

Preparation of birch bark

Birch bark (*Betula*) was collected in Vienna in summer 2012, frozen with liquid nitrogen and milled to a particle size of 1 µm.

Yew samples

Needles of the European yew tree (*Taxus baccata*) were collected in Vienna in summer 2014, frozen with liquid nitrogen and milled to a particle size of 1 µm.

Maize samples

Maize variety RWA38 was kindly provided by the Institute of Biotechnology in Plant Production at IFA-Tulln, Austria and ground either to a powder of gross particle size with an Oster blender (Boca Raton, FL, USA) or to a fine powder with a Retsch ball mill MM301 (Haan, Germany). Maize variety Bt-11 was bought from the Institute for Reference Materials and Measurements of the European Union. Samples were stored at 4 °C when received until usage.

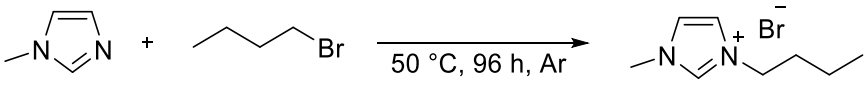
Meat samples

Authentic fresh meat samples from beef (*Bos taurus*), pork (*Sus scrofa*), chicken (*Gallus gallus*), horse (*Equus ferus caballus*), as well as traditional Austrian products like garlic sausage, gouchos, leberkäse, blunzen and knacker were bought in a local supermarket in Vienna, Austria and were kept frozen at -20 °C until further usage. Model sausages were prepared by mixing beef, pork, bacon (20%), salt (2%), pepper (0.7%), Worcester sauce (0.7%), vinegar (0.7%), garlic (0.7%), as well as different herbs and spices. The mixture was thoroughly homogenised with portions of horsemeat fixed at 0%, 0.1%, 0.5%, 1% and 5% by using a meat cutter and filled into sheep gut. Sausages were later cooked for 20 min and were kept frozen at -20 °C until further use.

7.2 Synthesis of ionic liquids

7.2.1 Synthesis of ionic liquids *via* alkylation

7.2.1.1 1-Butyl-3-methylimidazolium bromide [C$_4$mim]Br (1)



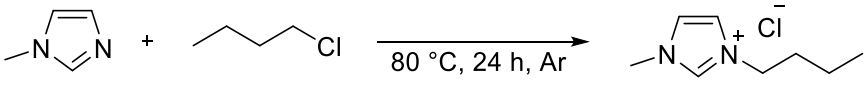
$C_8H_{15}BrN_2$
219.13
1

To freshly distilled 1-methylimidazole (41.0 g, 0.50 mol) 1-butylbromide (87.0 g, 0.64 mol, 1.3 eq.) was added dropwise. The mixture was refluxed at 50 °C for 96 h under argon until NMR control indicated complete conversion. After cooling to RT the solution was washed with EtOAc (3 × 10 ml) and dried *in vacuo* ($1 \cdot 10^{-2}$ mbar) at 80 °C for 24 h. A dark yellow oil was obtained that was recrystallized in a mixture of EtOAc and ACN. The product was yielded as colorless crystals in 95% (103.9 g) yield.[350]

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H = 0.88 (t, *J* = 7.59, 3H, N-CH_2-$(CH_2)_3$-C*H*$_3$), 1.33 (sext, *J* = 6.06, 2H, N-CH_2-CH_2-C*H*$_2$-CH_3), 1.84 (quin, *J* = 3.86, 2H, N-CH_2-C*H*$_2$-CH_2-CH_3), 4.06 (s, 3H, NCH_3), 4.27 (t, *J* = 7.32, 2H, N-C*H*$_2$-$(CH_2)_2$-CH_3), 7.48 (t, *J* = 1.76, 1H, H-4), 7.64 (t, *J* = 1.76, 1H, H-5), 10.29 (s, 1H, H-2).

Analytical data was in accordance with literature.[350]

7.2.1.2 1-Butyl-3-methylimidazolium chloride [C$_4$mim]Cl (2)



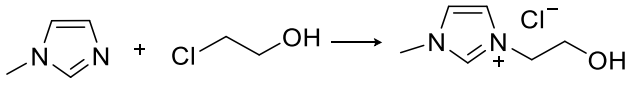
$C_8H_{15}ClN_2$
174.67
2

To freshly distilled 1-methylimidazole (20.5 g, 0.25 mol) 1-chlorobutane (22.5 g, 0.30 mol, 1.2 eq.) was added. The mixture was refluxed at 50 °C for 96 h under argon until NMR control indicated complete conversion. After cooling to RT the solution was washed with EtOAc (3 × 10 ml) and dried *in vacuo* ($1 \cdot 10^{-2}$ mbar) at 80 °C for 24 h. A dark yellow oil was obtained that was recrystallized in a mixture of EtOAc and ACN. The product was yielded as colorless crystals in 94% (41.0 g) yield.[351]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H = 0.88 (t, *J* =7.2, 3H, N-$(CH_2)_3$-C*H*$_3$), 1.26 (sext, *J* = 7.54, 2H, N-CH_2-CH_2-C*H*$_2$-CH_3), 1.75 (quin, *J* = 7.23, 2H, N-CH_2-C*H*$_2$-CH_2-CH_3), 3.85 (s, 3H, N-CH_3), 4.17 (t, *J* = 7.24, 2H N-C*H*$_2$-CH_2-CH_2-CH_3), 7.79 (d, *J* = 14.64, 2H, H-4,H-5), 9.38 (s, 1H, H-2)

Analytical data was in accordance with literature.[351]

7.2.1.3 1-(2-Hydroxyethyl)-3-methylimidazolium chloride [C$_2$OHmim]Cl (3)



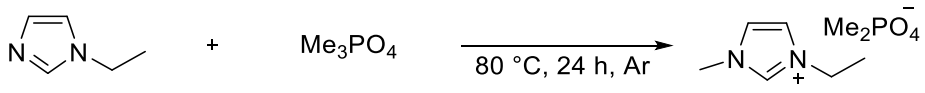
$C_6H_{12}ClN_2O$
163.63
3

Chloroethanol (10.80 g, 0.13 mol, 1.1 eq.) was added dropwise under stirring to freshly distilled 1-methylimidazole (10.02 g, 0.12 mol). The mixture was stirred at 80 °C for 96 h. The white solid was washed with EtOAc and with diethylether. Drying *in vacuo* ($1 \cdot 10^{-2}$ mbar) at 80 °C for 24 h yielded compound **3** as colorless solid in 99% (19.8 g).

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H = 2.83 (q, *J* = 5.09, 2H, -CH_2-C*H*$_2$-OH), 2.98 (s, 3H, -CH_3), 3.34 (t, *J* = 5.08, 2H, -C*H*$_2$-CH_2-OH), 4.47 (s, 1H, OH), 6.85 (m, 2H, H-4, H-5), 8.31 (s, 1H, H-2).

Analytical data was in accordance with literature.[352]

7.2.1.4 1-Ethyl-3-methylimidazolium dimethylphosphate [C_2mim]Me_2PO_4 (4)



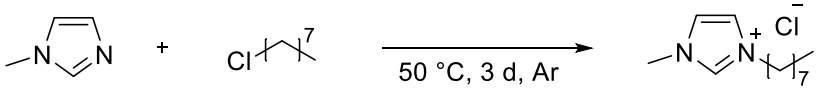
$C_8H_{17}N_2O_4P$
236.21
4

Trimethylphosphate (39 ml, 0.33 mol, 1.05 eq.) was added dropwise to 1-ethylimidazol (30.01 g, 0.32 mol) under argon atmosphere. The mixture was heated to 80 °C, stirred for 24 h and washed with EtOAc (3 × 50 ml). The solvent was evaporated to dryness and the residue was dried *in vacuo* ($1 \cdot 10^{-2}$ mbar) at 80 °C for 24 h. A yellow oil was obtained in 99% (75.1 g), which started to crystallize.[353]

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H = 1.52 (t, *J* = 7.33, 3H, N-CH_2-C*H*$_3$), 3.53 (d, *J* = 10.56, 6H, P(OCH_3)$_2$), 4.00 (s, 3H, NCH_3), 4.30 (q, *J* = 7.37, 2H, N-C*H*$_2$-CH_3), 7.38 (m, 2H, H-4, H-5), 10.50 (s, 1H, H-2).

Analytical data was in accordance with literature.[353]

7.2.1.5 1-Methyl-3-octylimidazolium chloride [C_8mim]Cl (50)



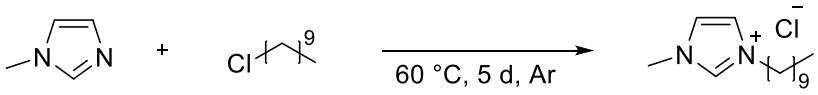
$C_{12}H_{23}ClN_2$
230.78
50

Freshly distilled 1-methylimidazole (30.0 g, 0.37 mol) and chlorooctane (59.7 g, 0.40 mol, 1.1 eq.) were stirred at 50 °C for 3 d under argon atmosphere. The residue was washed with diethylether, evaporated to dryness and dried *in vacuo* ($1 \cdot 10^{-2}$ mbar) overnight yielded 97% (81.8 g) of a yellowish liquid.[354]

^{1}H NMR ($CDCl_3$): *δ* = 0.78 (t, *J* = 6.36, 3H, -CH_2-C*H*$_3$), 1.17-1.24 (m, 10H, -C_5H_{10}-CH_3), 1.83 (t, *J* = 6.95, 2H, -C*H*$_2$-C_5H_{10}-CH_3), 4.05 (s, 3H, N-CH_3), 4.24 (t, *J* = 7.43, 2H, -C*H*$_2$-C_6H_{12}-CH_3), 7.39 (m, 1H, H-4), 7.60 (m, 1H, H-5), 10.52 (s, 1H, H-2)

Analytical data was in accordance with literature.[354]

7.2.1.6 1-Decyl-3-methylimidazolium chloride [C_{10}mim]Cl (51)



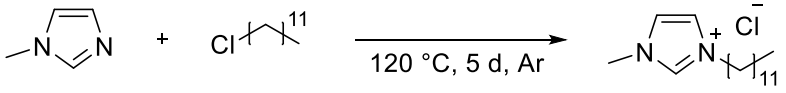
$C_{14}H_{27}ClN_2$
258.83
51

Freshly distilled 1-methylimidazole (30.0 g, 0.37 mol) and chlorodecan (70.9 g, 0.40 mol, 1.1 eq.) were stirred at 60 °C for 5 d under argon atmosphere. The residue was washed with diethylether, the solution was evaporated to dryness and dried *in vacuo* ($1 \cdot 10^{-2}$ mbar) overnight yielded 90% (85.1 g) of a yellowish liquid.[354]

^{1}H NMR ($CDCl_3$): δ = 0.79 (t, 3H, *J* = 6.36, -CH_2-C*H*$_3$), 1.18-1.26 (m, 14H, -C_7H_{14}-CH_3), 1.85 (t, *J* = 6.95, 2H, -C*H*$_2$-C_7H_{14}-CH_3), 4.05 (s, 3H, N-CH_3), 4.26 (t, *J* = 7.43, 2H, -C*H*$_2$-C_8H_{16}-CH_3), 7.40 (m, 1H, H-4), 7.60 (m, 1H, H-5), 10.52 (s, 1H, H-2)

Analytical data was in accordance with literature.[354]

7.2.1.7 1-Dodecyl-3-methylimidazolium chloride [C_{12}mim]Cl (52)



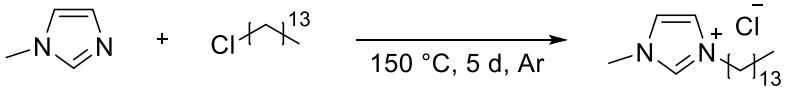
$C_{16}H_{31}ClN_2$
286.89
52

Freshly distilled 1-methylimidazole (30.0 g, 0.37 mol) and chlorododecan (82.3 g, 0.40 mol, 1.1 eq.) were stirred at 120 °C for 5 d under argon atmosphere. The residue was crystallized from THF, dried *in vacuo* ($1 \cdot 10^{-2}$ mbar) overnight and colorless crystals were obtained in 93% (97.5 g) yield.[354]

^{1}H NMR ($CDCl_3$): δ = 0.81 (t, *J* = 6.55, 3H, -CH_2-C*H*$_3$), 1.18-1.26 (m, 18H, -C_9H_{18}-CH_3), 1.84 (t, *J* = 6.95, 2H, -C*H*$_2$-C_9H_{18}-CH_3), 4.08 (s, 3H, N-CH_3), 4.24 (t, *J* = 7.43, 2H, -C*H*$_2$-$C_{10}H_{20}$-CH_3), 7.25 (m, 1H, H-4), 7.39 (m, 1H, H-5), 10.72 (s, 1H, H-2)

Analytical data was in accordance with literature.[354]

7.2.1.8 1-Methyl-3-tetradecylimidazolium chloride [C_{14}mim]Cl (53)



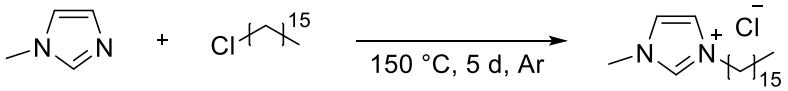
$C_{18}H_{35}ClN_2$
314.94
53

Freshly distilled 1-methylimidazole (30.0 g, 0.37 mol) and chlorotetradecan (93.5 g, 0.40 mol, 1.1 eq.) were stirred at 150 °C for 5 d under argon atmosphere. The residue was crystallized from THF, dried *in vacuo* ($1 \cdot 10^{-2}$ mbar) overnight and colorless crystals were obtained in 91% (104.7 g) yield.[354]

^{1}H NMR ($CDCl_3$): δ = 0.79 (t, *J* = 6.45, 3H, -CH_2-CH_3), 1.16 (m, 22H, -$C_{11}H_{22}$-CH_3), 1.81 (t, *J* = 6.65, 2H, -CH_2-$C_{11}H_{22}$-CH_3), 4.03 (s, 3H, N-CH_3), 4.23 (t, *J* = 7.33, 2H, -CH_2-$C_{12}H_{24}$-CH_3), 7.36 (m, 1H, H-4), 7.57 (m, 1H, H-5), 10.32 (s, 1H, H-2)

Analytical data was in accordance with literature.[354]

7.2.1.9 1-Methyl-3-hexadecylimidazolium chloride [C_{16}im]Cl (54)



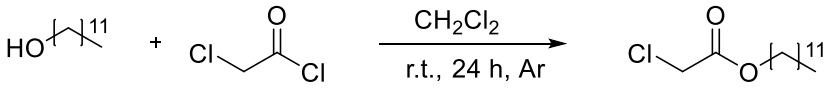
$C_{20}H_{39}ClN_2$
343.00
54

Freshly distilled 1-methylimidazole (6.1 g, 0.074 mol) and chlorohexadecan (19.2 g, 0.074 mol, 1 eq.) were stirred at 150 °C for 5 d under argon atmosphere. The residue was crystallized from THF twice, dried *in vacuo* ($1\cdot10^{-2}$ mbar) overnight and colorless crystals were obtained in 92% (23.4 g) yield.[354]

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H=0.85 (t, *J* = 6.36, 3 H, -CH_2-CH_3), 1.16-1.38 (m, 26 H, -N-CH_2-CH_2-(CH_2)$_{13}$-CH_3), 1.88 (quin, *J* = 7.92, 2H, N-CH_2-CH_2-CH_2), 4.11 (s, 3 H, -N-CH_3), 4.29 (t, *J* = 7.53, 2 H, -N-CH_2-CH_2-), 7.21 (s, 1H, H-4), 7.30 (s, 1H, H-5), 10.86 (s, 1H, H-2))

Analytical data was in accordance with literature.[354]

7.2.1.10 Dodecyl 2-chloroacetate (55)



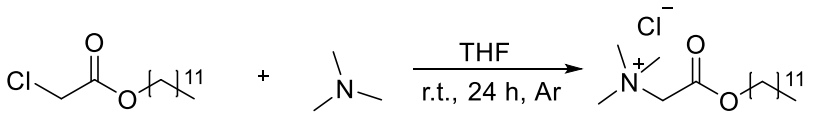
$C_{14}H_{27}ClO_2$
262.82
55

Dodecanol (14.2 g, 76.10 mmol) and chloroacetyl chloride (11.2 g, 98.90 mmol, 1.3 eq.) were dissolved in 50 ml of anhydrous dichloromethane under argon and chilled to 0 °C. Triethylamine (10.00 g, 98.90 mmol) was added dropwise. The solution was stirred at room temperature overnight until TLC indicated full conversion. The solution was diluted with 50 ml of water and extracted with dichloromethane. The combined organic layers were successively washed with 2 N HCl, saturated $NaHCO_3$ and brine. The solution was dried over Na_2SO_4, filtered and evaporated to dryness. Dodecyl 2-chloroacetate was obtained as a light yellow oil in 95 % (19.0 g) yield and used as obtained.

^{1}H NMR ($CDCl_3$): δ = 0.88 (t, *J* = 6.40, 3H, -$C_{11}H_{22}$-CH_3), 1.26 (m, 18H, -C_2H_4-C_9H_{18}-CH_3), 1.67 (t, *J* = 6.62, 2H, -CH_2-CH_2-$C_{10}H_{21}$), 4.06 (s, 2H, Cl-CH_2-CO), 4.19 (t, *J* = 7.43, 2H, -CH_2-$C_{11}H_{23}$).

Analytical data was in accordance with literature.[355]

7.2.1.11 2-(Dodecyloxy)-N,N,N-trimethyl-2-oxoethanaminium chloride [C_{12}betaine]Cl (56)



$C_{14}H_{27}ClO_2$
262.82
55

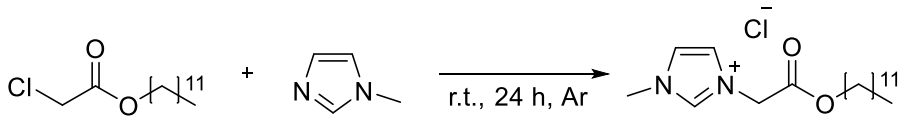
$C_{17}H_{36}ClNO_2$
321.93
56

Dodecyl 2-chloroacetate (**55**, 5.39 g, 20.51 mmol) was dissolved in 15 ml anhydrous THF. A solution of trimethylamine in THF (102.50 mmol, 5 eq.) was added dropwise at room temperature. After stirring overnight the precipitate was collected *via* filtration and washed with anhydrous THF and anhydrous diethyl ether. After drying *in vacuo* ($2 \cdot 10^{-2}$ mbar) overnight [C_{12}betaine]Cl was obtained as colorless crystals in 88 % (5.8 g) yield.

^{1}H NMR ($CDCl_3$): δ = 0.81 (t, *J* = 7.02, 3H, -$C_{11}H_{22}$-C*H*$_3$), 1.19 (m, 18H, -C_2H_4-C_9H_{18}-CH_3), 1.58 (t, *J* = 6.74, 2H, -CH_2-C*H*$_2$-$C_{10}H_{21}$), 3.60 (s, 9H, N-$(CH_3)_3$), 4.10 (t, *J* = 7.04, 2H, -C*H*$_2$-$C_{11}H_{23}$), 5.01 (s, 2H, Cl-C*H*$_2$-CO)

Analytical data was in accordance with literature.[356]

7.2.1.12 3-(2-(Dodecyloxy)-2-oxoethyl)-1-methylimidazolium chloride [C_{12}COmim]Cl (57)



$C_{14}H_{27}ClO_2$
262.82
55

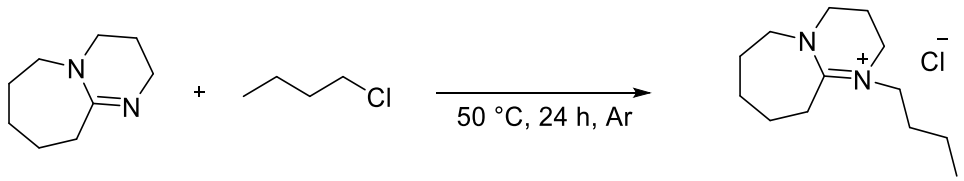
$C_{18}H_{33}ClN_2O_2$
344.92
57

1-Methylimidazol (1.19 g, 14.49 mmol) and **55** (3.81 g, 14.49 mmol) were stirred at ambient temperature for 24 h. After crystallization with THF a white solid was yielded in 81% (4.1 g).

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H=0.86 (t, *J* = 6.36, 3 H, -CH_2-C*H*$_3$), 1.18-1.36 (m, 18 H, -O-CH_2-CH_2-(C*H*$_2$)$_9$-CH_3), 1.64 (quint, *J* = 6.55, 2 H, -O-CH_2-C*H*$_2$-$(CH_2)_9$-CH_3), 4.06 (s, 3 H, N-CH_3), 4.16 (t, *J* = 6.84, 2 H, -O-C*H*$_2$-CH_2-$(CH_2)_9$-CH_3), 5.47 (s, 2 H, N-CH_2-COO-), 7.40 (s, 1H, H-4), 7.51 (s, 1H, H-5), 10.58 (s, 1 H, H-2)

Analytical data was in accordance with literature.[357]

7.2.1.13 1-Butyl-1,8-diazabicyclo[5.4.0]undec-7-enium chloride [C_4DBU]Cl (11)



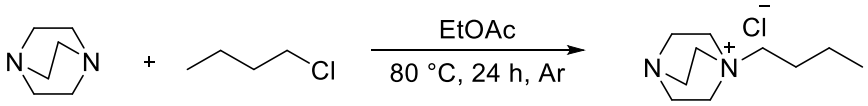
$C_{13}H_{25}ClN_2$
244.81
11

1,8-Diazabicyclo[5.4.0]undec-7-en (6.63 g, 43.55 mmol) and chlorobutane (4.43 g, 47.90 mmol, 1.1 eq.), were heated under argon at 50 °C for 24h. The mixture was washed with EtOAc, and dried *in vacuo* ($1 \cdot 10^{-2}$ mbar). 9.244 g (87 %) of an orange oil, which started to crystallize was yielded.[358]

^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.89 (t, *J* = 7.14, 3H), 1.31 (sext, *J* = 7.32, 2H), 1.56 (quint, *J* = 7.63, 2H), 1.76 (m, 8H), 2.12 (quint, *J* = 5.92, 2H), 2.89-2.93 (m, 2H), 3.45-3.76 (m, 8H)

Analytical data was in accordance with literature.[358]

7.2.1.14 1-Butyl-1,4-diazabicyclo[2,2,2]octane-1-ium-chlorid [C_4DABCO]Cl (12)



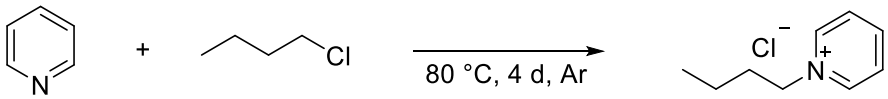
$C_{10}H_{21}ClN_2$
204.74
12

1,4-Diazabicoclyo[2,2,2]octane (10.01 g, 0.089 mol) was dried *in vacuo* for 1 h and then dissolved in 50 ml EtOAc under argon atmosphere. Chlorobutane (8.18 g, 0.096 mol, 1.1 eq.) in 50 ml EtOAc was added dropwise to the solution within 30 min. The solution was refluxed overnight and NMR showed full conversion of the starting material. After cooling to r.t. the white solid was removed *via* filtration, washed with cold Et_2O and dried *in vacuo* ($1\cdot10^{-2}$ mbar) overnight. A white solid was obtained in 48% (8.80 g) yield.

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H = 0.94 (t, *J* = 7.74, 3H, -CH_3), δ_H = 1.27 (sext, *J* = 7.36, 2H, -CH_2-CH_3), δ_H = 1.71 (sext, *J* = 6.49, 2H, -CH_2-CH_2-CH_3), δ_H = 3.23 (t, *J* = 7.44, 2H, -CH_2-CH_3), δ_H = 3.49 (t, *J* = 8.61, 2H, -N-CH_2-CH_2-CH_2-CH_3), δ_H = 3.64 (t, *J* = 7.53, 2H, -CH_2-CH_3)

Analytical data was in accordance with literature.[359]

7.2.1.15 1-Butylpyridinium chloride [C_4Pyr]Cl (13)



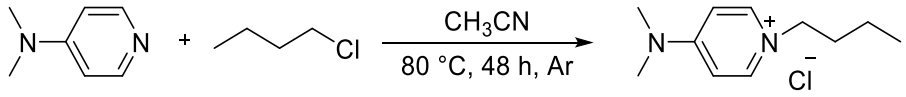
$C_9H_{14}ClN$
171.67
13

A mixture of pyridine (107.8 g, 1.36 mol) and butylchloride (151.4 g 1.64 mol, 1.2 eq.) were refluxed for 4 days. As NMR indicated no full conversion, 0.1 eq butylchloride was added and refluxed overnight. A solid precipitated and was filtered, washed with EE and Et_2O and dried *in vacuo* for 48 h. An off white solid was obtained in 82% (191 g) yield.[360]

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H = 0.89 (t, J= 7.23, 3H, -CH_3), 1.36 (sext, *J* = 7.51, 2H, -CH_2-CH_3), 1.99 (quint, *J* = 7.53, 2H,-CH_2-CH_2-CH_3), 5.00 (t, *J* = 7.33, 2H, -CH_2-CH_2- CH_2-CH_3), 8.09 (t, *J* = 7.04, 2H, H-3, H-5), 8.44 (t, *J* = 7.83, 1H, H-4), 9.67 (d, *J* = 5.48, 2H, H-2, H-6)

Analytical data was in accordance with literature.[360]

7.2.1.16 1-Butyl-4(dimethylamino)pyridin-1-ium-chlorid [C4DMAP]Cl (14)



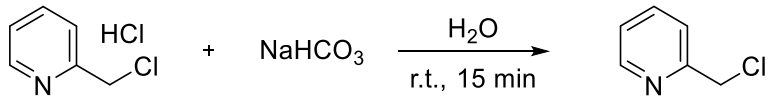
$C_{11}H_{19}ClN_2$
214.74
14

4-Dimethylaminopyridine (3.01 g, 0.025 mol) were dissolved in 10 ml anhydrous ACN. Chlorobutane (3.83 g, 0.042 mol, 1.6 eq.) was added and the solution was stirred at 80 °C for 48 h under argon. The white solid was removed *via* filtration, washed with ACN and dried *in vacuo* ($1 \cdot 10^{-2}$ mbar) overnight. A white solid was obtained in 67% (3.64 g) yield.

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H = 0.80 (t, *J* = 7.24, 3H, -CH_3), δ_H = 1.22 (sext, *J* = 7.47, 2H, -C*H*$_2$-CH_3), δ_H = 1.74 (sext, *J* = 6.38, 2H, -C*H*$_2$-CH_2-CH_3), δ_H = 3.14 (s, 3H, CH_3), δ_H = 4.21 (t, *J* = 7.28, 2H, -N-C*H*$_2$-CH_2-CH_2-CH_3), δ_H = 6.93 (d, *J* = 7.83, 2H, -C*H*$_2$-CH_3), δ_H = 8.43 (d, *J* = 7.83, 2H, -C*H*$_2$-CH_3)

Analytical data was in accordance with literature.[361]

7.2.1.17 2-(Chloromethyl)pyridine (19)

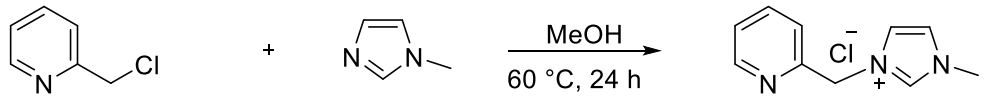


C_6H_6ClN
127.57
19

2-Picolylchloride, hydrochloride (7.49 g, 45.66 mmol) was dissolved in water, $NaHCO_3$ (5.75 g, 68.49 mmol, 1.5 eq.) was added and the solution stirred for 15 min at r.t. The solution was extracted with DCM three times, the combined organic layers washed with brine and dried over Na_2SO_4, filtered and evaporated to dryness. A red oil was obtained in 87 % yield. (5.07 g)[362]

The unstable compound **19** was directly used in a next step.

7.2.1.18 1-Methyl-3-(pyridin-2-ylmethyl)-1H-imidazol-3-ium chloride [C1PyrImd]Cl (20)



19

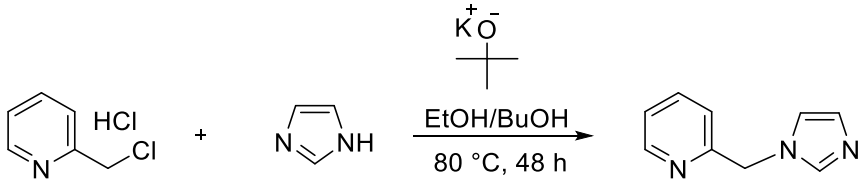
$C_{10}H_{12}ClN_3$
209.68
20

Freshly prepared **19** (5.07 g, 39.70 mmol, 1.1 eq.) was dissolved in anhydrous 30 ml methanol under argon atmosphere. 1-Methylimdazole (2.96 g, 36.09 mmol) was added dropwise. The solution was stirred at 60 °C overnight. The dark slurry was crystallized from EtOAc to yield slightly brown crystals in 98% yield. (7.39 g)

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H = 3.93 (s, 3H, -CH_3), 5.63 (s, 2H, N-C*H*$_2$-Pyr), 7.11-7.18 (m, 2H, Pyr-3, Pyr-5), 7.49 (s, 1H), 7.56-7.67 (m, 2H), 10.49 (s, 1H, N-H-N)

Analytical data was in accordance with literature values.[363]

7.2.1.19 2-((1H-imidazol-1-yl)methyl)pyridine (22)



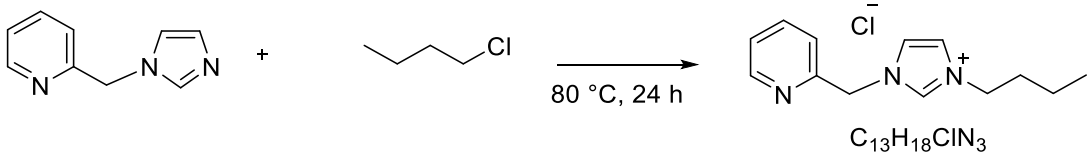
$C_9H_9N_3$
159.19
22

In a dried flask imidazole (3.42 g, 50.19 mmol) was dissolved in 20 ml butanol under argon atmosphere. Potassium-*tert*-butoxid (11.2 g, 100.38 mmol, 2 eq.) was added and diluted with 30 ml butanol at 80 °C. 2-Picolylchloride, hydrochloride (8.23 g, 1 eq.) was dissolved in 40 ml anhydrous ethanol and added dropwise. The solution was refluxed for 48 h. The brown residue was purified using column chromatography (75 g. SiO_2, $CHCl_3$ (+ NEt_3):MeOH = 20:1-15:1-10:1. A brown oil was obtained in 64% yield. (5.11 g)

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H = 5.25 (s, 2H, N-CH_2-Pyr), 6.92-6.99 (m 2H), 7.11 (s, 1H), 7.20-7.22 (m, 1H), 7.60-7.69 (m, 2H), 8.58 (d, *J* = 4.89, 1H, N-H-N)

Analytical data was in accordance with literature values. [364]

7.2.1.20 1-Butyl-3-(pyridin-2-ylmethyl)- imidazolium chloride [C_4PyrImd]Cl (23)



22

$C_{13}H_{18}ClN_3$
251.76
23

To **22** (1.92 g, 12.00 mmol) butylchloride was added (1.20 g, 13.22 mmol, 1.1 eq.) and the solution refluxed overnight. The solution was washed with EtOAc to obtain **23** (2.55 g, 84%) as a brown viscous oil.

^{1}H-NMR: (400 MHz, d_4-MeOD): δ_H = 1.01 (t, *J* = 7.38, 3H, -CH_3), 1.41 (sext, *J* = 7.47, 2H,C*H*$_2$-CH_3), 1.92 (quin, *J* = 7.46, 2H, C*H*$_2$-CH_2-CH_3), 4.29 (t, *J* = 7.28, 2H, N- C*H*$_2$-C_3H_7), 4.88 (s, 2H, N-CH_2-Pyr), 7.43 (dd, J_1 = 5.07, J_2 = 7.02, 1H, pyr-5), 7.58 (d, *J* = 7.76, 1H, Pyr-3), 7.71 (d, *J* = 6.20, 2H, Imd-4, Imd-5), 7.68 (ddd, J_1 = 1.64, J_2 = 3.85, 1H), 8.57 (d, *J* = 4.68, 1H, Pyr-6), 9.24 (s, 1H, N-H-N)

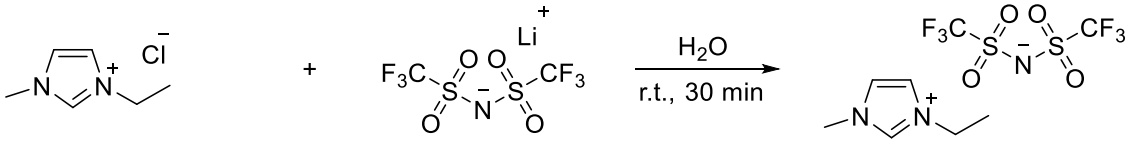
^{13}C-NMR (100 MHz, d_4-MeOD): δ_C = 12.3, 19.0, 31.7, 49.3, 53.4, 122.4, 122.8, 123.0, 124.0, 136.8, 137.8, 149.6, 152.9

ν^{max}/cm^{-1}: 3380, 3136, 3065, 2960, 2874, 1669, 1595, 1563, 1439, 1361, 1160, 1099, 1052, 1033, 997, 840, 755, 647, 625, 587, 563, 555, 538, 523, 513, 507

HR-ESI-TOF-MS *m/z*: calcd. 216.1495 for $C_{13}H_{18}N_3^+$, found 216.1489; calcd. 73.9320 $[Cl+K]^+$, found 73.9322

7.2.2 Synthesis of ionic liquids *via* metathesis

7.2.2.1 1-Ethyl-3-methylimidazolium bis(trifluoromethane)sulfonimide [C_2mim]N(Tf)$_2$ (6)



5 **6**

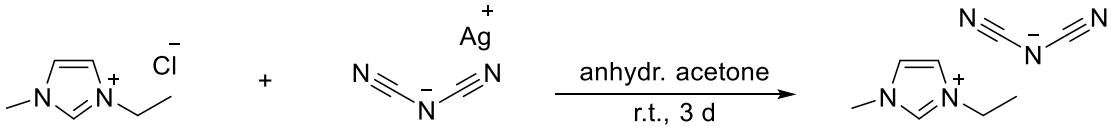
$C_8H_{11}F_6N_3O_4S_2$
391.31

To a solution of **5** (5.00 g, 34.11 mmol) in water lithium bis(trifluoromethane)sulfonimide (LiN(Tf)$_2$) (10.76 g, 37.52 mmol, 1.1 eq.) was added. The reaction mixture was stirred vigorously for 30 min at room temperature. The formation of a second phase was observed. The mixture was extracted 3 times with DCM and the combined organic layers were subsequently washed with water until no chloride could be detected in the organic phase (tested with $AgNO_3$ solution). The organic phase was dried over Na_2SO_4, filtered and the solvent was evaporated. After drying *in vacuo*, a colorless liquid was obtained in 98 % (13. 1 g) yield.[365]

^{1}H NMR (200 MHz, d_6-DMSO): δ= 1. (t, *J* = 7.24, 3H, -CH_2-C*H*$_3$), 3.82 (s, 3H, -N-CH_3), 4.18 (q, *J* = 7.24, 2H, -N-CH_2), 7.62 (s, 1H, H-4), 7.75 (s, 1H, H-5),9.10 (s, 1H, H-2).

Analytical data was in accordance in with literature.[366]

7.2.2.2 1-Ethyl-3-methylimidazolium dicyanamide [C_2mim]DCA (7)



5 **7**

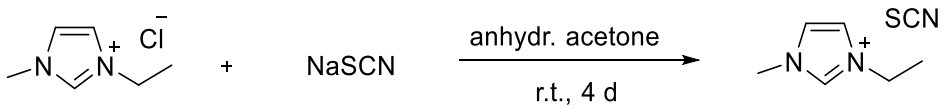
$C_8H_{11}N_5$
177.21

In a 100 ml round bottom flask [C_2mim]Cl (**5**, 3.26 g, 22.23 mmol) was dissolved in 35 ml anhydr. MeOH. To the solution AgN(CN)$_2$ (4.25 g, 24.44 mmol, 1.1 eq., available in the lab) was added and the flask was covered with aluminium foil. The solution was stirred 3 days at room temperature under argon atmosphere. The turbid solution was filtered over a batch of silica, washed with methanol. The solvent was evaporated and the ionic liquid dried *in vacuo* overnight. The product was yielded in 93 % (3.7 g) as a yellow liquid. [367]

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H = 1.56 (t, *J* = 7.43, 3H, -CH_2-C*H*$_3$), 3.97 (s, 3H, NCH_3), 4.26 (q, *J* = 7.48, 2H, -C*H*$_2$-CH_3), 7.37 (m, 2H, H-4, H-5), 9.10 (s, 1H, H-2).

Analytical data was in accordance with literature. [367]

7.2.2.3 1-Ethyl-3-methylimidazolium thiocyanate [C_2mim]SCN (8)



$C_7H_{11}N_3S$
169.25

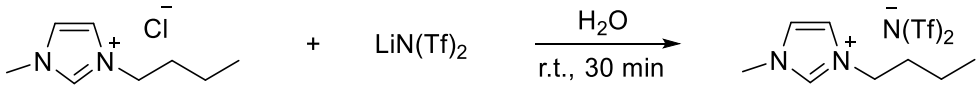
5 **8**

[C_2mim]Cl (**5**) was dried *in vacuo* at 110 °C overnight prior to use. NaSCN was dried *in vacuo* for 3 h prior to use. To **5** (3.31 g, 22.59 mmol) NaSCN (2.02 g, 24.85 mmol, 1.1 eq.) was added and dried *in vacuo* for 2 h. Anhydrous acetone (10 ml) were added and the reaction was stirred at room temperature under argon atmosphere for 4 days. The turbide solution was filtered over a batch of silica and the solvent was evaporated. The obtained producted was dissolved in anhydrous DCM, dried with Na_2SO_4 and filtered over a batch of silica. A yellow liquid was obtained in 99% (3.79 g) yield.[368]

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H = 1.42 (t, *J* = 7.34, 3H, -CH_2-C*H_3*), 3.94 (3H, s, NCH_3), 4.18 (q, *J* = 7.30, 2H,-C*H_2*-CH_3), 7.60-7.76 (m, 2H, H-4, H-5), 9.09 (s, 1H, H-2).

Analytical data were in accordance with literature values.[369]

7.2.2.4 1-Butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide [C_4mim]N(Tf)$_2$ (9)



$C_{10}H_{15}F_6N_3O_4S_2$
419.36

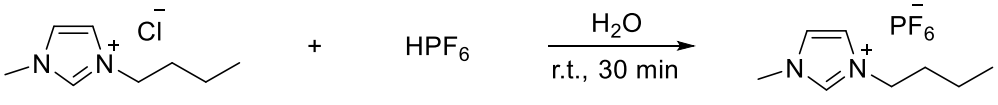
2 **9**

According to procedure 7.2.2.1 compound **9** was prepared from **2** (11.98 g, 68.59 mol) and LiN(Tf)$_2$ (16.5 g, 75.45 mmol, 1.1 eq.) to yield a colorless liquid in 98% (28.2 g).[365]

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H = 0.89 (t, *J* = 7.24, 3H, -CH_2-C*H_3*), 1.29 (sext, *J* = 7.43, 2H, -C*H_2*-CH_3), 1.78 (quint, *J* = 7.53, 2H, -C*H_2*-CH_2-CH_3), 3.87 (s, 3H, N-CH_3), 4.10 (t, 2H, -C*H_2*-CH_2-CH_2-CH_3), 7.29 (m, 2H, H-4, H-5), 8.75 (s, 1H, H-2).

Analytical data was in accordance in with literature.[365]

7.2.2.5 1-Butyl-3-methylimidazolium hexafluorophosphate [C_4mim]PF_6 (10)



$C_8H_{15}F_6N_2P$
284.19

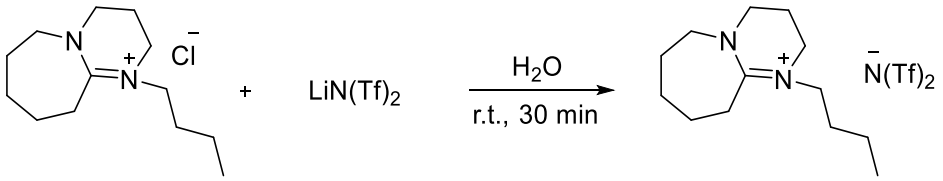
2 **10**

[C_4mim]Cl (**2**, 17.5 g, 0.10 mol) and 17.5 ml (60%, 0.12 mol, 1.1 eq.) hexafluorophosphoric acid 200 ml water at room temperature for 30 min. The formation of a second phase was observed. The mixture was extracted 3 times with DCM, washed twice with water (pH was checked), dried over Na_2SO_4, and the solvent was evaporated. After drying *in vacuo* overnight an orange liquid was obtained in 57% yield. (16.2 g) [365]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H = 0.90 (t, *J* = 7.21, 3H, N-$(CH_2)_3$-C*H*$_3$), 1.28 (sext, *J* = 7.54, 2H, N-CH_2-CH_2-C*H*$_2$-CH_3), 1.77 (quin, 2H, *J* = 7.22, N-CH_2-C*H*$_2$-CH_2-CH_3), 3.84 (s,3H, N-CH_3), 4.16 (t, *J* = 7.23, 2H, N-C*H*$_2$-CH_2-CH_2-CH_3), 7.70 (d, *J* = 14.6, 2H, H-4,H-5), 9.07 (s, 1H, H-2)

Analytical data was in accordance in with literature.[365]

7.2.2.6 1-Butyl-1,8-diazabicyclo[5.4.0]undec-7-enium bis(trifluoromethane)sulfonimide [C_4DBU]N(Tf)$_2$ (15)



$C_{15}H_{25}F_6N_3O_4S_2$
489.49

11 **15**

According to procedure 7.2.2.1 compound **15** was prepared from **11** (4.41 g, 18.01 mmol) and LiN(Tf)$_2$ (5.71 g, 19.82 mmol, 1.1 eq.) to yield the product as yellowish oil in 96 % (8.46 g).

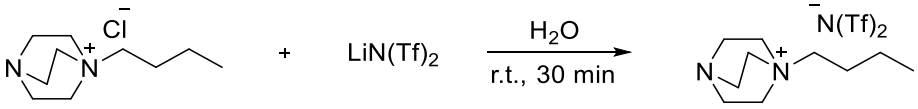
^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.96 (t, *J* = 7.27, 3H,), 1.35 (sext, *J* = 7.1, 2H,), 1.60 (quint, *J* = 5.97, 2H), 1.73-1.81 (m, 6H), 2.11 (quint, *J* = 5.95, 2H), 2.79-2.82 (m, 2H), 3.43-3.64 (m, 6H), 3.62-3.64 (m, 2H)

^{13}C-NMR (100 MHz, $CDCl_3$): δ_C = 13.6, 19.7, 19.9, 23.1, 25.9, 28.3, 28.5, 30.6, 47.1, 49.1, 53.9, 55.3, 119.9 (q, *J* = 321.4), 166.5

ν^{max}/cm^{-1}: 2938, 2876, 1651, 1619, 1529, 1449, 1349, 1328, 1226, 1175, 1134, 1053, 984, 915, 845, 788, 762, 739, 653, 614, 600, 569, 538, 512, 502

HR-ESI-TOF-MS *m/z*: calcd. 209.2012 for $C_{13}H_{25}N_2^+$, found 209.2005; calcd. 279.9167 for $C_2F_6NO_4S_2^-$, found 279.9179

7.2.2.7 1-Butyl-1,4-diazabicyclo[2,2,2]octanium bis(trifluoromethane)sulfonimide [C_4DABCO]N(Tf)$_2$ (16)



$C_{12}H_{21}F_6N_3O_4S_2$
449.43

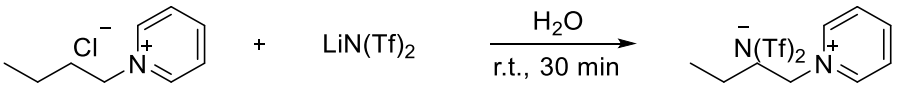
12 **16**

According to procedure 7.2.2.1 compound **16** was prepared from **12** (2.00 g, 9.77 mmol) and LiN(Tf)$_2$ (3.10 g, 10.75 mmol, 1.1 eq.) to yield the product as white solid in 99% (4.43 g).

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H = 0.94 (t, *J* = 7.30, 3H, -CH_3), 1.31 (sext, *J* = 7.27, 2H, -C*H*$_2$-CH_3), 1.64 quin, *J* = 7.85, (2H, -C*H*$_2$-CH_2-CH_3), 3.03 (t, *J* = 6.98, 2H), 3.17 (2H, t, *J* = 7.46, -C*H*$_2$-CH_2-CH_2, CH_3), 3.26 (2H, t, *J* = 7.41)

Analytical data was in accordance in with literature.[370]

7.2.2.8 1-Butylpyridinium bis(trifluoromethane)sulfonimide [C_4Pyr]N(Tf)$_2$ (17)



$C_{11}H_{14}F_6N_2O_4S_2$
416.35

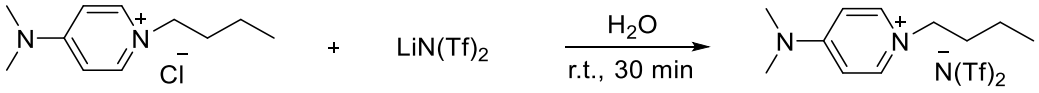
13 **17**

According to procedure 7.2.2.1 compound **17** was prepared from **13** (3.01 g, 17.53 mmol) and LiN(Tf)$_2$ (5.54 g, 19.29 mol, 1.1 eq.) to yield the product as colorless liquid in 94% (6.86 g).

^{1}H NMR (200 MHz, d_6-DMSO) 0.92 (t, *J* = 7.22, 3H, -CH_3), 1.30 (sext, *J* = 7.47, 2H, -C*H*$_2$-CH_3), 1.91 (quin, *J* = 7.53, 2H,-C*H*$_2$-CH_2, CH_3), 4.61 (t, *J* = 7.46, 2H, N-C*H*$_2$-), 8.16 (t, *J* = 7.06, 2H, H-3, H-5), 8.61 (t, *J* = 7.80, 1H, H-4), 9.09 (d, *J* = 5.44, 2H, H-2, H-6)

Analytical data was in accordance in with literature.[371]

7.2.2.9 1-Butyl-4-(dimethylamino)pyridinium bis(trifluoromethane)sulfonimide [C_4DMAP]N(Tf)$_2$ (18)



$C_{13}H_{19}F_6N_3O_4S_2$
459.42

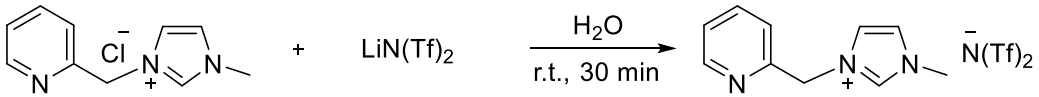
14 **18**

According to procedure 7.2.2.1 compound **18** was prepared from **14** (1.55 g, 7.12 mmol) and LiN(Tf)$_2$ (2.29 g, 7.83 mmol) to yield the product as yellowish liquid in 96% (3.14 g).

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H = 0.94 (t, *J* = 7.24, 3H,CH_2-C*H*$_3$), δ_H = 1.33 (sext, *J* = 7.43, 2H, -C*H*$_2$-CH_3), δ_H = 1.81 (sext, *J* = 6.34, 2H, -C*H*$_2$-CH_2-CH_3), δ_H = 3.22 (s, 6H,N-CH_3), δ_H = 4.10 (t, *J* = 7.33, 2H, -N-C*H*$_2$-CH_2-CH_2-CH_3), δ_H = 6.85 (d, *J* = 7.63, H-3, H-5), δ_H = 7.96 (d, *J* = 7.63, H-2, H-6)

Analytical data was in accordance in with literature.[372]

7.2.2.10 1-Methyl-3-(pyridine-2-ylmethyl) imidazolium bis(trifluoromethane)sulfonimide [C_1Pyrlmd]N(Tf)$_2$ (21)



$C_{12}H_{12}F_6N_4O_4S_2$
454.36

20 **21**

According to procedure 7.2.2.1 compound **21** was prepared from **20** (2.02g, 9.63 mmol) and LiN(Tf)$_2$ (3.07 g, 10.56 mmol) to yield the product as brown liquid in 95% (4.16 g).

^{1}H-NMR (400 MHz, d_6-DMSO): δ_H = 3.90 (s, 3H), 5.57 (s, 2H), 7.41 (dd, J_1= 4.88, J_2 = 6.83, 1H), 7.49 (d, *J* = 7.80, 1H), 7.72 (t, *J* = 1.68, 1H), 7.78(t, *J* = 1.90, 1H), 7.86 (ddd, J_1=1.74, J_2=3.85), 8.56 (s, 1H), 8.57 (s, 1H), 9.23 (s, 1H)

^{13}C-NMR (100 MHz, d_6-DMSO): δ_C = 36.3, 53.5, 119.9 (q, *J* = 321.9), 122.9, 123.6, 124.1, 137.8, 137.9, 150.0, 154.1

ν^{max}/cm^{-1}: 3158, 1597, 1575, 1479, 1441, 1347, 1329, 1178, 1132, 1051, 998, 830, 789, 752, 740, 720, 654, 612, 599, 569, 533, 514, 507

HR-ESI-TOF-MS *m/z*: calcd. 174.1026 for $C_{10}H_{12}N_3^+$, found 174.1021; calcd. 279.9167 for $C_2F_6NO_4S_2^-$, found 279.9178

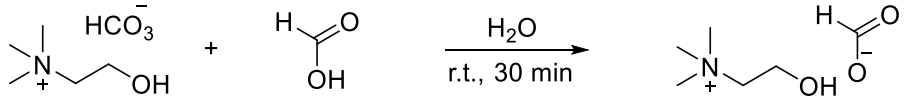
7.2.3 Synthesis of ionic liquids *via* acid-base reaction

7.2.3.1 Synthesis of choline derivatives

7.2.3.1.1 General procedure for choline-ionic liquids *via* neutralization

The exact concentration of choline hydrogen carbonate was determined *via* titration prior to use. Ionic liquids were prepared by dropwise addition of the base to the corresponding acid (1:1) in an appropriate solvent, e.g. water or methanol. The solution was stirred at ambient temperature and pressure for 30 min to 4 h. The solvent was removed and the ionic liquid was dried *in vacuo* (0.01 mbar) overnight.

7.2.3.1.2 Choline formate [chol]fom (25)



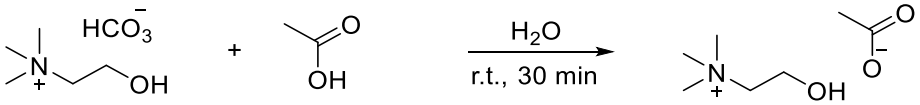
24 $C_6H_{15}NO_3$ 149.19 **25**

Synthesis was accomplished according to the general procedure using formic acid (3.03 g, 65.85 mmol), choline bicarbonate (**24**, 77% solution in water) (14.13 g, 65.85 mmol) and 5 ml water. A colorless solid was obtained in quantitative yield (9.82 g).[373]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H =3.12 (s, 9H, $N(CH_3)_3$), 3,40-3.49 (m, 2H, N-CH_2), 3.80-3.87 (m, 2H, O-CH_2), 8.52 (s, 1H, HCOO)

Analytical data was in accordance with literature.

7.2.3.1.3 Choline acetate [chol]OAc (26)



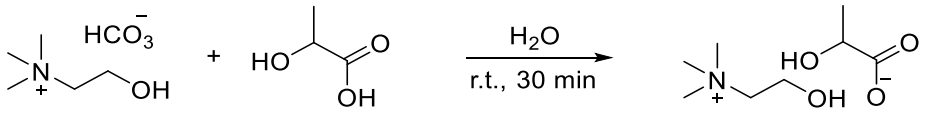
24 $C_7H_{17}NO_3$ 163.22 **26**

Synthesis was accomplished according to the general procedure using HOAc (1.00 g, 16.66 mmol), choline bicarbonate (**24**, 77% solution in water) (3.57 g, 16.66 mmol) and 3 ml water. A colorless jelly-like solid was obtained in quantitative yield (2.72 g).[373]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H = 1.58 (s, 3H, CH_3COO), 3.12 (s, 9H, $N(CH_3)_3$), 3.39-3.44 (m, 2H, N-CH_2), 3.78-3.86 (m, 2H, O-CH_2)

Analytical data was in accordance with literature.[373]

7.2.3.1.4 Choline lactate [chol]lac (27)



$C_8H_{19}NO_4$
193.24

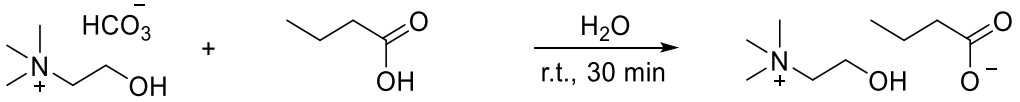
24 **27**

Synthesis was accomplished according to the general procedure using lactic acid (monomerized with water at reflux for 24 h) (0.99 g, 1.10 g 90 %, 11.04 mmol), choline bicarbonate (**24**, 77% solution in water) (2.37 g, 11.04 mmol) and 3 ml water. A colorless oil was obtained in quantitative yield (2.13 g).[373]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H = 1.05 (d, *J* = 6.65, 3H, CH-C*H*$_3$), 3.10 (s, 10H, N(CH$_3$)$_3$, OH), 3.33-3.45 (m, 3H, N-CH$_2$, C*H*-CH$_3$), 3.79-3.87 (m, 2H, O-CH$_2$)

Analytical data was in accordance with literature.[373]

7.2.3.1.5 Choline butyrate [chol]but (28)



$C_9H_{21}NO_3$
191.27

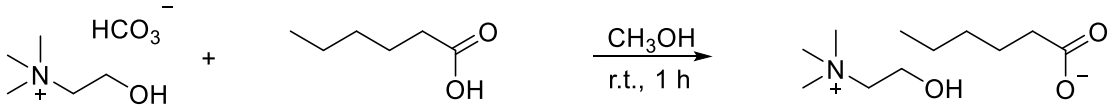
24 **28**

Synthesis was accomplished according to the general procedure using butyric acid (0.96 g, 10.90 mmol), choline bicarbonate (**24**, 77% solution in water) (2.337 g, 10.90 mmol) and 3 ml water. A colorless liquid was obtained in quantitative yield (2.08 g).[374]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H = 0.79 (t, *J* = 7.33, 3H, -CH$_2$-C*H*$_3$), 1.39 (sext, *J* =7.32, 2H, -C*H*$_2$-CH$_3$), 1.79 (t, *J* = 7.14, 2H, -C*H*$_2$-CH$_2$-CH$_3$), 3.12 (s, 9H, N(CH$_3$)$_3$), 3.40-3.48 (m, 2H, N-CH$_2$), 3.80-3.85 (m, 2H, O-CH$_2$)

Analytical data was in accordance with literature.[374]

7.2.3.1.6 Choline hexanoate [chol]hex (29)



$C_{11}H_{25}NO_3$
219.32

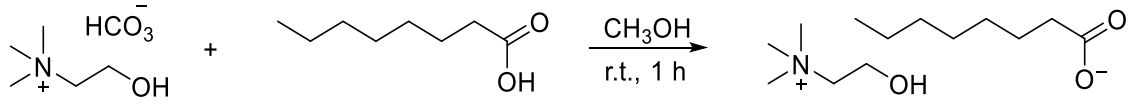
24 **29**

Synthesis was accomplished according to the general procedure using hexanoic acid (10.37 g, 89.28 mmol), choline bicarbonate (**24**, 77% solution in water) (19.154 g, 89.28 mmol) and 15 ml methanol. A colorless gel was obtained in quantitative yield (19.58 g).[374]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H = 0.83 (t, *J* = 6.65, 3H, -CH$_2$-C*H*$_3$), 1.13-1.20 (m, 4H, C*H*$_2$-C*H*$_2$-CH$_3$), 1.25-1.45 (m, 2H, COO-CH$_2$- C*H*$_2$), 1.79 (t, *J* = 7.34, 2H, COO-C*H*$_2$-), 3.11 (s, 9H, N(CH$_3$)$_3$), 3.37-3.43 (m, 2H, N-CH$_2$), 3.80-3.94 (m, 2H, O-CH$_2$)

Analytical data was in accordance with literature.[374]

7.2.3.1.7 Choline octanoate [chol]oct (30)



$C_{13}H_{29}NO_3$
247.38

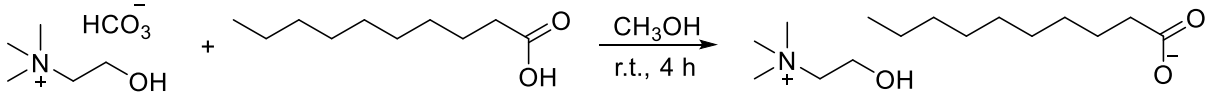
24 **30**

Synthesis was accomplished according to the general procedure using 1.59 g (10.79 mmol) octanoic acid, choline bicarbonate (**24**, 77% solution in water) (2.32 g, 10.79 mmol) and 15 ml methanol. A colorless gel was obtained in quantitative yield (2.67 g).[374]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H = 1.35 (t, *J* = 6.86, 3H, -CH_2-C*H*$_3$), 1.77-1.78 (m, 8H, -C_4H_8-CH_3), 2.04 (t, *J* = 6.65, 2H, COO-CH_2-C*H*$_2$), 2.60 (t, *J* = 7.60, 2H, COO-C*H*$_2$-), 3.67 (s, 9H, N(CH_3)$_3$), 3.93-3.95 (m, 2H, N-CH_2), 4.44-4.47 (m, 2H, O-CH_2)

Analytical data was in accordance with literature.[374]

7.2.3.1.8 Choline decanoate [chol]dec (31)



$C_{15}H_{33}NO_3$
275.43

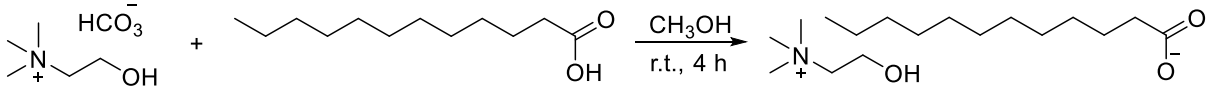
24 **31**

Synthesis was accomplished according to the general procedure using decanoic acid (4.00 g, 23.43 mmol), choline bicarbonate (**24**, 80% solution in water) (4.84 g%, 23.43 mmol) choline bicarbonate and 15 ml methanol. A colourless solid was obtained in quantitative yield (6.54 g).[374]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H =0.90 (t, *J* = 6.46, 3H, -CH_3), 1.30 (s, 12H, (C*H*$_2$)$_6$-CH_3), 1.60 (t, *J* =6.95, 2H, OOC-CH_2-C*H*$_2$-), 2.14 (t, *J* = 7.53, 2H, OOC-C*H*$_2$-CH_2-), 3.22 (s, 9H, N(CH_3)$_3$), 3.47-3.52 (m, 2H, N-CH_2), 3.96-4.04 (m, 2H, O-CH_2)

Analytical data was in accordance with literature. [374]

7.2.3.1.9 Choline dodecanoate [chol]dod (32)



$C_{17}H_{37}NO_3$
303.49

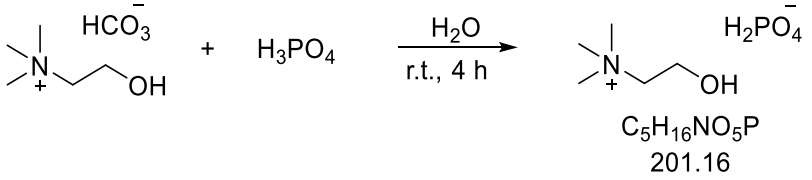
24 **32**

Synthesis was accomplished according to the general procedure using dodecanoic acid (3.94 g, 19.66 mmol), choline bicarbonate (**24**, 80% solution in water) (4.102 g, 19.66 mmol) and 15 ml methanol. A colorless solid was obtained in quantitative yield (5.97 g).[374]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H =0.92 (t, *J* = 3.72, 3H, -CH_3), 1.30 (s, 16H, (C*H*$_2$)$_8$-CH_3), 1.59 (t, *J* = 4.99, 2H, OOC-CH_2-C*H*$_2$-), 2.16 (t, *J* = 7.53, 2H, OOC-C*H*$_2$-CH_2-), 3.23 (s, 9H, N(CH_3)$_3$), 3.48-3.53 (m, 2H, N-CH_2), 3.97-4.05 (m, 2H, O-CH_2)

Analytical data was in accordance with literature.[374]

7.2.3.1.10 Choline dihydrogen phosphate [chol]dhp (33)



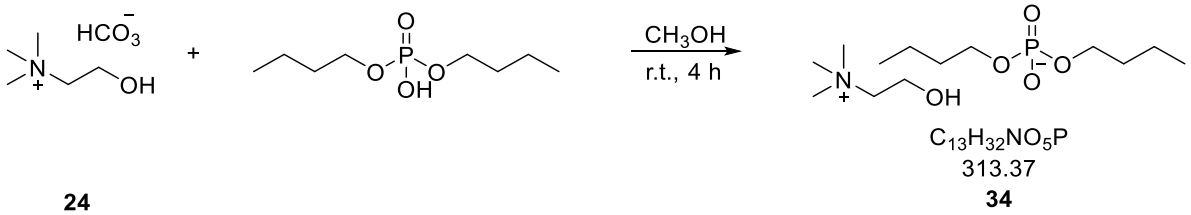
24 **33**

Synthesis was accomplished according to the general procedure using phosphoric acid (89% solution in water) (1.022 g 89%, 9.29 mmol), choline bicarbonate (**24**, 77% solution in water) (1.99 g, 9.29 mmol) and 5 ml water. A colorless solid was obtained in quantitative yield (1.87 g).[375]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H = 3.22 (s, 9H, N(CH_3)$_3$), 3.51 (m, 2H, N-CH_2), 4.01 (m, 2H, O-CH_2)

Analytical data was in accordance with literature.

7.2.3.1.11 Choline dibutyl phosphate [chol]dbp (34)



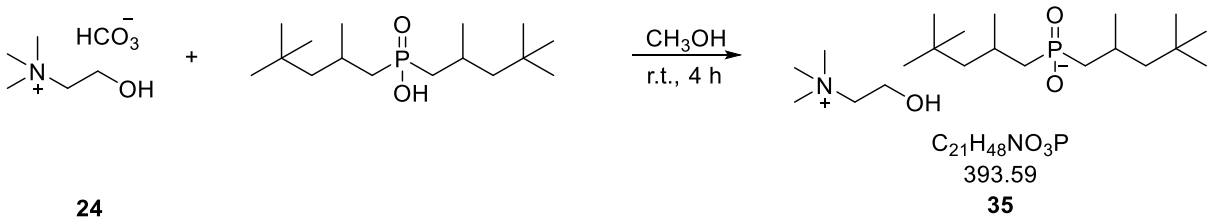
24 **34**

Synthesis was accomplished according to the general procedure using dibutylphosporic acid (1.52 g, 1.02 g 89%, 7.23 mmol) and **24** (1.50 g, 1.99 77%, 7.23 mmol) choline bicarbonate and 5 ml water. A colorless solid was obtained in quantitative yield (1.52 g).[375]

^{1}H-NMR (400 MHz, d_4-MeOD): δ_H= 0.94 (t, *J* = 7.24, 6H,-CH_3), 1.43 (sext, *J* = 7.32, 4H, -C*H*$_2$-CH_3), 1.61 (quint, *J* = 7.25, 4H, -C*H*$_2$-CH_2-CH_3), 3.23 (9H, s, N(CH_3)$_3$), 3.50 (2H, t, *J* = 4.79, N-CH_2), 3.85 (4H, quint, *J* = 6.26, P-O-CH_2), 3.99-4.04 (2H, m, O-CH_2)

Analytical data was in accordance with literature. [375]

7.2.3.1.12 Choline bis(2,4,4-trimethylpentyl) phosphinate [chol]dop (35)



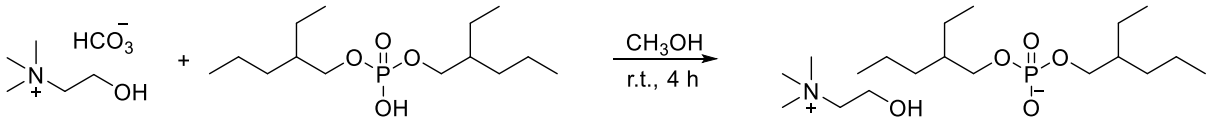
24 **35**

Synthesis was accomplished according to the general procedure using bis(2,4,4-trimethylpentyl) phosphinate (90%, 1.53 g, 4.75 mmol), choline bicarbonate (**24**, 77% solution in water) (1.02 g, 4.75 mmol) and 5 ml methanol. A colorless gel was obtained in quantitative yield (1.76 g).[375]

^{1}H-NMR (400 MHz, d_4-MeOD): δ_H= 0.95 (s, 18H, -CH-(C*H*$_3$)$_3$), 1.33-1.54 (m, 14H, -CH_2-CH_2-CH-CH_2-), 3.23 (s, 9H, N(CH_3)$_3$), 3.50 (m, 2H, N-CH_2), 3.76-3.79 (m, 4H, P-O-CH_2), 3.99-4.04 (m, 2H, O-CH_2)

Analytical data was in accordance with literature.[375]

7.2.3.1.13 Choline bis(2-ethylhexyl) phosphate [chol]bep (36)



24

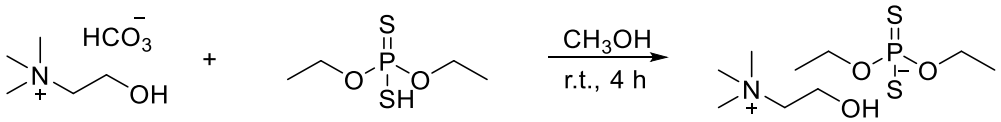
$C_{19}H_{44}NO_5P$
397.54
36

Synthesis was accomplished according to the general procedure using bis(2-ethylhexyl) phosphate (97%, 1.62 g, 4.86 mmol) and choline bicarbonate (**24**, 77% solution in water) (1.04 g, 4.86 mmol) and 5 ml methanol. A colorless viscous oil was obtained in quantitative yield (1.93 g).

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H= 0.93 (t, *J* = 7.31, 12H, -CH_3), 1.33-1.54 (m, 14 H, P-O-CH_2-C*H*-(-C*H*$_2$-CH_3)-C*H*$_2$-C*H*$_2$-CH_3), 3.23 (s, 9H, N(CH_3)$_3$), 3.49-3.52 (m, 2H, N-CH_2), 3.75-3.78 (m, 4H, P-O-CH_2), 3.99-4.03 (m, 2H, O-CH_2)

Analytical data was in accordance with literature.[375]

7.2.3.1.14 Choline O,O-diethyl dithiophosphate [chol]dtp (37)



24

$C_9H_{24}NO_3PS_2$
289.39
37

Synthesis was accomplished according to the general procedure using (1.76 g, 94.20 mmol) O,O-diethyl dithiophosphate and choline bicarbonate (**24**, 77% solution in water) (2.02 g, 94.20 mmol) choline bicarbonate and 5 ml methanol. A colorless viscous oil was obtained in quantitative yield (2.73 g).

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H =1.10, (t, *J* = 7.04, 6H, -CH_2-C*H*$_3$), 3.10 (s, 9H, N(CH_3)$_3$), 3.36-3.42 (m, 2H, N-CH_2), 3.70-3.86 (m, 6H, O-CH_2, -C*H*$_2$-CH_3)

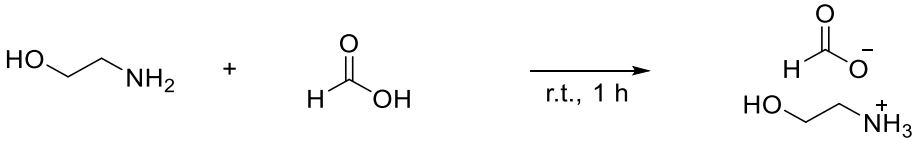
Analytical data was in accordance with literature.[375]

7.2.3.2 Synthesis of ethanolammonium derivatives

7.2.3.2.1 General procedure

Ionic liquids were prepared by dropwise addition of the base to the corresponding acid (1:1). The solution was stirred at ambient temperature and pressure for 1 h to 4 h. The ionic liquid was dried *in vacuo* (0.01 mbar) overnight.

7.2.3.2.2 *N*-(2-Hydroxyethyl) ammonium formate [N_{002OH}]fom (38)



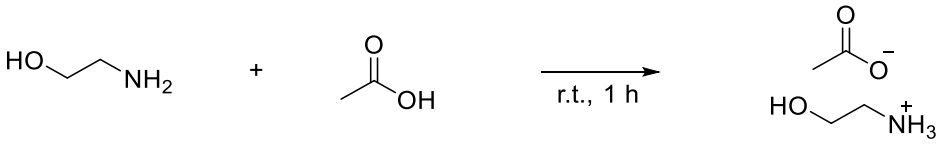
$C_3H_9NO_3$
107.11
38

Synthesis was accomplished according to the general procedure using formic acid (3.76 g, 81.70 mmol) and ethanolamine (4.99 g, 81.70 mmol). A yellowish oil was obtained in quantitative yield (8.75 g).[376]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H= 2.78 (t, *J* = 5.28, 2H, -N-CH_2), 3.53 (t, *J* = 5.38, 2H, -O-CH_2), 5.8 (broad), 8.49 (s, 1H, HCOO)

Analytical data was in accordance with literature.[376]

7.2.3.2.3 N-(2-Hydroxyethyl) ammonium acetate [N_{002OH}]OAc (39)



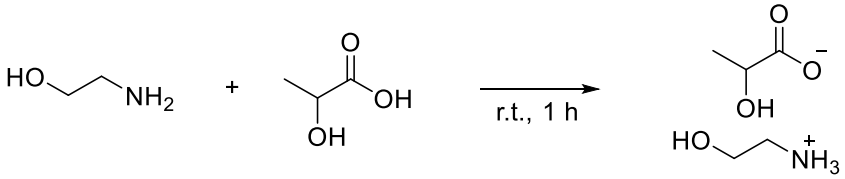
$C_4H_{11}NO_3$
121.14
39

Synthesis was accomplished according to the general procedure using HOAc (4.56 g, 74.64 mmol) and (4.48 g, 74.64 mmol) ethanolamin. A yellowish oil was obtained in quantitative yield (9.04 g).[376]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H= 1.74, (s, 3H, -CH_3), 2.69 (t, *J* = 5.38, 2H,-N-CH_2), 5.48 (t, 2H, *J* = 5.48, -O-CH_2), 6.01 (broad)

Analytical data was in accordance with literature.[376]

7.2.3.2.4 *N*-(2-Hydroxyethyl) ammonium lactate [N_{002OH}]lac (40)



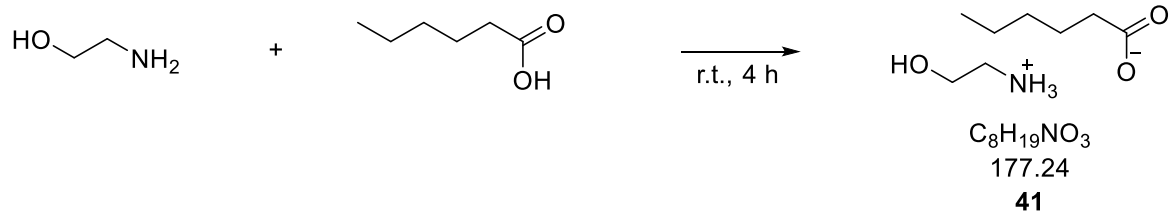
$C_5H_{13}NO_4$
151.16
40

Synthesis was accomplished according to the general procedure using (7.02 g. 114.91 mmol) ethanolamine and lactic acid (10.34 g, 114.91 mmol). A colorless oil was obtained in quantitative yield (17.35 g).[376]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H= 1.11 (d, *J* = 6.65, 3H, -CH_3), 2.78 (t, *J* = 5.27, 2H, t -N-CH_2), 3.53 (t, *J* = 5.38, 2H, -O-CH_2), 3.63 (q, *J* = 6.78, 1H, -CH), 6.08 (s, broad)

Analytical data were in accordance with literature.[376]

7.2.3.2.5 *N*-(2-Hydroxyethyl) ammonium hexanoate [N_{002OH}]hex (41)



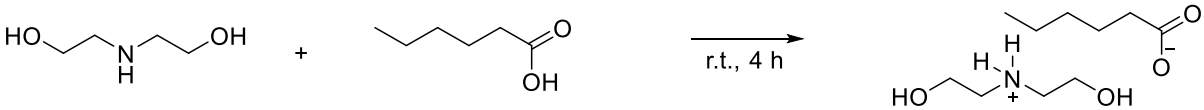
$C_8H_{19}NO_3$
177.24
41

Synthesis was accomplished according to the general procedure using ethanolamine (4.99 g, 81.70 mmol) and caproic acid (9.49 g, 81.70 mmol). A yellowish oil was obtained in quantitative yield (14.48 g).[376]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H= 0.91 (t, *J* = 6.55, 3H, -CH_3), 1.30-1.36 (m, 4H, -C*H*$_2$-C*H*$_2$-CH_3), 1.59 (quint, *J* = 7.24, 2H, COO-CH_2-C*H*$_2$), 2.16 (t, *J* = 7.63, 2H, COO-C*H*$_2$-),3.01 (t, *J* = 5.28, 2H, -N-CH_2), 3.75 (t, *J* = 5.18, 2H, -O-CH_2), 5.23 (broad)

Analytical data were in accordance with literature.[376]

7.2.3.2.6 Bis(2-hydroxyethyl)ammonium hexanoate [$N_{00(2OH)2}$]hex b-(41)



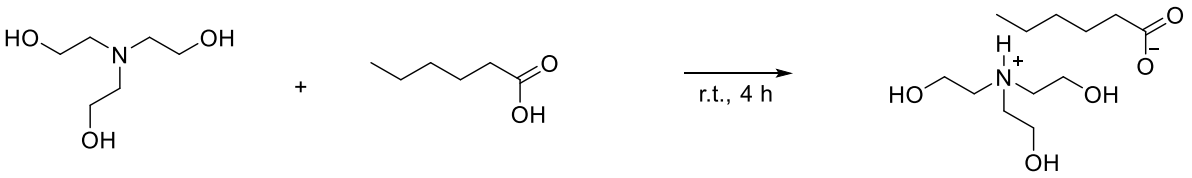
$C_{10}H_{23}NO_4$
221.30
b-**41**

Synthesis was accomplished according to the general procedure using caproic acid (5.00 g, 43.07 mmol) and (4.53 g, 43.07 mmol) diethanolamine. A yellowish oil was obtained in quantitative yield (9.53 g).[376]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H= 0.84 (t, *J* = 6.75, 3H, -CH_3), 1.21-1.27 (m, 4H, -C*H*$_2$-C*H*$_2$-CH_3), 1.46 (quint, *J* = 7.14, 2H, COO-CH_2-C*H*$_2$), 2.10 (t, *J* = 7.24, 2H, COO-C*H*$_2$-), 2.64 (m, *J* = 5.28, 4H, -N-CH_2), 3.47 (t, *J* = 5.57, 4H, -O-CH_2), 5.34 (broad)

Analytical data was in accordance with literature.[376]

7.2.3.2.7 Tris(2-hydroxyethyl)ammonium hexanoate [$N_{0(2OH)3}$]hex t-(41)



$C_{12}H_{27}NO_5$
265.35
t-**41**

Synthesis was accomplished according to the general procedure using caproic acid (4.62 g, 39.77 mmol) and triethanolamine (5.93 g, 39.77 mmol). A yellowish oil was obtained in quantitative yield (10.55 g).[376]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H= 0.84 (t, *J* = 6.65, 3H, -CH_3), 1.21-1.27 (m, 4H, -C*H*$_2$-C*H*$_2$-CH_3), 1.48 (quint, *J* = 7.09, 2H, COO-CH_2-C*H*$_2$), 2.16 (t, *J* = 7.33, 2H, COO-C*H*$_2$-), 2.54 (m, *J* = 6.16, 6H, -N-CH_2), 3.39 (t, *J* = 6.15, 6H, -O-CH_2),

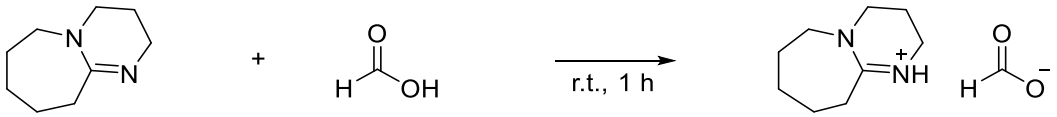
Analytical data was in accordance with literature.[376]

7.2.3.3 Synthesis of 1,8-diazabicyclo[5.4.0]undec-7-en derivatives

7.2.3.3.1 General procedure

Ionic liquids were prepared by dropwise addition of the base to the corresponding acid (1:1). The solution was stirred at ambient temperature and pressure for 30 min to 4 h. The ionic liquid was dried *in vacuo* (0.01 mbar) overnight.

7.2.3.3.2 Pyrimido[1,2-*a*]azepine, 2,3,4,6,7,8,9,10-octahydro-, formate [DBU]fom (42)



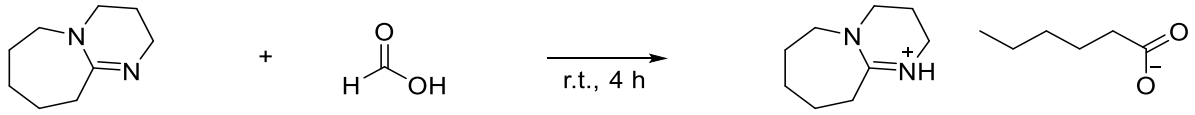
$C_{10}H_{18}N_2O_2$
198.27
42

Synthesis was accomplished according to the general procedure using formic acid (1.50 g, 32.65 mmol) and DBU (5.04 g, 32.65 mmol). A colourless solid was obtained in quantitative yield (6.47 g).[377]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H= 1.73-1.82 (m, 6H), 2.06 (quint, *J* = 5.85, 2H), 2.69-2.72 (m, 2H), 3.36 (m, 3H), 3.57 (t, *J* = 5.84, 2H), 3.62-3.64 (m, 2H), 8.58 (s, 1H)

Analytical data were in accordance with literature.[377]

7.2.3.3.3 Pyrimido[1,2-*a*]azepine, 2,3,4,6,7,8,9,10-octahydro-, hexanoate [DBU]hex (43)



$C_{15}H_{28}N_2O_2$
268.40
43

Synthesis was accomplished according to the general procedure using caproic acid (3.82 g, 32.84 mmol) and (5.00 g, 32.84 mmol) 1,8-diazabicyclo[5.4.0]undec-7-en. A colorless oil was obtained in quantitative yield (8.81 g).[378]

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H= 0.83 (t, *J* = 6.55, 3H), 1.14-1.25 (m, 4H), 1.32-1.47 (m, 2H), 1.56-1.60 (m, 6H), 1.89-1.78 (m, 4H), 2.70 (d, *J* = 9.19, 2H), 3.20 (t, *J* = 5.67, 2H), 3.37-3.48 (m, 4H)

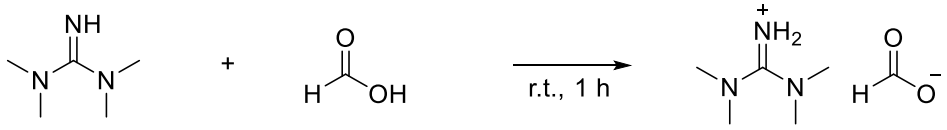
Analytical data were in accordance with literature.[378]

7.2.3.4 Synthesis of tetramethylguanidinium derivatives

7.2.3.4.1 General procedure for *N,N,N,N*-tetramethylguanidinium derivatives

Ionic liquids were prepared by dropwise addition of *N,N,N,N*-tetramethylguanidine to the corresponding acid (1:1) in an appropriate solvent or without solvent. The solution was stirred at ambient temperature and pressure for 30 min to 4 h. The solvent was evaporated and the ionic liquid dried *in vacuo* (0.01 mbar) for 3 h.[379]

7.2.3.4.2 *N,N,N,N* -tetramethylguanidinium formate [guan]fom (44)



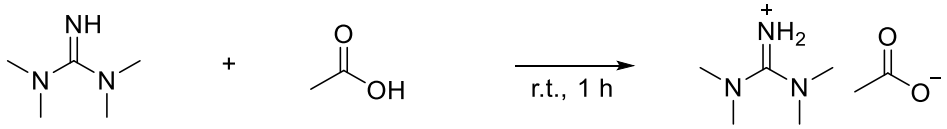
$C_6H_{15}N_3O_2$
161.20
44

Synthesis was accomplished according to the general procedure using formic acid (0.406 g, 8.69 mmol) and *N,N,N,N*-tetramethylguanidine (1.015 g, 8.69 mmol). A colorless solid was obtained in quantitative yield (1.40 g).[379]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H = 2.99 (s, 12H, N-CH_3), 8.55 (s, 1H, HCOO)

Analytical data was in accordance with literature.[379]

7.2.3.4.3 *N,N,N,N* -tetramethylguanidinium acetate [guan]OAc (45)



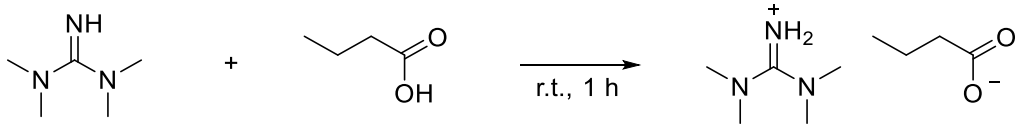
$C_7H_{17}N_3O_2$
175.23
45

Synthesis was accomplished according to the general procedure using HOAc (0.541 g, 8.99 mmol) and *N,N,N,N*-tetramethylguanidine (1.036 g, 8.99 mmol). A colorless solid was obtained in quantitative yield (1.58 g).[379]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H = 1.89 (s, 3H, -CH_3), 2.99 (s, 12H, N-CH_3),

Analytical data was in accordance with literature.[379]

7.2.3.4.4 *N,N,N,N* -tetramethylguanidinium butyrate [guan]but (46)



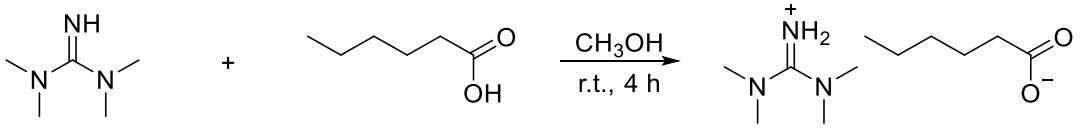
$C_9H_{21}N_3O_2$
203.29
46

Synthesis was accomplished according to the general procedure using butyric acid (0.96 g, 10.91 mmol) and *N,N,N,N*-tetramethylguanidine (1.26 g, 10.91 mmol). A colorless solid was obtained in quantitative yield (2.22 g).[379]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H = 0.94 (t, *J* = 7.34, 3H, -CH_3), 1.62 (sex, *J* = 7.39, 2H, -C*H*$_2$-CH_3), 2.14 (t, *J* = 7.43, 2H, -C*H*$_2$-CH_2-CH_3), 2.99 (s, 12H, N-CH_3)

Analytical data was in accordance with literature.[379]

7.2.3.4.5 *N,N,N,N* -tetramethylguanidinium hexanoate [guan]hex (47)



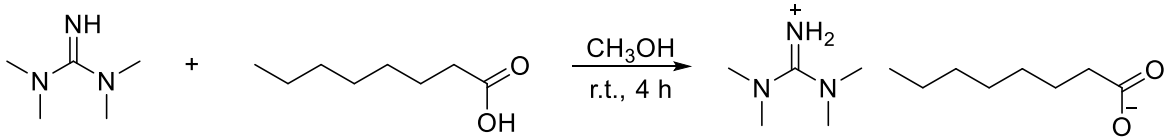
$C_{11}H_{25}N_3O_2$
231.34
47

Synthesis was accomplished according to the general procedure using hexanoic acid (1.79 g, 15.37 mmol), *N,N,N,N*-tetramethylguanidine (1.77 g, 15.37 mmol) and 5 ml methanol. A colorless solid was obtained in quantitative yield (3.55 g).[379]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H = 0.91 (t, *J* = 6.65, 3H, -CH_3), 1.32 (m, 4H, -(C*H*$_2$)$_2$-CH_3), 1.60 (m, 2H, -C*H*$_2$-(CH_2)$_2$-CH_3), 2.16 (t, *J* = 7.53, 2H, -C*H*$_2$-(CH_2)$_3$-CH_3), 2.99 (s, 12H, N-CH_3)

Analytical data was in accordance with literature.[379]

7.2.3.4.6 *N,N,N,N* -tetramethylguanidinium octanoate [guan]oct (48)



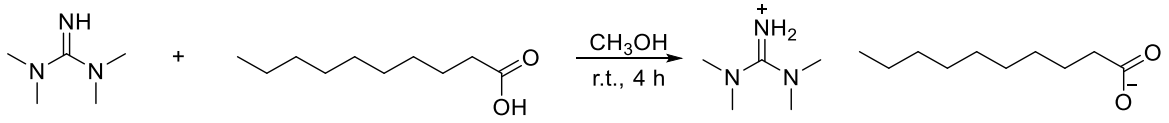
$C_{13}H_{29}N_3O_2$
259.39
48

Synthesis was accomplished according to the general procedure using octanoic acid (0.949 g, 6.58 mmol), *N,N,N,N*-tetramethylguanidine (0.758 g, 6.58 mmol) and 5 ml methanol. A colorless solid was obtained in quantitative yield (1.71 g).[379]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H = 0.90 (m, 3H, -CH_3), 1.31 (m, 8H, -(C*H*$_2$)$_4$-CH_3), 1.59 (m, 2H, -C*H*$_2$-(CH_2)$_4$-CH_3), 2.15 (t, *J* = 7.53, 2H, -C*H*$_2$-(CH_2)$_5$-CH_3), 2.99 (s, 12H, N-CH_3)

Analytical data was in accordance with literature.[380]

7.2.3.4.7 *N,N,N,N* -tetramethylguanidinium decanoate [guan]dec (49)



$C_{15}H_{33}N_3O_2$
287.45
49

Synthesis was accomplished according to the general procedure using decanoic acid (0.906 g, 5.14 mmol), *N,N,N,N*-tetramethylguanidine (0.592 g, 5.14 mmol) and 5 ml methanol. A colourless solid was obtained in quantitative yield (1.48 g).[379]

^{1}H-NMR (200 MHz, d_4-MeOD): δ_H = 0.90 (t, *J* = 6.36, 3H, -CH_3), 1.30 (m, 12H, -(C*H*$_2$)$_6$-CH_3), 1.59 (m, 2H, -C*H*$_2$-$(CH_2)_6$-CH_3), 2.15 (t, *J* = 7.53, 2H, -C*H*$_2$-$(CH_2)_7$-CH_3), 2.99 (s, 12H, N-CH_3);

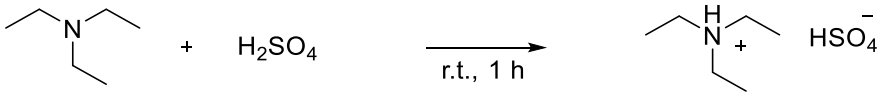
^{13}C-NMR (100 MHz, d_4-MeOD): δ_C = 14.44, 23.73, 27.72, 30.46, 30.67, 30.71, 30.85, 33.07, 39.05, 39.91, 161.90, 182.60

ν^{max}/cm^{-1}: 2921, 2852, 1549, 1455, 1434, 1407, 1388, 1308, 1238, 1103, 1066, 1035, 884, 722, 539, 518, 506

Elemental analysis: calculated: w-% C: 62.68, w-% H: 11.57, w-%N: 14.62, calculated: 0.60xH_2O: w-% C: 60.14, w-% H: 11.56, w-%N: 14.09, measured: w-% C: 60.59, w-% H: 11.28, w-%N: 13.70

7.2.4 Ammonium and phosphonium based ionic liquids

7.2.4.1 Triethylammonium hydrogen sulphate [Et_3NH]HSO_4 (74)



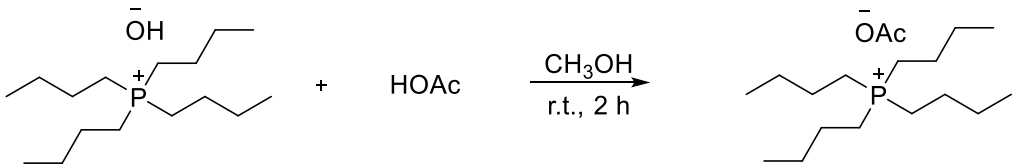
$C_6H_{17}NO_4S$
199.27
74

To triethylamine (10.00 g, 0.098 mol) sulphuric acid (10.09 g, 0.098 mol) was slowly added at 0°C. The reaction mixture was stirred at room temperature for 1 h. After drying *in vacuo* for 15 min the product was obtained in quantitative yield (19.53 g) as white crystals.[27]

^{1}H NMR (200 MHz, d_6-DMSO): δ= 1.06 (t, *J* = 11.07, 9H, N-CH_2-C*H*$_3$), 2.97 (m, 6H, N-CH_2), 4.79 (s, 1H, N-H).

Analytical data was in accordance with literature.[27]

7.2.4.2 Tetrabutylphosphonium acetate [P_{4444}]OAc (92)



$C_{18}H_{39}O_2P$
318.48
92

Tetrabutylphosphonium hydroxide (5.01 g, 18.00 mmol as a 40% solution) and HOAc (1.08 g, 18.00 mmol) were dissolved in 10 ml methanol and the reaction mixture was stirred for 2 h at room temperature. After evaporation of the solvent and drying *in vacuo* colorless crystals were obtained in quantitative yield. (5.73 g)

^{1}H NMR (200 MHz, $CDCl_3$): δ= 0.90 (t, *J* = 6.85, 12H, -CH_2-C*H*$_3$), 1.43-1.47 (m, 16H, -C*H*$_2$-C*H*$_2$-CH_3), 1.87 (s, 3H, COO-CH_3), 2.27-2.42 (m, 8H,-P-CH_2).

Analytical data was in accordance with literature.[381]

7.3 Extraction procedures for the isolation of piperine

7.3.1 Preparation of standard calibration

For aqueous solutions

A stock solution of 10 mg piperine (**58**) in 10 ml methanol (solution A) was prepared. Solution B - G were prepared by dilution. (Table 61)

Table 61: Solutions for standard calibration

Solution	[ml]	Solution	Diluted to [ml]	Piperine [mg/ml]
A	-	-	-	1
B	1	A	2	0.5
C	0.5	A	2	0.2
D	1	A	10	0.1
E	1	B	10	0.05
F	1	C	10	0.02
G	1	D	10	0.01
H	1	E	10	

A sample of 1 ml was taken from the solution and 0.2 ml of a phenol stock solution (60.0 mg phenol in 100 mL of water) were added. The samples were filtered and directly analyzed *via* HPLC according to Method A.

For organic solutions

The standard calibration of piperine for organic solvents was accomplished according to 7.3.1 using a phenol stock in methanol. (60.0 mg phenol in 100 ml methanol) The samples were filtered over 0.2 μm syringe filter and directly analyzed *via* HPLC according to Method A.

7.3.2 Extraction procedure for ionic liquid solutions

7.3.2.1 Optimization of conditions

A suspension of black pepper in aqueous 50 mM [C_{12}mim]Cl (**52**) solution (1 wt%: 10.0 mg pepper, 990 mg ionic liquid solution, 5 wt%: 50 mg pepper, 950 mg ionic liquid solution, 10 wt%: 100 mg pepper, 900 mg ionic liquid solution) was stirred at 25 °C for 3 h. A sample of 100 µl was taken from the supernatant and diluted to 5 mL with the ionic liquid solution. An aliquot of 1 mL was taken, mixed with 200 µl of internal standard (60.0 mg phenol in 100 mL of water) filtered over 0.2 μm syringe filter and immediately analyzed *via* HPLC. (Method A) Results are based on three independent experiments.

Time screening was performed using a 5 wt% black pepper in aqueous 50 mM [C_{12}mim]Cl (**52**) solution (50 mg pepper, 950 mg ionic liquid solution) at r.t. with sample taking after 1,3,6 and 24 h.

7.3.2.2 Influence of different ionic liquids

A suspension of 5 wt% black pepper in aqueous ionic liquid solution (50 mg pepper, 950 mg ionic liquid solution) was stirred at 25 °C for 3 h. A sample of 100 µl was taken from the supernatant and diluted to 5 mL with the ionic liquid solution. An aliquot of 1 mL was taken, mixed with 200 µl of internal standard (60.0 mg phenol in 100 mL of water) filtered over 0.2 µm syringe filter and immediately analysed *via* HPLC. (Method A) Results are based on three independent experiments. Screening was accomplished using following ionic liquids with following concentrations: (Table 62)

Table 62: Aqueous ionic liquids solutions tested for the extraction of piperine

Entry	[C_{10}mim]Cl (51) [mM]	[C_{12}mim]Cl (52) [mM]	[C_{14}mim]Cl (53) [mM]	[C_{12}betaine]Cl (56) [mM]
1	1	1	0.5	1
2	2	5	1	5
3	5	10	3	10
4	10	15	5	15
5	15	25	7	25
6	25	50	10	50
7	40	100	15	100
8	50	250	50	250
9	75		100	
10	100		250	
11	250			

Screening of the influence of the anion was accomplished using following ionic liquids: [C_{12}mim]Cl (**52**), [C_{12}mim]Br (**59**), [C_{12}mim]OTf (**60**), [C_{12}mim]N$(CN)_2$ (**61**) and [C_{12}mim]OAc (**62**).

7.3.3 Extraction procedure using pure ionic liquids

A suspension of 5 wt% black pepper in pure ionic liquid solution (50 mg pepper, 950 mg ionic liquid) was stirred at 80 °C for 3 h. The mixture was diluted to 10 ml with methanol. An aliquot of 1 mL was taken, mixed with 200 µl of internal standard (60.0 mg phenol in 100 mL of water) filtered over 0.2 µm syringe filter and immediately analysed *via* HPLC. (Method A) Results are based on three independent experiments.

The screening was performed using [C_{10}mim]Cl (**51**), [C_{12}mim]Cl (**52**) and [C_{14}mim]Cl (**53**).

7.3.4 Extraction procedure for conventional solvents

A suspension of black pepper in solvent (50 mg pepper, 950 mg solvent) was stirred at 25 °C for 3 h. A sample of 100 µl was taken from the supernatant and diluted to 5 mL with the solvent. An aliquot of 1 mL was taken, mixed with 200 µl of internal standard (60.0 mg phenol in 100 mL of water or methanol) filtered over 0.2 µm syringe filter and immediately analysed *via* HPLC. (Method A) Results are based on three independent experiments.

Screening was accomplished using water, 50 mM aqueous NaCl solution, BuOAc, methanol, toluene, DCM and chloroform.

7.3.5 Scaled procedure for the isolation of piperine

Ground black pepper (1.000 g) was stirred with 19 mL of a 50 mM solution of [C_{12}betaine]Cl (**56**) in water at 25 °C for 3 h. After filtration the solution was extracted 3 times with a total volume of 5 mL of BuOAc. Samples of 100 µl were taken from the aqueous layer, and HPLC was measured before and after extraction with BuOAc to quantify the amount of piperine in the micellar solution. (Method A) The remaining micellar solution was directly used for the next extraction step without further purification.

For isolation of piperine, the combined organic layers were dried over Na_2SO_4, filtered and evaporated to dryness. Results are based on two independent experiments.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.59-1.63 (m, 6H, N-CH_2-C*H*$_2$-C*H*$_2$-C*H*$_2$-CH_2-), 3.54-3.64 (m, 4H, N-C*H*$_2$-CH_2-CH_2-CH_2-C*H*$_2$-), 5.98 (s, 2H, O-CH_2-O), 6.43 (d, 1H, *J* = 15.1, N-CO-CH), 6.72-6.99 (m, 5H,Ph-*H*, Ph-C*H*-C*H*), 7.35-7.54 (1H, m, N-CO-CH-C*H*)

Analytical data was in accordance with literature.[382]

7.4 Towards the synthesis of isoeugenol (63)

7.4.1 Standard calibrations

Standard calibration for eugenol using HPLC

The standard calibration of eugenol (**63**) was accomplished according to 7.3.1 using a phenol stock in methanol. (66.7 mg phenol in 100 ml water) The samples were directly analyzed *via* HPLC according to Method B.

*Standard calibration for eugenol (**63**) and isoeugenol (**64**) using GC*

A stock solution of 10 mg eugenol (**63**) in 10 ml EtOAc containing the internal standard methylbenzoat (49.7 mg methylbenzoat/100 ml EtOAc, solution A) was prepared. Solution B - G were prepared by dilution. (Table 63)

Table 63: Solutions for standard calibration

Solution	[ml]	Solution	Diluted to [ml]	Eugenol (**63**) [mg/ml]
A	-	-	-	1
B	1	A	2	0.5
C	0.5	A	2	0.2
D	1	A	10	0.1
E	1	B	10	0.05
F	1	C	10	0.02
G	1	D	10	0.01
H	1	E	10	

1 ml of each solution was taken and directly analyzed *via* GC. (*Method J*)

7.4.2 Extraction procedure of eugenol (63) from cloves

7.4.2.1 Optimization of conditions

In a 1.5 ml Eppendorf-vial 50 ± 1 mg ground cloves 950 µl aqueous [C_{12}mim]Cl (**52**) was added. The suspension was stirred for 1/3/5/24 h at r.t. The sample was centrifuged at 13000 rpm for 5 min and 100 µl of the supernatant was taken. The clear solution was diluted with 900 µl aqueous ionic liquid solution and 200 µl of standard, (66.7 mg phenol/100 ml water) filtered and analyzed *via* HPLC. (Method B)

Optimization of biomass loading

In a 1.5 ml Eppendorf-vial 1 wt%, 2.5 wt%, 5 wt% and 10 wt% solutions of ground cloves in aqueous [C_{12}mim]Cl (**52**) solution (1 wt%: 10 ± 1 mg in 900 µl ionic liquid solution, 2.5 wt% 25 ± 1 mg in 975 µl ionic liquid solution, 5 wt%: 50 ± 1 mg in 950 µl ionic liquid solution and 10 wt%: 100 ± 1 mg in 900 µl ionic liquid solution) were stirred for 3 h at r.t. The sample was centrifuged at 13000 rpm for 5 min and 100 µl of the supernatant was taken. The clear solution was diluted with 900 µl aqueous ionic liquid solution and 200 µl of standard, (66.7 mg phenol/100 ml water) filtered and analyzed *via* HPLC. (Method B)

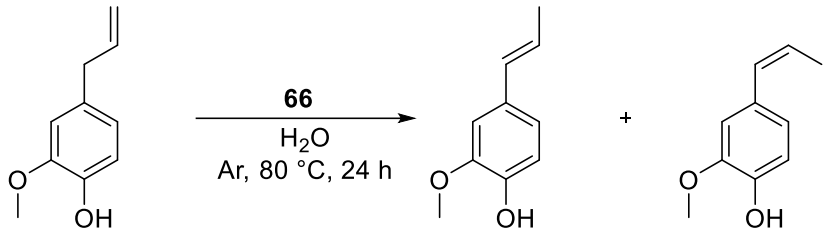
7.4.2.2 Influence of ionic liquids on the extraction

In a 1.5 ml Eppendorfvial 25 ± 1 mg ground cloves 975 µl aqueous ionic liquid solution was added. The suspension was stirred for 1 h at r.t. The sample was centrifuged at 13000 rpm for 5 min and 100 µl of the supernatant was taken. The clear solution was diluted with 900 µl aqueous ionic liquid solution and 200 µl of standard, (66.7 mg phenol/100 ml water) filtered and analyzed *via* HPLC. (Method B)

*Table 64: Aqueous ionic liquids solutions tested for the extraction of eugenol (**63**)*

Entry	[C_{10}mim]Cl (51) [mM]	[C_{12}mim]Cl (52) [mM]	[C_{14}mim]Cl (53) [mM]	[C_{16}mim]Cl (54) [mM]	[$C_{12}m_2$im]Cl (65) [mM]	[C_{12}betaine]Cl (56) [mM]
1	5	5	0.5	5	5	5
2	10	10	1	15	10	10
3	25	15	3	50	15	15
4	40	25	5		25	25
5	50	35	7		35	50
6	60	50	10		50	75
7	75	75	15		75	100
8	80	100	50		100	150
9	100	150	75		250	200
10	150	250	100			250
11	200		150			
12	250		250			

7.4.3 General procedure for the isomerization of eugenol using 66



63

$C_{10}H_{12}O_2$
164.20
(*E*)-**64**

$C_{10}H_{12}O_2$
164.20
(*Z*)-**64**

In a 5 ml screw cap vial dichlorotris(triphenylphosphine)ruthenium(II) (**66**), the ionic liquid and eugenol (**63**) were added under argon atmosphere. Degassed water (1 ml) was added and the solution was stirred under argon at 100 °C for 24 h. A sample of 100 µl was taken, extracted with 5 ml EtOAc/standard (49.7 mg methylbenzoat/100 ml EtOAc), filtered over SiO_2 and analyzed *via* GC. (Method J)

Variation of the ionic liquid concentration

In a 5 ml screw cap vial 1 mol% catalyst, [C_{12}mim]Cl (**52**) and 12 mg eugenol (**63**) were added under argon atmosphere. Degassed water (1 ml) was added to obtain a 5/15/25/25/50/100 mM solution of **52** in water and the solution was stirred under argon at 100 °C for 24 h.

*Concentration of eugenol (**63**)*

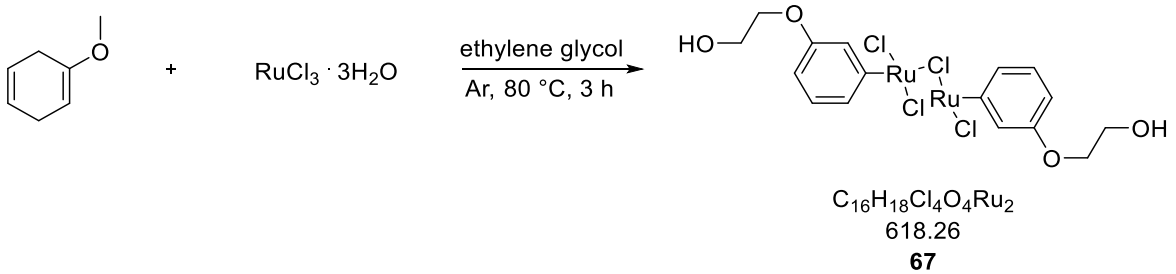
In a 5 ml screw cap vial 1 mol% catalyst, 1 ml of a 50 mM [C_{12}mim]Cl (14 mg, 0.05 mmol) and 6/12/24/48 mg eugenol (**63**) were added under argon atmosphere.

Amount of catalyst

For the variation of the amount of catalyst 0.5/1/2 mol% catalyst, 1 ml of a 50 mM [C_{12}mim]Cl (**52**) solution and 48 mg eugenol (**63**) were used.

7.4.4 Synthesis of (Trimethoxyphosphin)(phenoxyethanol)ruthenium(II)dichloride (68)

7.4.4.1 [{RuCl(μ-Cl)(η^6-$C_6H_5OCH_2CH_2OH$)}$_2$] (67)



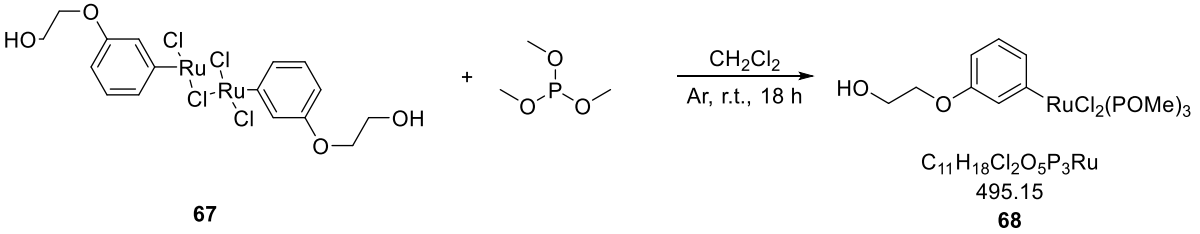
$C_{16}H_{18}Cl_4O_4Ru_2$
618.26
67

To rutheniumtrichloride trihydrate (0.973 g, 3.72 mmol) and 15 ml ethylene glycol 1-methoxy-1,4-cyclohexadien (4.36 ml, 37.2 mmol) was added and the solution was stirred at 80 °C for 3 h under argon atmosphere. A red-brown precipitate was removed *via* filtration and dried *in vacuo*. A red-brown solid was obtained in 65% (515 mg) yield.

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H= 3.70 (t, *J* = 4.79, 2H, -CH_2-OH), 4.19 (t, *J* = 4.79, 2H, -OCH_2), 5.35 (t, *J* = 5.18, 1H, H_{para}), 5.52 (2H, d, *J* = 6.06, H_{ortho}), 6.13 (2H, t, *J* = 5.67, H_{meta})

Analytical data was in accordance with literature.[383]

7.4.4.2 (Trimethoxyphosphin)(phenoxyethanol)ruthenium(II)dichloride (68)



67

$C_{11}H_{18}Cl_2O_5P_3Ru$
495.15

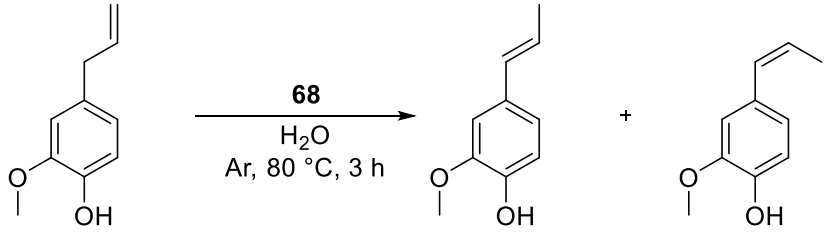
68

67 (355.5 mg, 0.821 mmol) and 162 µl (1.97 mmol, 2.4 eq.) trimethoxyphosphine were dissolved in anhydrous DCM and stirred under argon atmosphere for 18 h at r.t. The solvent was evaporated and the brown residue was purified *via* column chromatography (5 g SiO_2, DCM:acetone=25:1-15:1-10:1-5:1). A red solid was obtained in 47 % yield. (264 mg)

^{1}H-NMR (200 MHz, $CDCl_3$): δ_H= 3.79 (d, *J* = 11.15, 9H, -OMe), 4.01 (m, 2H, -OCH_2), 4.38 (m, 2H -OCH_2), 4.92 (t, *J* = 4.99, 1H, H_{para}), 5.37 (d, *J* = 4.89, 2H, H_{ortho}),5.84 (m, 2H, H_{meta})

Analytical data was in accordance with literature.[384]

7.4.5 General procedure for the isomerization of eugenol using 68



63

$C_{10}H_{12}O_2$
164.20
(*E*)-**64**

$C_{10}H_{12}O_2$
164.20
(*Z*)-**64**

In a 5 ml screw cap vial (trimethoxyphosphin)(phenoxyethanol)ruthenium(II)dichloride (**68**), the ionic liquid and eugenol (**63**) were added under argon atmosphere. 1 ml degassed water was added and the solution was stirred under argon at 80 °C for 1 h. 100 µl was taken, extracted with 5 ml EtOAc/standard (49.7 mg methylbenzoat/100 ml EtOAc), filtered over SiO_2 and analyzed *via* GC. (Method J)

Concentration of eugenol (**63**) was investigated using 1 mol% catalyst, [C_{12}mim]Cl (**52**) (14 mg, 0.05 mmol)/without ionic liquid and 17/86/165 mg eugenol in 1 ml degassed water.

Concentration of the ionic liquid solution was investigated using 1 mol% catalyst and 1 ml of a 10/50/100/250 mM solution of ionic liquid (**52**) in water.

7.5 Towards the synthesis of betulinic acid (70) and bevirimat (71)

7.5.1 Extraction procedure for the isolation of betulin (69)

7.5.1.1 Standard calibration for betulin (69)

Standard calibrations of betulin (**69**) were performed according to 7.3.1 using a 1-methyl-1-cyclohexene stock in methanol. (200 mg in 100 ml in methanol) The samples were directly analyzed *via* HPLC according to Method F.

7.5.1.2 Microwave assisted extraction

In a 5 ml microwave vial 100 mg (± 5 mg) ground birch bark and 900 mg (± 50 mg) of the ionic liquid or the ionic liquid solution were heated for 15 min at 100 °C using high absorption level. 2 ml of water and 3 ml of BuOAc were added and the mixture was again heated for 15 min at 100 °C. The organic layer was separated and diluted to 10 ml with HPLC MeOH. A sample of 100 µl was taken, 900 µl of HPLC MeOH and 200 µl of internal standard (200 mg 1-methyl-1-cyclohexen in 100 ml MeOH) were added. The solution was filtered and analyzed *via* HPLC. (Method F) Results are based on three independent experiments.

Screening was accomplished using following solvents (Table 65):

*Table 65: Solvents used for the microwave assisted extraction of betulin (**69**) from birch bark*

Entry	Imidazolium ionic liquids[a]	Ammonium ionic liquids	Phosphonium ionic liquids[a]	Conv. solvents[a]
1	$[C_2mim]OAc$ (**72**)	$[HNEt_3]HSO_4$	$[P_{4444}]Br$ (**75**) (40%)	water
2	$[C_2mim]Me_2PO_4$ (**4**)		$[P_{4444}]OH$ (**76**) (40%)	sat. NaCl solution
3	$[C_2mim]Me_2PO_4$ (**4**, 40%)		$[P_{4444}]OH$ (**76**) (20%)	NaOH (40%)
4	$[C_2mim]MeSO_3$ (**73**)		$[P_{4444}]OH$ (**76**) (10%)	
5	$[C_4mim]Cl$ (**2**)		$[P_{4444}]OH$ (**76**) (5%)	

[a] Aqueous solutions in round brackets.

7.5.1.3 Extraction at room temperature

In a 20 ml screw-cap vial 100 mg (± 5 mg) ground birch bark and 900 mg (± 50 mg) of the solvent were stirred for 24 h at room temperature. 2 ml of water and 3 ml of BuOAc were added and the mixture was again stirred for 1 h at room temperature. The organic layer was separated and diluted to 10 ml with HPLC MeOH. A sample of 100 µl was taken, 900 µl of HPLC MeOH and 200 µl of internal standard (200 mg 1-methyl-1-cyclohexen in 100 ml MeOH) were added. The solution was filtered and analysed *via* HPLC. (Method F) Results are based on three independent experiments.

Screening was performed using $[P_{4444}]Br$ (**75**, 40%), $[P_{4444}]OH$ (**76**, 40%), water, saturated NaCl solution and NaOH solution (40%). For investigation of time screening 900 mg (± 50 mg) of $[P_{4444}]OH$ (**76**, 40%) were stirred for 0.5/1/3/6/24 h at room temperature.

7.5.1.4 Isolation of betulin (69) from birch bark

In a 20 ml screw-cap vial 101.0 mg ground birch bark and 900 mg 40% $[P_{4444}]OH$ (**76**) were stirred for 15 min at 100 °C under MW irradiation. 2 ml of water and 3 ml of BuOAc were added and the mixture was again subjected to MW irradiation. The organic layer was separated. After purification *via* column

chromatography (7 g SiO_2, PE:EtOAc = 20:1-10:1-5:1-2:1) 25.4 mg betulin (**69**) were obtained as colorless crystals. (25 wt% corresponding to birch bark)

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.74 (s, 3H), 0.80 (s, 3H), 0.94 (s, 3H), 0.95 (s, 3H), 0.99 (s, 3H), 1.24-1.27 (m, 8H), 1.35-1.42 (m, 6H), 1.48-1.68 (m, 12H), 1.80-1.97 (m, 3H), 2.32-2.40 (m, 1H), 3.16 (dd, J_1 = 4.88, J_2 = 11.1, 1H), 3.31 (d, J = 10.9, 1H), 3.77 (d, J = 10.9, 1H), 4.55 (s, 1H), 4.65 (s, 1H)

Analytical data was in accordance with literature values.[249]

Lupeol (**77**) was obtained in 3 mg (3 wt% corresponding to birch bark) as colorless crystals.

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.74 (s, 3H), 0.76 (s, 3H), 0.80 (s, 3H), 0.91 (s, 3H), 0.94 (s, 3H), 1.10-1.65 (m, 30H), 1.84-1.94 (m, 1H), 2.32-2.38 (m, 1H), 3.16 (dd, J_1 = 5.1, J_2 = 10.9, 1H), 4.56 (s, 1H), 4.68 (s, 1H)

^{13}C-NMR (100 MHz, $CDCl_3$): δ_C = 13.5, 14.3, 14.9, 15.1, 16.9, 17.3, 18.3, 19.9, 24.1, 26.4, 26.4, 26.9, 28.8, 33.2, 34.5, 36.1, 37.0, 37.7, 37.8, 38.9, 39.8, 41.8, 41.9, 46.9, 47.3, 49.4, 54.3, 77.9, 108.3, 149.9

Analytical data was in accordance with literature.[385]

7.5.2 Pretreatment of plane bark

7.5.2.1 Preatratment of plane bark using ionic liquid/water solutions

200 mg ground plane bark were stirred with 1800 mg solvent at 80 °C for 1 h. The solution was filtered and the wood residues were dried overnight *in vacuo* at 50 °C.

Table 66: Ionic liquid/water solutions used for the pretreatment of plane bark

Entry	Ionic liquid	Concentration in water (wt%)	Ionic liquid	Concentration in water (wt%)
1	$[C_2mim]OAc$ (**72**)	0	$[HNEt_3]HSO_4$ (**74**)	0
2	$[C_2mim]OAc$ (**72**)	10	$[HNEt_3]HSO_4$ (**74**)	10
3	$[C_2mim]OAc$ (**72**)	25	$[HNEt_3]HSO_4$ (**74**)	25
4	$[C_2mim]OAc$ (**72**)	50	$[HNEt_3]HSO_4$ (**74**)	50
5	$[C_2mim]OAc$ (**72**)	75	$[HNEt_3]HSO_4$ (**74**)	75
6	$[C_2mim]OAc$ (**72**)	90	$[HNEt_3]HSO_4$ (**74**)	90
7	$[C_2mim]OAc$ (**72**)	100	$[HNEt_3]HSO_4$ (**74**)	100

7.5.2.2 Extraction of betulinic acid (70) from (pretreated) plane bark

100 mg of (pretreated) plane bark was stirred with 900 mg BuOAc for 1 h at room temperature in an Eppendorf vial. The solution was centrifuged for 5 min at 13 000 rpm. 100 µl of the supernatant were taken and diluted with 900 µl MeOH and 200 µl ISTD. The solution was filtered and analyzed *via* HPLC. (Method E)

7.5.2.3 MW assisted extraction of betulinic acid (70) from plane bark

100 mg plane bark and $[C_2mim]OAc$ (**72**)/water/BuOAC were heated under microwave irradiation at 80 °C for 15 min. Optionally water and BuOAc were added ant the solution heated again at 80 °C for 15 min. 100 µl of the organic/aqueous layer were taken and diluted with 900 µl MeOH and 200 µl ISTD.

The solution was filtered and analyzed *via* HPLC. (Method E) Results are based on three independent experiments.

*Table 67: MW irradiation for the extraction of betulinic acid (**70**)*

Entry	MW 1, 80 °C, 15 min			MW 2, 80 °C, 15 min	
	[C₂mim]OAc (72) [mg]	water [mg]	BuOAc [mg]	water [mg]	BuOAc [mg]
1	450	450			900
2	900			900	900
3	450	450	900		
4			900		
5		900			
6	900				
7	900				900
8	450	450			

7.5.3 Synthesis of reference materials

7.5.3.1 Lup-20(29)-ene-3β,28-diol, betulin (69)

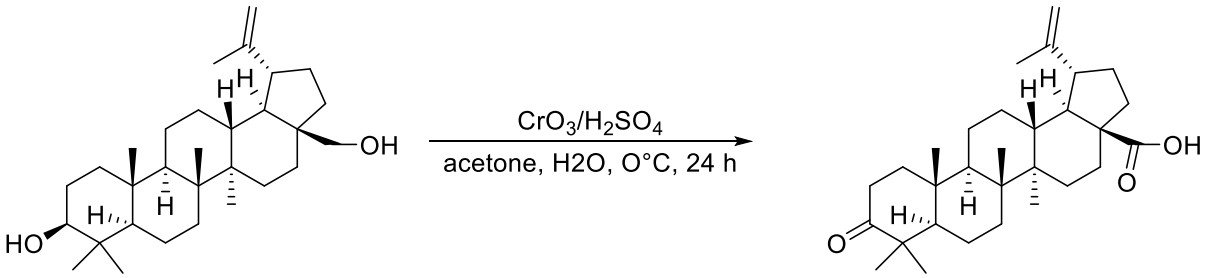
Small pieces of birch bark (10 g) were refluxed in methanol and removed *via* filtration. The solution was concentrated and 3 g yellowish solid was obtained. The residue was purified using column chromatography. (150 g SiO_2, PE:EtOAc = 30:1-20:1-10:5:1-1:1)

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.65-2.05 (m, 21H), 2.39 (m, 1H), 3.19 (d, *J* = 5.08, 1H), 3.32 (d, *J* = 10.82, 1H), 3.80 (d, *J* = 10.9, 1H), 4.59 (s, 1H), 4.69 (s, 1H).

^{13}C-NMR (100 MHz, $CDCl_3$): δ_C =14.7, 15.3, 15.9, 16.1, 18.3, 18.4, 19.1, 20.8, 25.2, 27.0, 27.3, 27.9, 29.1, 29.7, 34.0, 34.2, 37.1, 37.3, 38.7, 38.8, 40.9, 42.7, 47.7, 48.7, 50.4, 55.2, 60.5, 79.0, 109.7, 150.4

Analytical data was in accordance with literature values.[249]

7.5.3.2 Lup-20(30)-en-28-oic acid, 3-oxo- (7Cl), betulonic acid (80)



69

$C_{30}H_{46}O_3$
454.69
80

Betulin (**69**, 1.37 g, 3.09 mmol) was dissolved in 70 ml acetone. The solution was cooled to 0 °C. To 9.2 ml water sulfuric acid (1.77 g, 18.5 mmol, 6 eq.) and chromium trioxide (1.85 g, 18.5 mmol, 6 eq.) were subsequently added under cooling. The Jones reagent was added dropwise to the betulin solution at 0 °C and stirred for 24 h. TLC showed full conversion of the starting material. The reaction was

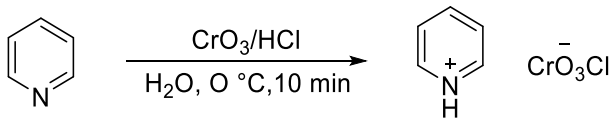
quenched with 30 ml methanol and 50 ml water. The organic solvent was removed and the aqueous layer was extracted with EtOAc for five times. The combined organic layers were washed with brine and dried over Na_2SO_4. After filtration and evaporation the crude product was purified *via* column chromatography. (50 g SiO_2, PE:EtOAc = 30:1-20:1-15:1-10:1-5:1-2:1) The product was obtained as colorless solid in 45% yield (0.63 g).

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.90 (s, 3H), 0.95 (s, 3H), 0.97 (s, 3H), 0.99 (s, 3H), 1.04 (s, 3H), 1.17-1.52 (m, 17H), 1.67 (s, 3H), 1.68-1.73 (m, 1H), 1.86-1.89 (m, 1H), 1.96-198 (m, 2H), 2.17-2.28 (m, 2H), 2.41-2.47 (m, 2H), 2.98-3.00 (m, 1H), 4.59 (s, 1H), 4.72 (s, 1H)

^{13}C-NMR (100 MHz, $CDCl_3$): δ_C = 13.5, 14.7, 14.9, 18.3, 18.5, 19.9, 20.3, 24.5, 25.9, 28.6, 29.5, 31.0, 32.5, 33.1, 35.8, 36.0, 37.4, 38.5, 39.5, 41.4, 45.8, 46.3, 48.1, 48.8, 53.9, 55.3, 108.7, 149.3, 180.9, 217.27

Analytical data was in accordance with literature.[249]

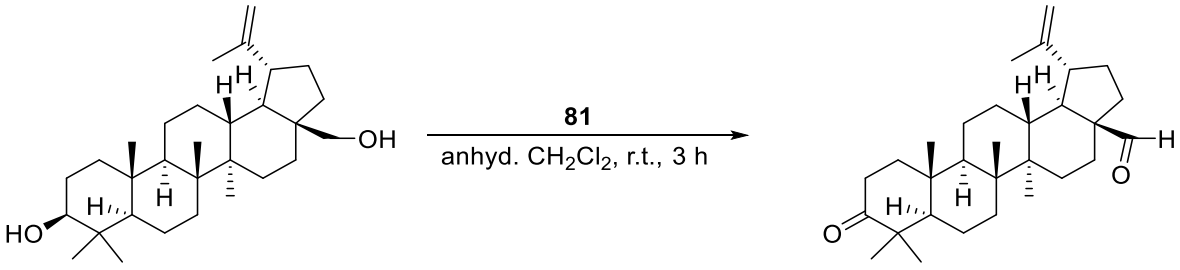
7.5.3.3 Pyridinium chlorochromate (81)



$C_5H_6ClCrNO_3$
215.55
81

To 8.5 ml hydrochloric acid (50.88 mmol) chromium trioxide (4.625 g, 46.25 mmol) was added and stirred for 10 min at r.t. The solution was cooled to 0 °C and pyridine (3.650 g, 46.24 mmol) were added over 15 min. The solid was removed *via* filtration and washed with cold water. An orange solid was obtained in 24% yield (2.36 g).[386]

7.5.3.4 Lup-20(30)-en-28-al, 3-oxo- (6Cl,7Cl), betulonic aldehyde (79)



69

$C_{30}H_{46}O_2$
438.69
79

Betulin (**69**, 30.9 mg, 0.0698 mmol) and **81** (90.3 mg, 0.419 mmol, 6 eq.) were dissolved in 5 ml anhydrous DCM and stirred for 3 h at r.t. (Full conversion) 3 ml Et_2O were added and the solution was filtered over Al_2O_3, washed with 2 N HCl and water. The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The product was obtained as colorless solid in 87% yield. (26.5 mg)

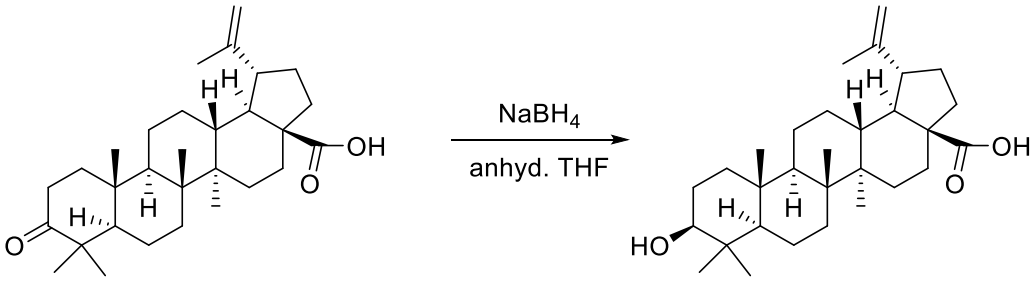
^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.93 (s, 3H) 0.97 (s, 3H), 1.00 (s, 3H), 1.04 (s, 3H), 1.08 (s, 3H), 1.26-1.46 (m, 15H), 1.71 (s, 3H), 1.75-1.80 (m, 3H), 1.89-1.92 (m, 2H), 2.03-2.12 (m, 2H), 2.41-2.51 (m, 2H), 2.87-2.89 (m, 1H), 4.65 (s, 1H), 4.77 (s, 1H), 9.65 (s, 1H)

^{13}C-NMR (100 MHz, $CDCl_3$): δ_C = 14.2, 15.7, 15.9, 19.0, 19.6, 21.0, 21.3, 25.5, 26.6, 28.8, 29.1, 29.7, 29.8, 33.1, 33.6, 34.1, 36.9, 38.8, 39.6, 40.8, 42.6, 47.3, 47.5, 47.9, 49.8, 54.9, 59.3, 110.2, 149.6, 206.5, 218.0

Analytical data was in accordance with literature.[387]

7.5.3.5 (3β)-3-Hydroxy-lup-20(29)-en-28-oic acid, betulinic acid (70)

*Reduction of betulonic acid (**80**) to betulinic acid (**70**)*



80

$C_{30}H_{48}O_3$
456.71
70

Compound **80** (0.86 g, 1.89 mmol) was dissolved in 35 ml anhydrous THF and cooled to 0 °C. $NaBH_4$ (1.15 g, 30.40 mmol) was added and the solution was stirred for 24 h at r.t. TLC did not show full conversion. The reaction was quenched with 5 ml 2N HCl and evaporated to 20 ml. The solution was extracted three times with EtOAc. The combined organic layer were washed three times with water, with brine and dried over Na_2SO_4. The solvent was evaporated to dryness. The colorless solid was crystallized in methanol to yield 0.5 g (56%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.75 (s, 3H), 0.82 (s, 3H), 0.94 (s, 3H), 0.97 (s, 3H), 0.99 (s, 3H), 1.10-1.57 (m, 20H), 1.62 (s, 3H), 1.87-1.97 (m, 2H), 2.09-2.21 (m, 2H), 2.89-2.96 (m, 1H), 3.12 (dd, J_1 = 4.96, J_2 = 11.24, 1H), 4.54 (s, 1H), 4.67 (s, 1H)

^{13}C-NMR (100 MHz, $CDCl_3$): δ_C =13.7, 14.3, 15.0, 15.1, 17.3, 18.4, 19.8, 24.5, 26.4, 26.9, 28.7, 29.5, 31.1, 33.3, 35.9, 36.2, 37.4, 37.7, 37.8, 39.7, 41.4, 45.9, 48.3, 49.5, 54.3, 55.2, 77.9, 108.7, 149.4, 180.3

Analytical data was in accordance with literature.[249]

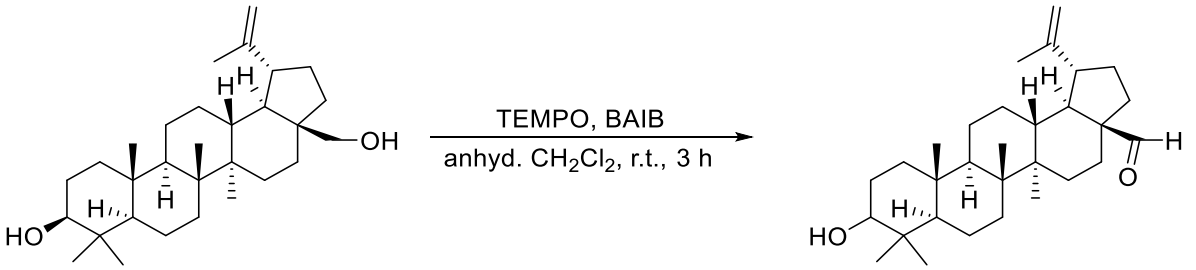
*Isolation of betulinic acid (**70**) of plane bark*

100 g plane bark were stirred in 950 ml refluxing DCM for 2 h. The bark was removed *via* filtration, washed with DCM and the solvent was evaporated to 100 ml. Crystallization was performed at 0 °C. The colorless solid was removed *via* filtration and crystallized in MeOH. A colorless solid was yielded in 0.7 wt%.

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.76 (s, 3H), 0.83 (s, 3H), 0.94 (s, 3H), 0.97 (s, 3H), 0.98 (s, 3H), 1.10-1.57 (m, 20H), 1.62 (s, 3H), 1.87-1.97 (m, 2H), 2.09-2.21 (m, 2H), 2.89-2.96 (m, 1H), 3.12 (dd, J_1 =4.88, J_2 = 11.1, 1H), 4.59 (s, 1H), 4.73 (s, 1H)

Analytical data was in accordance with literature.[249]

7.5.3.6 Lup-20(29)-en-28-al, 3β-hydroxy- (8Cl), betulinic aldehyde (78)



69

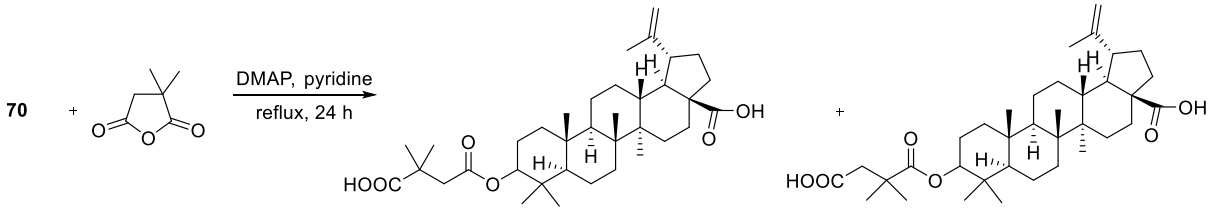
$C_{30}H_{46}O_2$
438.69
78

Betulin (**69**, 50.0 mg, 0.1129 mmol), TEMPO (17.6 mg, 1 eq.) and BAIB (109 mg, 0.3388 mmol, 3 eq.) were dissolved in anhydrous DCM and stirred for 3 h. After TLC showed full conversion the solution was quenched with saturated sodium thiosulfate solution, extracted with DCM three times. The combined organic layers were dried over Na_2SO_4, filtered and evaporated to dryness. The colorless solid was flashed over 1 g SiO_2 using PE and PE:EtOAc = 1:1. A colorless solid was obtained in 80 % yield, which seemed to be unstable.

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.68 (s, 3H), 0.75 (s, 3H), 0.84 (s, 3H), 0.89 (s, 3H), 0.90 (s, 3H), 1.09-2.04 (m, 18H), 2.72-2.87 (m, 1H), 3.07-3.15 (m, 1H), 4.56 (s, 1H), 4.68 (s, 1H), 9.61 (s, 1H)

Analytical data was in accordance with literature.[249]

7.5.3.7 3-O-(3',3'-Dimethylsuccinyl)betulinic acid, bevirimat (71)



$C_{36}H_{56}O_6$
584.84
71

$C_{36}H_{56}O_6$
584.84
iso-**71**

For the isolation of bevirimat several charges of esterifications were combined and purified *via* prep HPLC. (MeOH:H2O(0.1% TFA)=83:17, 210 nm, 20 ml/min. The regioisomer iso-(**71**) was synthesized using $[C_4DBU]N(Tf)_2$ as solvent and purified applying the same method.

The separated regioisomers iso-(**71**) were then crystallized from methanol.

Characterization of bevirimat (**71**):

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H =0.73 (s, 3H), 0.77 (s, 3H), 0.82 (s, 3H), 0.89 (s, 3H), 0.93 (s, 3H), 1.16-1.61 (m, 29H), 1.84-2.02 (m, 2H), 2.02-2.10 (m, 1H), 2.10 (s, 1H), 2.21-2.24(m, 2H), 2.34 (d, *J* = 15.20, 1H), 2.79 (d, *J* = 15.20, 1H), 2.93-2.99 (m, 1H), 4.45-4.96 (m, 1H), 4.53 (s, 1H), 4.65 (s, 1H)

^{13}C-NMR (100 MHz, $CDCl_3$): δ_C =13.4, 15.6, 16.0, 16.8, 17.4, 18.4, 19.9, 22.6, 22.7, 24.1, 26.3, 27.6, 28.9, 29.5, 31.2, 32.7, 36.1, 36.2, 36.6, 36.8, 37.4, 39.4, 39.7, 41.2, 44.7, 45.9, 48.2, 48.3, 53.9, 55.8, 80.1, 108.6, 149.4, 169.4, 182.3, 182.4

Analytical data was in accordance with literature.[388]

m/z: 585.6 (positive) 583.5 (negative)

Characterization of the undesired regioisomer iso-(**71**):

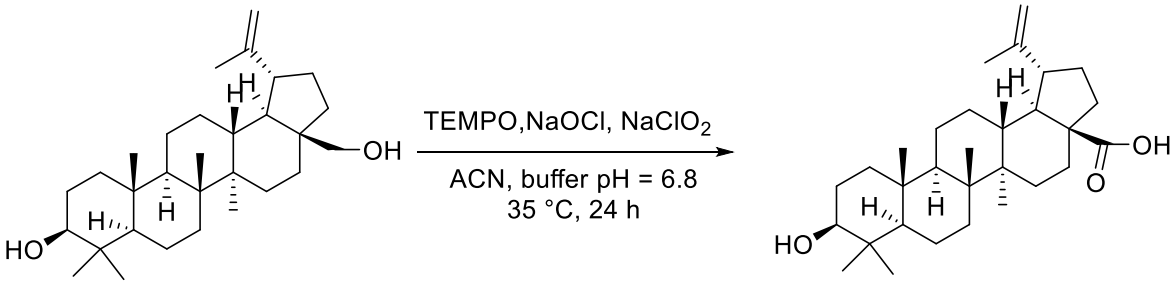
^{1}H-NMR (400 MHz, d_6-DMSO): δ_H = 0.79 (s, 3H), 0.80 (s, 3H), 0.81 (s, 3H), 0.88 (s, 3H), 0.95 (s, 3H), 1.16 (s, 3H), 1.17 (s, 3H), 1.30-1.61 (m, 28H), 1.65 (s, 3H), 1.79-1.81 (m, 2H), 2.10-2.13 (m, 1H), 2.19-2.25 (m, 1H), 2.49 (m, 2H), 2.92-2.99 (m, 1H), 4.31-4.35 (m, 1H), 4.57 (s, 1H), 4.69 (s, 1H), 12.1 (s, 2H)

^{13}C-NMR (100 MHz, d_6-DMSO): δ_C = 20 mal Carbon 14.88, 16.2, 16.3, 16.9, 18.2, 19.4, 20.9, 23.4, 25.1, 25.6, 25.7, 28.1, 29.7, 30.6, 32.2, 34.2, 36.8, 37.1, 37.9, 38.1, 40.5, 40.6, 40.7, 42.4, 43.9, 47.1, 49.0, 50.1, 55.1, 55.8, 80.1, 110.1, 150.8, 172.8, 176.0, 177.7

m/z: 585.4 (positive) 583.4 (negative)

7.5.4 TEMPO/NaOCl/$NaClO_2$ oxidation

Procedure according to Zhao et al.



69

$C_{30}H_{48}O_3$
456.71
70

Betulin (**69**, 20 mg, 0.05 mmol) and TEMPO (0.7 mg, 0.0038 mmol, 7mol%) were stirred at 35 °C in a solution of 10 ml ACN and 1 ml buffer. (phosphate buffer, pH = 6.8) To the suspension sodium chlorite (9.1 mg of 80% sodium chlorite dissolved in 100 µl water, 2 eq.) and sodiumhypochlorite (10.6 µl of a 0.327 M solution, 7 mol%) were added simultaneously but not mixed. The solution was stirred at 35 °C overnight, diluted with water and 2 N NaOH, poured into saturated Na_2SO_3 solution at 0 °C and stirred for 30 min. The solution was extracted with EtOAc three times. The combined organic layers were washed with water and brine and dried over Na_2SO_4, filtered and concentrated to dryness. A white solid was obtained which was identified as starting material containing impurities. (25.1 mg)[261]

Procedure according to Csuk *et al.*

To a 50 °C warm solution of betulin (**69**, 18.6 mg, 0.042 mmol), acetamido-TEMPO (0.6 mg, 0.0029 mmol, 7 mol%), Bu_4NBr in BuOAc (1 mg, 300 µl) and phosphate buffer (pH 7.6, 150 µl) were added. Sodium chlorite (9.5 mg of 80% sodium chlorite, 0.084 mmol, 2 eq.) dissolved in water and sodium hypochlorite (44 µl of a 10 wt% solution, 0.071 mmol, 1.7 eq.) were added simultaneously but not mixed. The solution was stirred at 50 °C overnight and poured into saturated Na_2SO_3 solution at 0 °C and stirred for 30 min. The solution was extracted with EtOAc three times. The combined organic layers were washed with water and brine and dried over Na_2SO_4, filtered and concentrated to dryness. [250]

NMR and HPLC (MeOH:H_2O=80:20, JASCO) confirmed that the main analyte is betulin (**69**).

Variations:

- Buffer pH 6.9
- Buffer pH 6.9 and $CHCl_3$ instead of BuOAc
- Chlorine scavenger
- Double amount of oxidation reagents

Procedure according to Noula et al.

Betulin (**69**, 10.4 mg, 0.0235 mmol) was dissolved in 3 ml DCM. Bu_4NBr and 3 ml of a 5% $NaHCO_3$ solution in water was added. The solution was cooled to 0 °C and KBr (2.6 eq., 7.3 mg, 0.611 mmol), TEMPO (2.6 eq., 9.5 mg) and NaOCl (2.6 eq., 38 µl of a 10 wt% solution) were added. The solution was stirred for 6 h at 0 °C and slowly chilled to room temperature. After 24 h stirring time the solution was extracted with EtOAc three times. The combined organic layers were washed with water and brine and dried over Na_2SO_4, filtered and concentrated to dryness.[264]

Procedure according to Zanka et al.

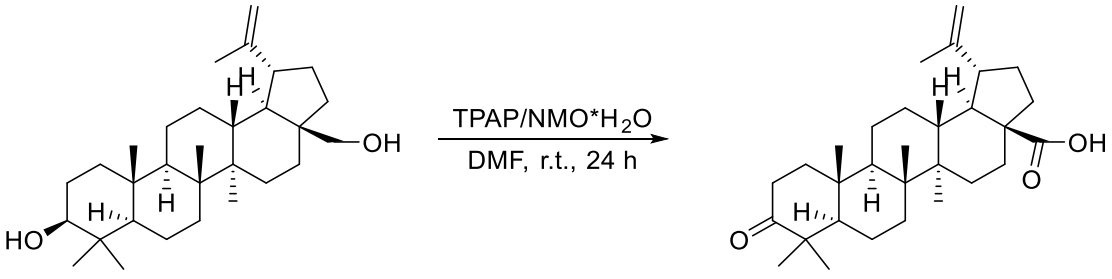
To a mixture of betulin (**69**, 88.4 mg, 0.2 mmol) in 25 ml EtOAc and KBr in 2 ml water (2.4 mg, 0.02 mmol, 10 mol%) 2 N NaOH was added and the pH adjusted to 8. The solution was cooled to 0 °C and NaOCl (140 µl, 0.25 mmol, 1.25 eq.) were slowly added over 30 min. The solution was stirred at room temperature for 2 h and acidified with 2 N HCl. (pH = 4) The solution was cooled to 0 °C and $NaClO_2$ in water (28.2 mg, 0.25 mmol, 12.5 eq.) slowly added. After 24 h stirring time the soltution was extracted with EtOAc three times. The combined organic layers were washed with water and brine and dried over Na_2SO_4, filtered and concentrated to dryness.[263]

7.5.5 TPAP/NMO oxidation

7.5.5.1 Standarcalibrations of betulin (69) and betulonic acid (80)

Standard calibration of betulin (**69**) and betulonic acid (**80**) were performed according to 7.3.1 using a 1-methyl-1-cyclohexene stock in methanol. (200 mg in 100 ml in methanol) The samples were directly analyzed *via* HPLC according to Method D.

7.5.5.2 General procedure for the TPAP/NMO oxidation



69

$C_{30}H_{46}O_3$
454.69
80

Betulin (**69**, 20 mg) and NMO were dissolved in 3 ml DMF. TPAP was added and the solution stirred at room temperature for 24 h. The solution was diluted to 10 ml, an aliquot of 1ml was taken and 200 µl ISTD were added, filtered and analyzed *via* HPLC. (Method D)

Time-screening was performed using 20 mg betulin (**69**) and 20 eq. of NMO and 3 ml DMF. 0.2 eq. TPAP were added and the solution stirred at room temperature/room temperature + 1 euqiv. Water/50 °C for 24 h. 50 µl sample was taken after 1/3/6/24/48/120 h, diluted with 950 µl MeOH and 200 µl ISTD, filtered and analyzed *via* HPLC. (Method D)

Variation of the oxidations reagents was performed using 10 mg betulin (**69**) and 2/5/10/20/40 eq. of NMO in 1.5 ml DMF.

Variation of the solvent

Betulin (**69**, 10 mg) and 20 eq.t of NMO were dissolved in 1.5 ml solvent. 0.2 eq. TPAP was added and the solution stirred at room temperature/50 °C/80 °C under air/O_2-atmosphere for 24 h.

Table 68: Solvents used for the TPAP/NMO oxidation

Entry	Solvent	Condition	Solvent	Condition
1	DMC	r.t.; 80 °C	$[C_2mim]DCA$ (**8**)	r.t.
2	DMSO	r.t.; 80 °C	$[C_2mim]NTf_2$ (**6**)	r.t.
3	*t*-BuOAc	r.t.; 80 °C	$[C_2mim]OAc$ (**72**)	r.t.
4	$CHCl_3$	r.t.; 80 °C	$[C_2mim]Me_2PO_4$ (**4**)	r.t.
5	EtOAc	0 °C; r.t.; 80 °C O °C, O_2-atm.; r.t., O_2-atm.	$[C_6mim]Cl$ (**82**)	50 °C
6	*iso*-propyl acetate	r.t.		
7	2-methyl-THF	r.t.		
8	BuOAc	r.t.		
9	DMF	r.t.; O °C, O_2-atm.		

7.5.5.3 Isolation of betulonic acid (80)/betulonic aldehyde (79) using the TPAP/NMO oxidation

Betulin (**69**, 20.6 mg, 0.04653 mmol) and NMO-monohydrate (126 mg, 0.9322 mmol, 20 eq.) were dissolved in anhydrous DMF. TPAP (3 mg, 0.008530 mmol, 0.2 eq.) were added and the solution was stirred for 24 h at room temperature. The reaction was quenched with 2-propanol. DMF was removed and the crude product was purified using 1.5 g SiO_2, and an eluent of PE:EE = 20:1-10:1-5:1-1:1. Betulonic acid (**80**) was obtained as a colorless solid in 37% yield. (7.9 mg).

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.90 (s, 3H), 0.98 (s, 3H), 0.99 (s, 3H), 1.02 (s, 3H), 1.07 (s, 3H), 1.17-1.52 (m, 17H), 1.67 (s, 3H), 1.68-1.73 (m, 1H), 1.86-1.89 (m, 1H), 1.96-198 (m, 2H), 2.17-2.28 (m, 2H), 2.41-2.47 (m, 2H), 2.97-3.10 (m, 1H), 4.62 (s, 1H), 4.74 (s, 1H)

The intermediate betulonic aldehyde (**79**) was obtained in 58% yield (11.8 mg).

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.93 (s, 3H) 0.97 (s, 3H), 1.00 (s, 3H), 1.04 (s, 3H), 1.08 (s, 3H), 1.26-1.46 (m, 15H), 1.71 (s, 3H), 1.75-1.80 (m, 3H), 1.89-1.92 (m, 2H), 2.03-2.12 (m, 2H), 2.41-2.51 (m, 2H), 2.87-2.89 (m, 1H), 4.65 (s, 1H), 4.77 (s, 1H), 9.65 (s, 1H)

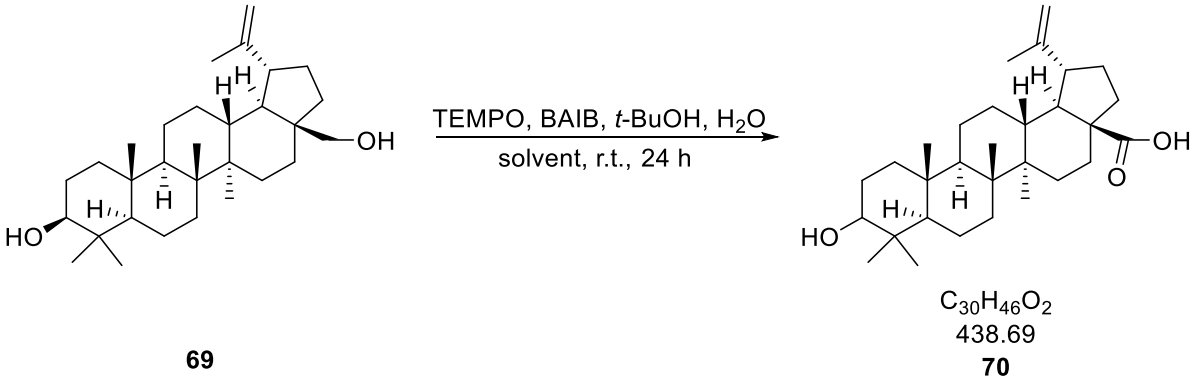
Analytical data were in accordance with literature.[249]

7.5.6 TEMPO/BAIB oxidation

7.5.6.1 Standard calibrations for betulin (69), betulinic acid (70) and beutlinic aldehyde (78)

Standard calibration of betulin (**69**), betulinic acid (**70**) and betulinic aldehyde (**78**) were performed according to 7.3.1 using a 1-methyl-1-cyclohexene stock in methanol. (200 mg in 100 ml in methanol) The samples were directly analyzed *via* HPLC according to Method G.

7.5.6.2 General procedure for the TEMPO/BAIB oxidation



69 **70**

Betulin (**69**, 10 mg), bis(acetoxy)iodobenzene (BAIB) and 2,2,6,6-tertramethylpiperidine-1-oxyl (TEMPO) were dissolved in 1 ml of the corresponding solvent. Water and 2 eq tertiary butanol (t-BuOH were added and the mixture was stirred at r.t. for 24 hours. After 24 hours reaction time the remaining solution was diluted with MeOH to 10 ml whereof 1 ml (plus 200 µl internal standard) were filtered and analyzed *via* HPLC.[273] (Method G)

Variation of the solvent was performed using 10 mg betulin (**69**), 3 eq. of BAIB and 1 eq. of TEMPO in 1ml of the particular solvent.[267] Following solvents were tested: DCM, DMC, DEC, BuOAC, EtOAc, *t*-BuOH and ACN.

Variation of the oxidation reagents was performed using 10 mg betulin (**69**), 1/2/3/4 eq. of BAIB and 0.05/0.1/0.2/1 eq. of TEMPO in 1ml BuOAc, 50 eq. of water and 2 eq. *t*-BuOH.

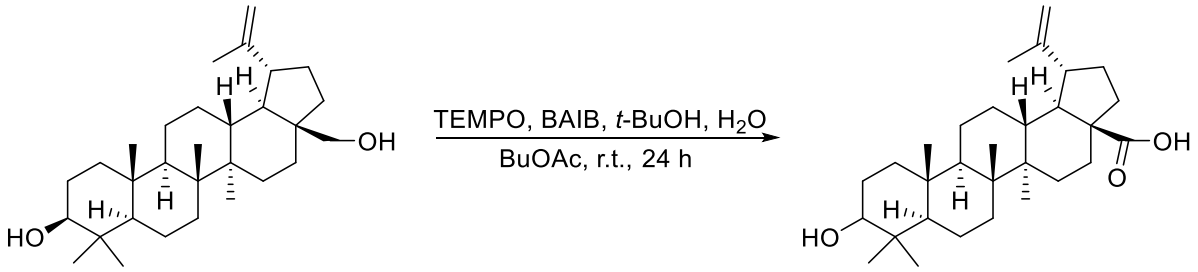
Variation of the concentration of betulin (**69**) was performed using 5/7.5/10/15 mg betulin, 3 eq. of BAIB and 0.2 eq. of TEMPO in 1ml BuOAc, 50 eq. of water and 2 eq. *t*-BuOH.

Variation of the temperature was performed using 10 mg betulin (**69**), 3 eq. of BAIB and 0.2 eq. of TEMPO in 1ml BuOAc, 50 eq. of water and 2 eq. *t*-BuOH.

Time screening

10 mg betulin, 3 eq. of BAIB and 0.2 eq. of TEMPO were dissolved in 1ml BuOAc and 50 eq. of water and 2 eq. *t*-BuOH were added and the mixture was stirred at r.t. 50 µl of the solution were taken after 1,2,3,4,5,6,7 h, diluted with 950 µl methanol and 200 µl internal standard, filtered and analyzed *via* HPLC. The remaining solution was diluted with MeOH to 10 ml whereof 1 ml (plus 200 µl internal standard) were filtered and analyzed *via* HPLC. (Method G)

7.5.6.2.1 Isolation of betulinic acid (70)



69

$C_{30}H_{46}O_2$
438.69
70

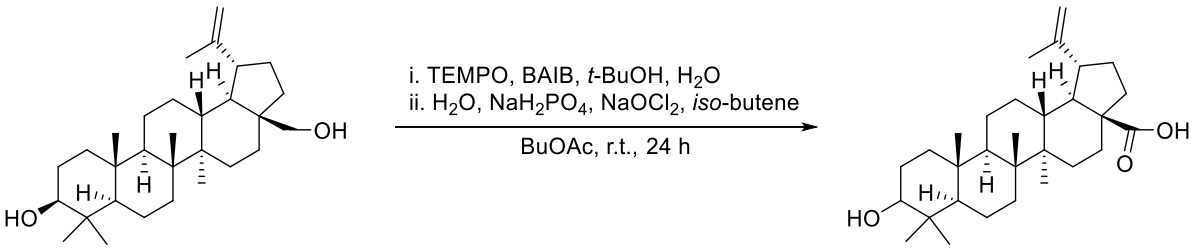
Betulin (**69**, 50 mg, 0.11 mmol), TEMPO (0.022 mmol, 0.2 eq.) and BAIB (111 mg, 0.34 mmol, 3 eq.) were dissolved in 700 µl HOAc, 21 µl *t*-BuOH, 100 µl water and 4.3 ml BuOAc. The solution was stirred for 24 h at r.t. The solution was quenched with saturated sodium thiosulfate solution and extracted with EtOAc three times, washed with sat. $NaHCO_3$ solution and dried over Na_2SO_4. The solution was filtered and evaporated to dryness. The residue was purified using flash chromatography (1.5 g SiO2, PE:EtOAc = 1:0-1:1) to obtain betulinic acid (**70**) in 50% yield with 90% purity.

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.75 (s, 3H), 0.82 (s, 3H), 0.94 (s, 3H), 0.97 (s, 3H), 0.99 (s, 3H), 1.10-1.57 (m, 20H), 1.62 (s, 3H), 1.87-1.97 (m, 2H), 2.09-2.21 (m, 2H), 2.89-2.96 (m, 1H), 3.12 (dd, J_1 = 4.96, J_2 = 11.24, 1H), 4.54 (s, 1H), 4.67 (s, 1H)

^{13}C-NMR (100 MHz, $CDCl_3$): δ_C = 13.7, 14.3, 15.0, 15.1, 17.3, 18.3, 19.8, 24.5, 26.4, 26.9, 28.4, 28.7, 29.5, 31.1, 33.3, 36.0, 36.2, 37.4, 37.7, 39.7, 41.4, 45.9, 48.2, 49.5, 54.3, 55.3, 77.9, 108.7, 149.4, 179.3

Analytical data was in accordance with literature.[249]

7.5.6.3 Combination of TEMPO/BAIB and Pinnick oxidation

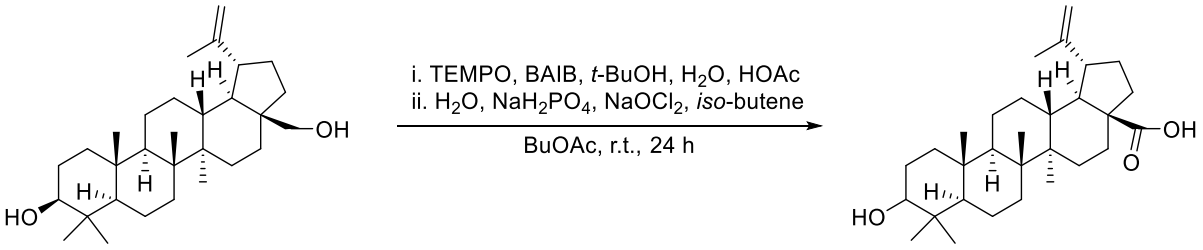


69

$C_{30}H_{46}O_2$
438.69
70

10 mg betulin (**69**), 3 eq. BAIB and 0.1/0.2 eq. of TEMPO were dissolved in 1 ml BuOAc and 50 eq. of water and 2 eq. *t*-BuOH were added and the mixture was stirred at r.t. for 6 h. After TLC showed full conversion 50 eq. of water, 4.6 eq. of *iso*-butene and 2.6 eq. of $NaOCl_2$ and NaH_2PO_4 are added. The solution changed its colour from yellow to brown and a white solid is precipitating. The solution is stirred at r.t. for 18 h.The remaining solution was diluted with MeOH to 10 ml whereof 1 ml (plus 200 µl internal standard) were filtered and analyzed *via* HPLC. (Mehtod G)

7.5.6.3.1 Isolation of betulinic acid (70)



69

$C_{30}H_{46}O_2$
438.69
70

Betulin (**69**, 99.5 mg, 0.2247 mmol), TEMPO (7.0 mg, 0.04495 mmol, 0.2 eq.) and BAIB (217 mg, 0.6742 mmol, 3 eq.) were dissolved in 10 ml BuOAc, 0.2 ml water and 42 µl *t*-BuOH. After 6 h stirring at room temperature TLC showed full conversion. To the clear yellow solution 0.2 ml water, 2-methyl-2-butene (72 µl, 1.03 mmol, 4.6 eq.), NaH_2PO_4 (70 mg, 0.584 mmol, 2.6 eq.) and $NaClO_2$ (66 mg with 80% purity, 0.584 mmol, 2.6 eq.) were added and a color change to brown was observed while a white solid was precipitating. The solution was quenched with saturated sodium thiosulfate solution and extracted with EtOAc three times, washed with sat. $NaHCO_3$ solution and dried over Na_2SO_4. The solution was filtered and evaporated to dryness. The white residue was crystallized from MeOH to yield **70** in 89% yield as colorless crystals.

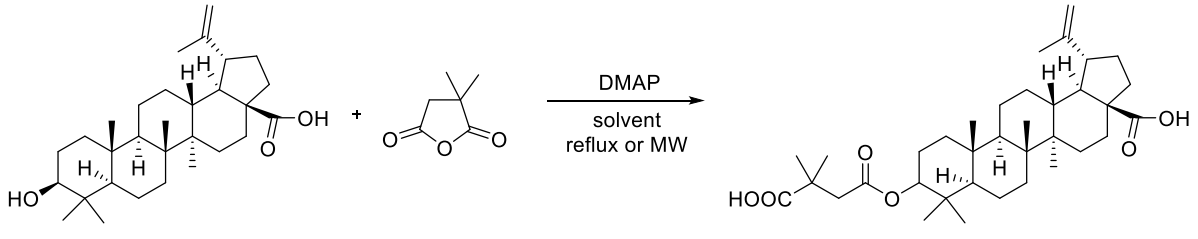
^{1}H-NMR (600 MHz, $CDCl_3$): δ_H = 0.74 (s, 3H), 0.81 (s, 3H), 0.93 (s, 3H), 0.96 (s, 3H), 0.97 (s, 3H), 1.10-1.57 (m, 20H), 1.68 (s, 3H), 1.94-1.98 (m, 2H), 2.24-2.27 (m, 2H),2.97-3.01 (m, 1H), 3.18 (dd, J_1 = 4.7, J_2 = 11.7, 1H), 4.73 (s, 1H), 4.60 (s, 1H)

^{13}C-NMR (150 MHz, $CDCl_3$): δ_C = 14.7, 15.4, 16.0, 16.1, 18.3, 19.4, 20.8, 25.5, 27.4, 27.9, 29.7, 30.5, 32.2, 34.3, 37.0, 37.2, 38.4, 38.7, 38.9, 40.7, 42.4, 46.9, 49.3, 50.5, 55.3, 56.3, 79.0, 109.7, 150.4, 179.9

LC-MS negativ *m*/*z* = 455.00

Analytical data was in accordance with literature.[249]

7.5.7 Esterification of betulinic acid (70)



70

$C_{36}H_{56}O_6$
584.84
71

7.5.7.1 Standard calibration for betulinic acid (70) and bevirimat (71)

Standard calibration of betulinic acid (**70**) and bevirimat (**71**) were performed according to 7.3.1 using a 1-methyl-1-cyclohexene stock in methanol. (200 mg in 100 ml in methanol) The samples were directly analyzed *via* HPLC according to Method H.

7.5.7.2 Conventional heating

Optimization of conditions

To 15 mg (± 0.5 mg) betulinic acid (**70**) 1/3/6 eq. dimethylsuccinic acid anhydride and 1/2 eq. DMAP were added in a screw-cap vial. 0.5 ml of pyridine or BuOAc was added and the mixture refluxed overnight. 20 µl of the solution were taken and mixed with 80 µl 2 N HCl, 900 µl methanol and 200 µl internal standard, filtered and analyzed *via* HPLC. (Method H)

Variation of the solvent was performed using 15 mg (± 0.5 mg) betulinic acid (**70**), 3 eq. dimethylsuccinic acid anhydride and 2 eq. DMAP. Screening was accomplished using pyridine, BuOAc, DCM, $N(Et)_3$, 2-Me-THF, dimethylcarbonate and chloroform.

For a time screening samples were taken after 1/2/3/4/5/6/8/24 h.

7.5.7.3 Microwave irradiation

In a 5 ml microwave vial 15 mg (± 0.5 mg) betulinic acid (**70**) 3 eq. dimethylsuccinic acid anhydride and 2 eq. DMAP were added and dissolved in 0.5 ml of BuOAc or pyridine and heated to 150 °C, 160 °C or 170 °C for 1/2 h. 20 µl of the solution were taken and mixed with 80 µl 2 N HCl, 900 µl methanol and 200 µl internal standard, filtered and analyzed *via* HPLC. (Method H)

Variation of the concentration of betulinic acid (**70**)

In a 5 ml microwave vial 15 mg (± 0.5 mg) betulinic acid (**70**) 3 eq. dimethylsuccinic acid anhydride and 2 eq. DMAP were added and 0.5/0.25/0.1 ml of BuOAc and heated to 160 °C for 1 h. To the solution 200 µl 2 N HCl was added and the solution was diluted to 10 ml with methanol. An aliquot of 1 ml was taken, 200 µl internal standard was added, filtered and analyzed *via* HPLC. (Method H)

Variation of solvents

Variation of solvents was performed using 0.1 ml of BuOAc, dimethylcarbonate and pyridine. Following ionic liquids were tested:

*Table 69: ionic liquids used for the esterification of betulinic acid (**70**)*

Entry	Ionic liquid	Amount [g]	Ionic liquid	Amount [g]
1	$[C_4DBU]Cl$ (**11**)	0.1	$[C_4DBU]N(Tf)_2$ (**15**)	0.1; 1
2	$[C_4DABCO]Cl$ (**12**)	0.1	$[C_4DABCO]N(Tf)_2$ (**16**)	0.1
3	$[C_4Pyr]Cl$ (**13**)	0.1	$[C_4Pyr]N(Tf)_2$ (**17**)	0.1
4	$[C_4DMAP]Cl$ (**14**)	0.1	$[C_4DMAP]N(Tf)_2$ (**18**)	0.1
5	$[C_1PyrImd]Cl$ (**20**)	0.1	$[C_1PyrImd]N(Tf)_2$ (**21**)	0.1
6	$[C_4PyrImd]Cl$ (**23**)	0.1		

7.5.8 *In situ* extraction and oxidation

In a 20 ml screw-cap vial 100 mg (± 5 mg) ground birch bark and 900 mg (± 50 mg) of the ionic liquid or the ionic liquid solution were stirred for 1 h at room temperature. 2 ml of water and 3 ml of BuOAc were added and the mixture was stirred vigorously for 1 h at room temperature. The organic layer was separated and an HPLC sample of 25 µl (24 mg) was taken, diluted with 975 µl methanol and 200 µl internal standard, and analyzed *via* HPLC. (Method F)

Different work-ups

- The solution was washed with water.
- The solution was filtered over SiO_2 and Na_2SO_4.
- The volume of the extract was adjusted to a concentration of 10 mg betulin (**69**)/ml solvent.
- Any combination of those possibilities.

The crude extract containing appr. 10 mg betulin (**69**) was oxidized according to 7.5.6.2. After 24 h stirring time the solution was diluted with methanol to 10 ml. An aliquot of 1 ml was taken, diluted with 200 µl ISTD, filtered and analyzed *via* HPLC. (Method G)

Variation of the amount of acetic acid

The concentration of betulin (**69**) in the mixture of BuOAc:HOAc was kept at 10 mg betulin (**69**)/ml.

7.5.8.1 Isolation of betulinic acid (70)

In a 20 ml screw-cap vial 200.2 mg ground birch bark and 1800 mg of a 40 wt% $[P_{4444}]OH$ (**76**) solution were stirred for 1 h at room temperature. 2 ml of water and 3 ml of BuOAc were added and the mixture was stirred vigorously for 1 h at room temperature. The organic layer was separated and filtered over a Pasteur pipette filled with SiO_2 and Na_2SO_4. The crude extract was concentrated to obtain 10 mg betulin/ml. TEMPO (3 mg, 0.021 mmol, 0.2 eq.), BAIB (99.5 mg, 0.31 mmol, 3 eq.) and 19 µl t-BuOH, 93 µl water and 1236 µl HOAc were added and the solution was stirred at room temperature for 6 h. Pinnick reagents were added, 2-methyl-2-butene (47 µl, 33 mg, 0.47 mmol, 4.6 eq.), $NaClO_2$ (30 mg of 80% purity, 0.27 mmol, 2.6 eq.) and NaH_2PO_4 (32 mg, 0.27 mmol, 2.6 eq.) and a color change to brown was observed while a white solid was precipitating. The solution was quenched with saturated sodium thiosulfate solution and extracted with EtOAc three times, washed with sat. $NaHCO_3$ solution and dried over Na_2SO_4. The solution was filtered and evaporated to dryness. The white residue was purified using column chromatography (3 g SiO_2, PE-EtOAc = 20:1-15:1-13:1-10:1-5:1-2:1) to yield **70** in 37.5 mg corresponding to 18 wt% yield as colorless crystals.

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.74 (s, 3H), 0.81 (s, 3H), 0.93 (s, 3H), 0.96 (s, 3H), 0.97 (s, 3H), 1.10-1.57 (m, 20H), 1.68 (s, 3H), 1.94-1.98 (m, 2H), 2.24-2.27 (m, 2H),2.97-3.01 (m, 1H), 3.18 (dd, J_1 = 4.7, J_2 = 11.7, 1H), 4.73 (s, 1H), 4.60 (s, 1H)

^{13}C-NMR (100 MHz, $CDCl_3$): δ_C = 14.7, 15.3, 16.0, 16.1, 18.3, 19.4, 20.8, 25.5, 27.4, 27.9, 29.7, 30.6, 32.2, 34.3, 37.0, 37.2, 38.4, 38.7, 38.9, 40.7, 42.5, 46.9, 49.3, 50.5, 55.3, 56.3, 79.0, 109.7, 150.4, 179.9

Analytical data was in accordance with literature.[249]

Identification of lupeol (**77**)

Lupeol (**77**) was obtained in 6.5 mg (3 wt% corresponding to birch bark) as colorless crystals.

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H = 0.74 (s, 3H), 0.76 (s, 3H), 0.80 (s, 3H), 0.91 (s, 3H), 0.94 (s, 3H), 1.10-1.65 (m, 30H), 1.84-1.94 (m, 1H), 2.32-2.38 (m, 1H), 3.16 (dd, J_1 = 5.1, J_2 = 10.9, 1H), 4.56 (s, 1H), 4.68 (s, 1H)

^{13}C-NMR (100 MHz, $CDCl_3$): δ_C = 13.5, 14.3, 14.9, 15.1, 16.9, 17.3, 18.3, 19.9, 24.1, 26.4, 26.4, 26.9, 28.8, 33.2, 34.5, 36.1, 37.0, 37.7, 37.8, 38.9, 39.8, 41.8, 41.9, 46.9, 47.3, 49.4, 54.3, 77.9, 108.3, 149.9

Analytical data was in accordance with literature.[385]

Isolation of betulinic acid (**70**) *via crystallization*

Procedure was performed as described above using 507.5 mg birch bark, 4.5 g 40% [P_{4444}]OH, 10 ml water and 15 ml BuOAc. For comparison 508.8 mg birch bark were used with 10 ml water and 15 ml BuOAc. Oxidation reagents were calculated on 130 mg betulin (**69**). The crude product was crystallized from a mixture of methanol/water.

*Table 70: Crystallization yields of betulinic acid (**70**)*

Entry	**[P_{4444}]OH**	**BuOAc**	**Remark**
fraction I			impurities
fraction II	111 mg (21.9 wt%)	78.8 mg (15.5 wt%)	including 3 wt% lupeol, purity > 90%
fraction III	36.9 mg (7 wt%)	28.4 mg (5.6 wt%)	including 3 wt% lupeol, purity < 90%
residue	44.0 mg	45.6 mg	

7.6 Isolation of limonene (83) and *in situ* multistep biocatalysis reaction

7.6.1 Standard calibrations

Standard calibration for GC

Standard calibration for GC analysis was performed according to 7.4.1 using methyl benzoate (100.0 mg in 500 ml EtOAc) as internal standard. (Method K)

Standard calibration for HPLC

Standard calibration for HPLC analysis was performed according to 7.3.1 using 1-methyl-1cyclohexen (100.0 mg in 500 ml methanol) as internal standard. (Method I)

7.6.2 Extraction of limonene (83)

GC analysis

350 mg of small pieces of orange peel (appr. 7 pieces) were stirred with 1 ml of extraction medium. (water, EtOAc plus ISTD, 250 mM ionic liquid solution) 100 µl were taken with a syringe, extracted with 5 ml EtOAc (incl. methylbenzoat) and analyzed *via* GC. For EtOAc 4.9 ml of EtOAc/methylbenzoat were added and shaken. (*Method K*) Alternatively the whole solution was extracted with EtOAc (plus ISTD) and analyzed.

HPLC analysis

200 mg of small pieces of orange peel (appr. 7 pieces) were stirred with 800 mg of extraction medium for 1.5 h at 80 °C. (water, EtOAc plus ISTD, 250 mM ionic liquid solution) 100 µl were taken with a syringe, the solution was diluted to 5 ml with methanol (incl. 1-methyl-1-cyclohexene), filtered and analyzed *via* HPLC. (Method I)

200 mg of small pieces of orange peel (appr. 10 pieces) were stirred with 800 µl of extraction medium. The solution was filled up to 10 ml methanol (incl. 1-methyl-1-cyclohexene) and analyzed *via* HPLC. (Method I)

Table 71: Ionic liquids tested for extraction experiments

Entry	Choline ionic liquids	Imidazolium ionic liquid
1	[chol]fom (**25**)	[C_2mim]OAc (**72**)
2	[chol]OAc (**26**)	[C_2mim]Cl (**5**)
3	[chol]lac (**27**)	[C_2mim]N(Tf)$_2$ (**6**)

7.6.3 Expression protocol

Table 72: Expression protocols[11]

Enzyme (origin)	Expression strain (plasmid)	Expression protocol	Ref.
CumDO (*Pseudomonas putida* PWD32)	*Pseudomonas putida* S12 (pBTBX-2)	TB(50µg/ml Kanamycin), 30 °C, 200 rpm; at OD590 0.6 + 2%(w/v) L-arabinose; 30 °C 200 rpm 6 h	
RR-ADH (*Rhodococcus ruber*)	*E. coli* BL21(DE3) (pRRADH)	LB_{Chl}, 37 °C, 120 rpm; at OD_{600} 0.4 + 1 mM $ZnCl_2$; 30 min 25 °C; + 0.1 mM IPTG, 20 h	Stampfer[312]
CHMO (*Acinetobacter calcoaceticus*)	*E. coli* BL21(DE3) (pET28a_CHMO)	LB_{Kan}, 37 °C, 180 rpm; at OD_{600} 0.6 + 0.1 mM IPTG; 20 h, 25 °C	Mallin[314]
OYE (*Saccharomyces* sp.)	*E. coli* BL21(DE3) (pDJB5)	LB_{Amp}, 37 °C, 180 rpm; at OD_{600} 0.6 + 0.1 mM IPTG; 20 h, 25 °C	Padhi[313]
RR-ADH (*Rhodococcus ruber*), OYE1 (*Saccharomyces sp*.) and CHMOAcineto (*Acinetobacter calcoaceticus*)	*E. coli* BL21(DE3) (pET28_CHMO_OYE + pRRADH)	TB(34µg/ml Chloramphenicol + 100µg/ml Kanamycin), 37 °C, 200 rpm; at OD590 0.3 + 1 mM $ZnCl_2$; 30 min 25 °C; at OD590 0.7 + 0.1 mM IPTG; 7 h, 25 °C	Oberleitner[282]

Resting cells

After expression of the enzymes, the cell cultures were centrifuged at 3600 xg at 4 °C for 15 min. The supernatant was discarded and the cell pellet gently washed in 50 mM TRIS-HCl pH=7.5 and

[11] Performed by Nikolin Oberleitner.

centrifuged again. After discarding the supernatant the cells were concentrated to a specific calculated OD_{590} and stored at 4° C until further use.

7.6.4 Bacterial growth

Growth curves were performed in 96-square-deep-well plates and analyzed using a plate reader at 595 nm. The ionic liquid was mixed with 1 ml of the resting cells or medium to obtain a concentration of either 50 mM or 100 mM. The well plate was shaken at 30 °C for *P. putida* and at 37 °C for *E. coli* at 200 rpm. A sample of 50 µl was taken every hour. When an OD_{595} > 0.3 was reached, dilution was performed.

A starting OD_{595} of 0.05 was used.

Table 73: Ionic liquids tested for bacterial growth tested for P. putida and E.coli

Entry	Choline ionic liquids	Imidazolium ionic liquid
1	[chol]fom (**25**)	[C_2mim]OAc (**72**)
2	[chol]OAc (**26**)	[C_2mim]Cl (**5**)
3	[chol]lac (**27**)	[C_4mim]Cl (**2**)
4	[chol]but (**28**)	[C_2mim]N(Tf)$_2$ (**6**)
5	[chol]hex (**29**)	[C_4mim]N(Tf)$_2$ (**9**)

E. coli strains were routinely cultured in LB medium (adapted from Bertani[389]): 10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl) and, if necessary, supplemented with ampicillin (100 µg/ml), chloramphenicol (34 µ/ml) or kanamycin (50 µg/ml), and incubated in baffled Erlenmeyer flasks in orbital shakers (InforsHT Multitron 2Standard) at 200 rpm and 37 °C.

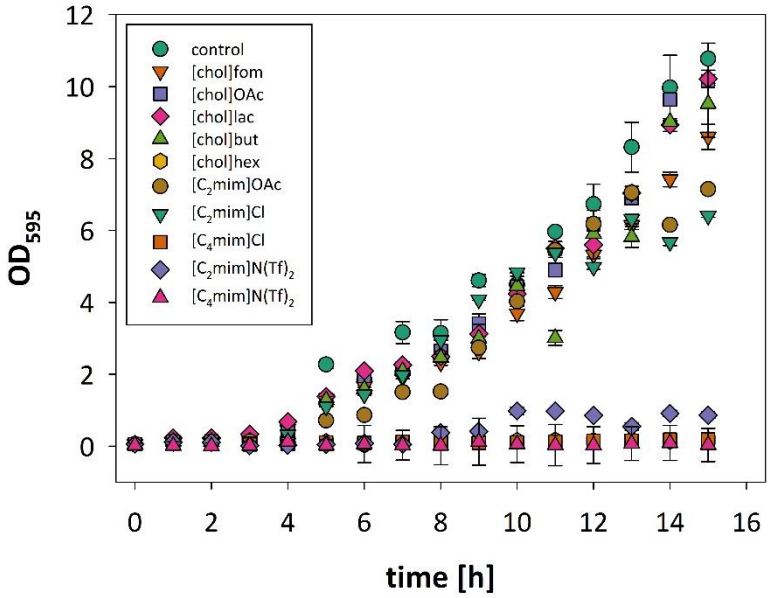


Figure 195: Growth curves of E. coli testing different ionic liquids at a 50 mM concentration

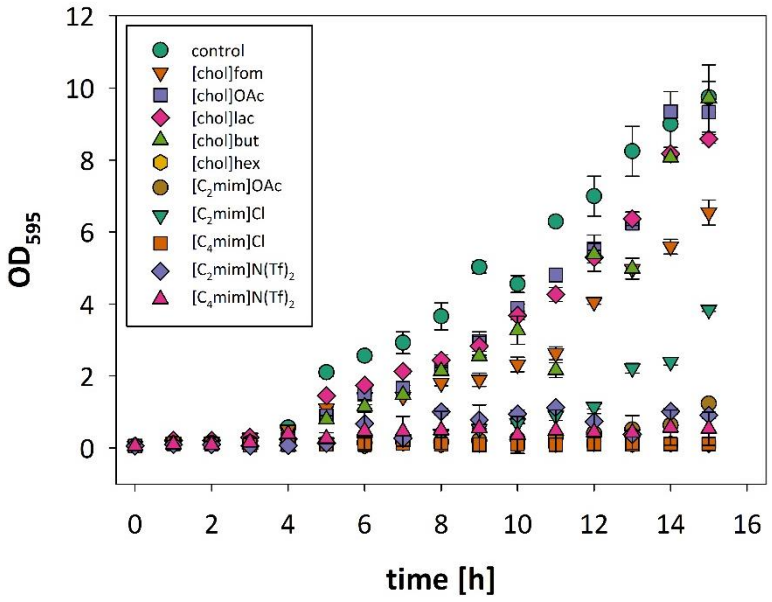


Figure 196: Growth curves of E. coli testing different ionic liquids at a 100 mM concentration

7.6.5 Biocatalysis

A 20 ml screw cap vial was loaded with the ionic liquid, 1 ml of the resting cells and 4 µl of an 1 M ethanolic stock of (R)-(+)-limonene (**83**) or (1*R*,5*R*)-carveol (**84**) to obtain a concentration of 4 mM. Resting cells of *P. putida* were usually used in an OD_{590} between 15 and 20 and the reaction was shaken at 30 °C at 200 rpm. *E. coli* resting cells (OD_{590} = 100) reactions were shaken at 30 °C at 200 rpm.

100 µl of the aqueous solution was taken and extracted with 500 µl EtOAc including 1mM methyl benzoate as internal standard. Alternatively the whole solution was extracted with 5 ml EtOAc, resp. diethyl ether for the bistriflimide ionic liquid.

7.6.6 Combination of extraction and biocatalysis

Approach I: Extraction was performed using 200 mg of orange peels and 800 mg of [C_2mim]OAc (**72**). The solution was diluted with resting cells to a volume of 100 ml and the reaction was performed as mentioned above.

Approach II: According to the procedure in 7.6.5 with the presence of orange peels.

7.7 Extraction procedures for the isolation of DNA from maize

7.7.1 Extraction procedure using ionic liquid and water

A 15 ml Falcon tube was loaded with 900 mg (± 50 mg) and 100 mg (± 1.0 mg) ground maize powder. The mixture was stirred for 15 min or 60 min at RT or 15 min at 80 °C. The solution was diluted to 5 ml with Milli-Q water. Denaturation was performed at 95 °C for 10 min. 1 ml of the solution was centrifuged (13 000 r min^{-1}, 10 min) and the supernatant was frozen. All experiments were carried out as duplicates or triplicates.

Screening was accomplished using following ionic liquids:

Table 74: Extraction of DNA from maize using ionic liquid/water mixtures

Ionic liquid used	$LiN(Tf)_2$	Conditions	Cq *ADH1* aver.	Cq *ADH1* STD
$[C_4DBU]Cl$ (**11**)	x	80 °C, 15 min	35.79	0.57
		80 °C, 15 min	35.00	0.69
	x	80 °C, 15 min	36.60	1.33
		80 °C, 15 min	34.17	0.53
$[C_{12}betaine]Cl$ (**56**)	x	80 °C, 15 min	-	-
		80 °C, 15 min	-	-
	x	80 °C, 15 min	-	-
		80 °C, 15 min	-	-
$[C_2OHmim]Cl$ (**3**)		80 °C, 15 min	38.14	-
		80 °C, 15 min	37.31	1.03
		80 °C, 15 min	38.54	0.94
		80 °C, 15 min	36.66	1.23
$[C_2mim]DCA$ (**7**)		RT, 1h	37.32	-
		RT, 1h	-	-
		RT, 15 min	38.43	-
		RT, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
$[C_2mim]OAc$ (**72**)		RT, 1h	33.64	0.93
		RT, 1h	44.32	1.85
$[C_2mim]Me_2PO_4$ (**4**)		80°C, 1h	45.98	0.37
$[C_4mim]Cl$ (**2**)		80°C, 1h	32.51	0.65
		80°C, 1h	-	-
	x	80 °C, 15 min	38.23	-
		80 °C, 15 min	39.18	0.25
	x	80 °C, 15 min	37.14	-
		80 °C, 15 min	39.33	-
$[C_4mim]N(Tf)_2$ (**9**)		RT, 1h	-	-
		RT, 1h	-	-
		RT, 15 min	-	-
		RT, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
$[C_4mim]PF_6$ (**10**)		RT, 1h	-	-
		RT, 1h	-	-
		RT, 15 min	-	-
		RT, 15 min	-	-
		80 °C, 15 min	-	-

		80 °C, 15 min	-	-
[C_4mim]Br (**1**)		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	46.43	-
[C_6mim]Cl (**82**)		80 °C, 15 min	-	-
		80 °C, 15 min	39.88	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
[C_8mim]Cl (**50**)		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
[C_{10}mim]Cl (**51**)	x	RT, 1h	39.37	2.61
		RT, 1h	-	-
	x	RT, 1h	-	-
		RT, 1h	-	-
	x	RT, 15 min	-	-
		RT, 15 min	-	-
	x	RT, 15 min	-	-
		RT, 15 min	38.51	-
	x	80 °C, 15 min	-	-
		80 °C, 15 min	-	-
	x	80 °C, 15 min	-	-
		80 °C, 15 min	-	-
[C_{12}mim]Cl (**52**)		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
[C_{14}mim]Cl (**53**)		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
[C_4Pyr]Cl (**13**)		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
		80 °C, 15 min	-	-
[chol]hdc (**24**)		RT, 1h	32.87	0.10
		RT, 1h	33.43	0.14
		RT, 15 min	34.90	0.61
		RT, 15 min	34.67	0.31

	80 °C, 15 min	35.38	0.71
	80 °C, 15 min	35.07	0.46
[DBU]fom (**42**)	80 °C, 15 min	29.31	0.42
[DBU]hex (**43**)	RT, 60 min	33.32	0.97
	RT, 15 min	34.79	0.39
	80 °C, 15 min	33.48	1.14
[N_{002OH}]fom (**38**)	RT, 60 min	30.27	0.22
	RT, 15 min	29.98	0.22
	80 °C, 15 min	29.73	0.14
[N_{002OH}]OAc (**39**)	RT, 60 min	29.71	0.33
	RT, 15 min	29.93	0.31
	80 °C, 15 min	29.82	0.45
[N_{002OH}]hex (**41**)	RT, 60 min	43.94	1.75
	RT, 15 min	40.58	1.18
	80 °C, 15 min	35.64	1.77
[$N_{00(2OH)_2}$]hex d-(**41**)	RT, 60 min	37.98	0.71
	RT, 15 min	41.17	4.50
	80 °C, 15 min	37.40	0.73
[$N_{0(2OH)_3}$]hex t-(**41**)	RT, 60 min	30.78	0.22
	RT, 15 min	31.97	0.43
	80 °C, 15 min	-	-

7.7.2 *In situ* formation of the $N(Tf)_2$ anion using ionic liquid and water

A 15 ml Falcon tube was loaded with 900 mg (± 50 mg) and 100 mg (± 1.0 mg) ground maize powder. The mixture was stirred for 15 min or 60 min at RT or 15 min at 80 °C. The solution was diluted to 5 ml with Milli-Q water and 1 eq. $LiN(Tf)_2$ in regard to the ionic liquid was added. Denaturation was performed at 95 °C for 10 min. 1 ml of the solution was centrifuged (13 000 r min^{-1}, 10 min) and the supernatant was frozen. All experiments were carried out as duplicates or triplicates. The ionic liquids used in this study are represented in Table 74.

7.7.3 Extraction procedure using ionic liquid/phosphate buffer

In a 1.5 ml Eppendorf-vial 100 mg (± 10.0 mg) ionic liquid were dissolved in 900 µl buffer and 100 mg (± 1.0 mg) of ground maize powder was added. The solution was stirred for 10 min at r.t. Denaturation was performed at 95 °C for 10 min in a thermo mixer. The mixture was centrifuged (13 000 r min^{-1}, 10 min) and the supernatant was frozen. All experiments were carried out as triplicates. (Table 75)

Table 75: Ionic liquids used for the extraction of DNA from maize using a buffer system

Entry	Choline alkylcarboxylate ionic liquids	Choline phosphate ionic liquids	Methylimidazolium ionic liquids	DBU based ionic liquids
1	[chol]fom (**25**)	[chol]dtp (**37**)	[C_2mim]Me_2PO_4 (**4**)	[C_4DBU]Cl (**11**)
2	[chol]OAc (**26**)	[chol]dhp (**33**)	[C_2mim]OAc (**72**)	
3	[chol]lac (**27**)	[chol]dbp (**34**)	[C_2mim]fom (**88**)	
4	[chol]but (**28**)	[chol]dop (**35**)	[C_2mim]DCA (**7**)	
5	[chol]hex (**29**)	[chol]bep (**36**)	[C_2mim]SCN (**8**)	
6	[chol]oct (**30**)		[C_4mim]Bu_2PO_4 (**90**)	
7	[chol]dec (**31**)		[amim]Cl (**89**)	
8	[chol]dod (**32**)			

Alternatively, in a 1.5 ml Eppendorf-vial 100 mg (± 10.0 mg) [C_2mim]Me_2PO_4 (**4**) were dissolved in 900 µl buffer and 100 mg (± 1.0 mg) of ground maize powder was added. Denaturation was performed at 95 °C for 10 min in a thermo mixer. The mixture was centrifuged (13 000 r min^{-1}, 10 min) and the supernatant was frozen. All experiments were carried out as triplicates.

7.7.4 Variation of the concentration of the ionic liquid

In a 1.5 ml Eppendorf-vial the [chol]fom (**25**) buffer solution (1 wt%: 10 mg ionic liquid and 990 µl buffer; 5 wt%: 50 mg ionic liquid and 950 µl buffer; 10 wt%: 100 mg ionic liquid and 900 µl buffer; 25 wt%: 250 mg ionic liquid and 750 µl buffer; 50 wt% 500 mg ionic liquid and 500 µl buffer) was stirred for 5-10 min until the ionic liquid was dissolved. 100 mg (± 1.0 mg) of ground maize powder was added. The solution was stirred for a defined time at room temperature or shaken in a thermo mixer 80 °C at 600 r min^{-1}. Denaturation was performed at 95 °C for 10 min in a thermo mixer. The mixture was centrifuged (13 000 r min^{-1}, 10 min) and the supernatant was frozen. All experiments were carried out as triplicates.

7.7.5 Extraction procedure using ionic liquid/phosphate buffer and Proteinase K

In a 1.5 ml Eppendorf-vial to a choline formate buffer solution containing 5 µl Proteinase K (1 wt%: 10 mg ionic liquid and 990 µl buffer; 5 wt%: 50 mg ionic liquid and 950 µl buffer; 10 wt%: 100 mg ionic liquid and 900 µl buffer; 25 wt%: 250 mg ionic liquid and 750 µl buffer; 50 wt% 500 mg ionic liquid and 500 µl buffer) 100 mg (± 1.0 mg) of ground maize powder was added. The solution was shaken for 10 min in a thermo mixer 80 °C at 600 rpm or stirred for 10 min at r.t. Denaturation was performed at 95 °C for 10 min in a thermo mixer. The mixture was centrifuged (13 000 r min^{-1}, 10 min) and the supernatant was frozen. All experiments were carried out as triplicates using [chol]fom (**25**) and [C_2mim]Me_2PO_4 (**4**).

7.7.6 Extraction procedure using ionic liquid/phosphate buffer using syringe filters

In a 1.5 ml Eppendorf-vial 100 mg (± 10.0 mg) [C_2mim]Me_2PO_4 (**4**) were dissolved in 900 µl buffer and 100 mg (± 1.0 mg) of ground maize powder was added. Denaturation was performed at 95 °C for 10 min in a thermo mixer. The mixture was filtered with a syringe filter and the supernatant was frozen. All experiments were carried out as triplicates.

7.7.7 CTAB extraction protocol[12]

Briefly, genomic DNA was extracted by performing a thermal lysis (65 °C, 1h) of the sample with the extraction buffer (1.4 M NaCl, 2% (w/v) cetyltrimethylammonium bromide (CTAB), 0.1M Tris-Base (pH=8), 0.02M EDTA (pH=8) and 1% polyvinyl pyrrolidone 40000). DNA was later precipitated with the precipitation buffer (1% (w/v) CTAB, 0.05 M Tris (pH=8), 0.01 M EDTA (pH=8)) and washed with 70% ethanol. Resuspension of the DNA was done afterwards in 10 mM Tris buffer at 65 °C for 20 min and stored at -20 °C until further use. All reagents were from Roth (Karlsruhe, Germany). Each extraction was done in triplicates and extracts were diluted 1:4.

7.7.8 Buffer preparation

Phosphate buffer was prepared by dissolving 32 mg NaH_2PO_4 and 3.499 g of Na_2HPO_4 in 200 ml Milli-Q water. The pH was adjusted to 8.5.

7.7.9 Real-time PCR assays[12]

The real-time PCR assays were performed on a RotorGene-Q cycler (Qiagen, Hilden, Germany). For each reaction, 2 µl template DNA were mixed with 13 µl reaction mix containing 7.5 µl Kapa^{TM} Probe® Fast (PeqLab, Erlangen, Germany), 4.78 µl sterile and nuclease free water and 0.24 µl for each of the forward and reverse primers (Sigma-Aldrich, St. Louis, USA) and for the JOE-labeled probe (Eurofins MWG Operon, Ebersberg, Germany) as well (each one at 6.25 pmol/µl). The Cq values obtained by quantitative PCR were used to assess the amount of amplifiable DNA.

The *ADH1* gene encoding for the alcohol dehydrogenase, which is present in both GM (Bt-11) and non-GM maize samples (RWA38), was used for the determination of the total amount of maize. In contrast, the cauliflower mosaic virus (CaMV) promoter *P35S* was utilized for the detection of the genetically modified sequences in maize. The *P35S* assay was used only when comparing the extraction performances for GM maize certified reference material (maize variety Bt-11) between the CTAB method and the here proposed IL-based method of extraction. The rest of the experiments were carried out with the RWA38 maize variety.

7.7.10 Calibration curves[12]

In addition, calibration curves with six points following two fold dilutions were made and linearity and PCR efficiency were calculated in order to assess the presence of qPCR inhibitors that might be coextracted. qPCR efficiency was calculated as follows:

$$\text{Efficiency}=10^{\left(\frac{-1}{\text{slope}}\right)}-1$$

Where slope is the slope of the linear regression in the calibration curve.

DNA concentrations of the extracts were photometrically measured in a NanoVue Plus spectrophotometer (GE Healthcare, Little Chalfont, USA). The yield was calculated as:

$$\text{Yield} = 10\text{c}(\text{DNA}) \cdot \text{V}$$

Where c(DNA) is the DNA concentration photometrically measured [ng/µl] and V is the total sample volume [µl]. Yield is expressed as µg DNA/g sample.

12 Performed by Eric Garcia.

7.8 Extraction procedures for the isolation of DNA from meat

7.8.1 Ionic liquid/buffer extraction procedure

In a 1.5 ml Eppendorf-vial 100 mg (± 10.0 mg) ionic liquid were dissolved in 900 µl buffer and 200 mg (± 15.0 mg) of meat were added. Optionally, the solution was stirred for 15 min at room temperature before further denaturation. Denaturation was performed at 95 °C for 10 min in a thermo mixer. The mixture was then centrifuged at 13000 rpm for 5 min and 400 µl of the supernatant were transferred to a new Eppendorf tube and kept frozen at -20 °C until further use. All experiments were carried out in triplicates.

Screening was accomplished using following ionic liquids: (Table 76)

Table 76: Ionic liquids used for the extraction of minced beef in combination with a phosphate buffer

Entry	Choline alkylcarboxylate ionic liquids	Choline phosphate ionic liquids	Guandidinium ionic liquids	Methylimidazolium ionic liquids
1	[chol]fom (**25**)	[chol]dhp (**33**)	[guan]fom (**44**)	[C_2mim]Me_2PO_4 (**4**)
2	[chol]OAc (**26**)	[chol]dbp (**34**)	[guan]OAc (**45**)	[C_2mim]OAc (**72**)
3	[chol]lac (**27**)	[chol]dop (**35**)	[guan]but (**46**)	[C_2mim]Cl (**5**)
4	[chol]but (**28**)		[guan]hex (**47**)	[C_4mim]Cl (**2**)
5	[chol]hex (**29**)		[guan]oct (**48**)	[C_6mim]Cl (**82**)
6	[chol]oct (**30**)		[guan]dec (**49**)	
7	[chol]dec (**31**)			
8	[chol]dod (**32**)			

Variation of the concentration of the ionic liquid

In a 1.5 ml Eppendorf-vial 1, 5, 10, 25, and 50 wt% choline hexanoate (**29**) buffer solutions (1 wt.: 10 mg ionic liquid and 990 µl buffer; 5 wt.: 50 mg ionic liquid and 950 µl buffer; 10 wt%: 100 mg ionic liquid and 900 µl buffer; 25 wt%: 250 mg ionic liquid and 750 µl buffer; 50 wt% 500 mg ionic liquid and 500 µl buffer) and 200 mg (± 15.0 mg) of meat were denaturated at 95 °C for 10 min in a thermo mixer. The mixture was then centrifuged at 13000 rpm for 5 min and 400 µl of the supernatant were transferred to a new Eppendorf tube and kept frozen at -20 °C until further use. All experiments were carried out in triplicates.

7.8.2 Buffer extraction procedure

Eppendorf-vials (1.5 ml) were charged with 1000 µl buffer and 200 mg (± 15.0 mg) of meat. Optionally, the samples were stirred for 15 min at room temperature before further denaturation. Denaturation was performed at 95 °C for 10 min in a thermo mixer. The mixture was then centrifuged at 13000 rpm for 5 min and 400 µl of the supernatant were transferred to a new Eppendorf tube and kept frozen at -20 °C until further use. All experiments were carried out in triplicates.

7.8.3 Buffer preparation

Phosphate buffer

Phosphate buffer was prepared by dissolving 32 mg NaH_2PO_4 and 3.499 g of Na_2HPO_4 in 200 ml Milli-Q water. The pH was adjusted to 8.5.

AMP buffer

AMP buffer was prepared by dissolving 446 mg of 2-amino-2-methyl-1-propanol in 100 ml Milli-Q water. The pH was adjusted to 8.5.

TRIS buffer

A 50 mM solution of Tris(hydroxymethyl)-aminomethane in Milli-Q water was prepared. The pH was adjusted to 8.5.

7.8.4 Pretreatment of meat

Pretreatment with heptane

Minced beef meat was stirred in heptane for one hour. The solvent was decanted and the meat was dried at ambient temperature and pressure.

Trituration in mortar

Minced beef meat was frozen with liquid nitrogen and triturated in a mortar.

7.8.5 Extraction with commercial kits[12]

7.8.5.1 SureFood® PREP Animal, from the company r-Biopharm.

400 µl of the lysis buffer and 40 µl of proteinase K were added to an Eppendorf tube containing 50 mg of meat, mixed and incubated at 52 °C for 30 min and 1400 rpm. The sample lysate was later centrifuged for 1 min at 12000 rpm for the pelleting of the unlysated ingredients. 350 µl of the supernatant were transferred to a new Eppendorf tube and 200 µl binding buffer were added to the supernatant. The mixture was later transferred onto a spin filter, incubated at room temperature for 1 min and centrifuged at 12000 rpm for 1 min. The filtrate was later discarded and the remaining ethanol in the filter was removed by another centrifugation step at 12000 rpm for 2 min. Afterwards, the DNA contained in the spin filter was eluted by adding 100 µl of preheated (52 °C) elution buffer directly onto the spin filter, incubated for 3 min at 52 °C and centrifuged at 10000 rpm for 1 min.

7.8.5.2 Wizard® Genomic DNA Purification Kit from the Promega Corporation.

20 mg of previously ground tissue tissue (in liquid nitrogen and using a mortar and pestle) were mixed with 600 µl of nuclei lysis solution in a 1.5 ml Eppendorf tube and incubated for 30 min at 65 °C. After this, 17.5 µl of proteinase K (20 mg/ml) were added and incubated for 3 h at 55 °C and 1400 rpm. After this digestion, 3 µl of RNase solution were added to the nuclear lysate and incubated for 30 min at 37 °C. The sample was later cooled down to room temperature during 5 min and 200 µl of protein precipitation solution were added and centrifuged for 4 min at 12500 rpm to form a tight white pellet from protein. 700 µl of the supernatant were then mixed with 600 µl room temperature isopropanol. The mixture was centrifuged for 1 min at 12500 rpm at room temperature, after which the pelleted DNA was visible. The supernatant was decanted and 600 µl of room temperature ethanol was added

to wash the DNA. This was later centrifuged for 1 min at 12500 rpm at room temperature. The ethanol was then removed and the pellet dried at 65 °C for 30 min. Afterwards, 100 µl of DNA rehydration solution were added and incubated at 65 °C for 1 h.

7.8.5.3 Extraction according to Amani *et al.*

The protocol from Amani *et al*., 2011 was performed with some modifications. 100 mg of the material was put on a 2 ml Eppendorf tube and 500µl of the preheated (70 °C) extraction buffer (200 mM Tris-HCl (pH=7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS) were added. The mixture was incubated at 70 °C and 1200 rpm for 30 min. Afterwards, 500 µl of chloroform:*iso*-amylalcohol (24:1) were added, vortexed and centrifuged for 5 min at 12500 rpm. 300 µl of the supernatant were then transferred to a new Eppendorf tube containing an equal volume of precooled (-20 °C) iso-popropanol. The mixture was vortexed and incubated at -20 °C for 90 min. After this, a centrifugation for 5 min at 12500 rpm was carried out. The supernatant was removed and the pellet was dried at 65 °C for 30 min. Later, it was dissolved in 100 µl TE buffer (pH=8).[341]

7.8.6 Real-time PCR assays[12]

Real time PCR assays were carried out in a RotorGene-Q cycler (Qiagen, Hilden, Germany) according to the following thermal cycling protocol: initial step of 2 min at 95 °C, followed by 50 cycles of 15 sec at 95 °C and 1 min at 62 °C. All amplification reactions were performed in triplicates. For each reaction, 2 µl template DNA were mixed with 13 µl reaction mix having 7.5 µl Kapa™ Probe® Fast (PeqLab, Erlangen, Germany), 4.78 µl sterile and nuclease free water and 0.24 µl for each of the forward and reverse primers and for the FAM-labeled probe (Eurofins MWG Operon, Ebersberg, Germany). Cq values obtained by quantitative PCR were used to assess the amount of amplifiable DNA. Primers and probes for all meat sorts were published by Köppel *et al*.[390]

7.8.7 Calibration curves[12]

In addition, calibration curves with 4 points following two or four fold dilutions were performed and linearity and PCR efficiencies were calculated to assess the presence of inhibitors that might hinder the amplification process during the qPCR. qPCR efficiency was determined as:

$$\text{Efficiency} = 10^{\left(\frac{-1}{\text{slope}}\right)} - 1$$

Where slope is the slope of the linear regression in the calibration curve.

The concentration of the DNA extracts were photometrically measured in a NanoVue Plus spectrophotometer (GE Healthcare, Little Chalfont, USA). The yield was calculated as follows:

$$\text{Yield} = \frac{\text{c(DNA)} \cdot \text{V}}{\text{m}}$$

Where c(DNA) is the concentration [ng/µl] of DNA photometrically measured, V is the total sample volume [µl] and m is the mass [mg] of the sample used for extraction. Yield is expressed as µg DNA/g sample.

7.9 10-Deacetyl-baccatin III (91)

7.9.1 Standard calibration

Standard calibration of 10-Deacetyl-baccatin III (**91**) was performed according to 7.3.1 using a methyl benzoate stock in methanol. (200 mg in 100 ml in methanol) The samples were directly analyzed *via* HPLC according to Method C.

7.9.2 Extraction procedure

450 mg of plant material powder and 1 g of ionic liquid was stirred at 25 °C for 1 h. The resulting solution was diluted to 5ml with MeOH. A sample of 1 mL was taken from the solution and 0.2 mL of internal standard (100.0 mg methyl benzoate in 100 mL methanol) was added. The solution was filtered over a 0.2 µm syringe filter and directly analysed *via* HPLC. (Method C) Results are based on three independent experiments.

450 mg of plant material powder and 2 mL of aqueous ionic liquid solution was stirred at 25 °C for 1 h. The resulting solution was centrifuged for 10 min at 13 000 min^{-1}. A sample of 0.1 mL was taken from the supernatant and 0.9 mL methanol, 0.2 mL of internal standard (100.0 mg methyl benzoate in 100 mL methanol) was added. The solution was filtered over a 0.2 µm syringe filter and directly analysed *via* HPLC. (Method C) Results are based on three independent experiments.

8 Appendix

8.1 List of abbreviations

[amim]	1-allyl-3-methylimidazolium
[C_{10}mim]	1-decyl-3-methylimidazolium
[C_{12}mim]	1-dodecyl-3-methylimidazolium
[C_{14}mim]	1-methyl-3-tetradecylimidazolium
[C_{16}mim]	1-hexdecyl-3-methylimidazolium
[C_1im]HSO_4	1-methylimidazolium hydrogensulfate
[C_2mim]	1-ethyl-3-methylimidazolium
[C_2OHmim]	1-(2-Hydroxyethyl)-3-methylimidazolium
[C_4mim]	1-butyl-3-methylimidazolium
[C_6mim]	1-hexyl-3-methylimidazolium
[C_8mim]	1-methyl-3-octylimidazolium
[$C_n$mim]	1-alkyl-3-methylimidazolium
[$HN(Et)_3$]HSO_4	triethylammonium hydrogensulfate
[$N_{0(2OH)3}$]	tris(2-hydroxyethyl)ammonium
[$N_{00(2OH)2}$]	bis(2-hydroxyethyl)ammonium
[N_{002OH}]	*N*-(2-hydroxyethyl) ammonium
[$N_{11(10)(10)}$]Cl	dimethyl-didecylammonium chloride
[P_{4444}]	tetrabutylphosphonium
ACN	acetonitrile
ADH	alcohol dehydrogenase
API	active pharmaceutical ingredients
ATPS	aqueous two-phase systems
bep	bis(2-ethylhexyl) phosphate
bistriflimide	bis(trifluoromethane)sulfonimide
BuOAc	butyl acetate
but	butyrate
c	concentration
carveol	(1*R*,5*S*)-carveol
carvone	(*S*)-(+)-carvone
[chol]	choline
CMC	critical micelle concentration
CTAB	cetyltrimethylammonium bromide
DABCO	1,4-diazabicyclo[2.2.2]octane
dbp	dibutyl phosphate
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
dec	decanoate
dihydrocarvone	(2*R*,5*S*)-dihydrocarvone
DIMCARB	*N*,*N*-dimethylammonium *N*'*N*'-dimethylcarbamate
DLLE	dispersive liquid-liquid extraction

DMAP	4-dimethylaminopyridine
DMCB	dimethylcarbonate
DMCEAP	*N*,*N*-dimethyl(cyanoethyl)ammonium propionate
DMHEEAP	*N*,*N*-dimethyl-*N*-(2-hydroxyethoxyethyl)ammonium propionate
DNA	deoxyribonucleic acid
dod	dodecanoate
dop	bis(2,4,4-trimethylpentyl) phosphinate
ds	double stranded
dtp	O,O-diethyl dithiophosphate
ERED	enoate reductase
EtOAc	ethyl acetate
fom	formate
FRET	Förstener/fluorescence resonance energy transfer
GMO	genetically modified organisms
HD	hydro distillation
hex	hexanoate
HPLC	high pressure liquid chromatography
IL	ionic liquid
IL-PLE	*Ionic liquid-based pressurized liquid extraction*
lac	lactate
lactone	carvolactone
limonene	(*R*)-(+)-limonene
MAE	microwave-assisted extraction
MPDE	multi-phase dispersive extraction
$N(CN)_2$	dicyanamide
$N(Tf)_2$	bis(trifluoromethane)sulfonimides
NMO	N-methylmorpholin-N-oxid
NMR	nuclear magnetic resonance
OAc	acetate
oct	octanoate
PE	petroleum ether
qPCR	quantitative real time polymerase chain reaction
RNase A	Ribonuclease A
RP	reversed phase
$scCO_2$	supercritical carbon dioxide
SiILs	silica-confined ionic liquids
slr.	solid:liquid-ratio
SPE	solid-phase extraction
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxy
TPAP	tetrapropylammoniumperruthenat
TRIS	tris(hydroxymethyl)-aminomethan
UAE	ultrasound-assisted extraction
VOCs	volatile organic compounds
WW	worldwide

8.2 Standard calibrations and HPLC chromatograms

8.2.1 Piperine (58)

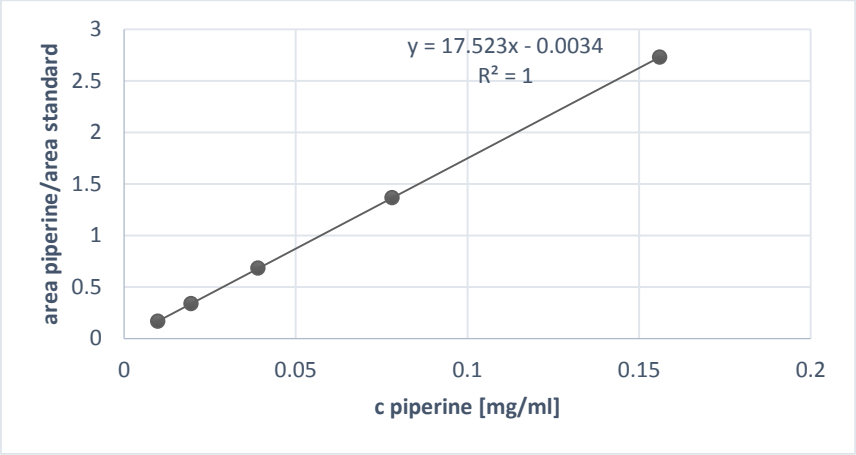


*Figure 197: Standard calibration of piperine (**69**)*

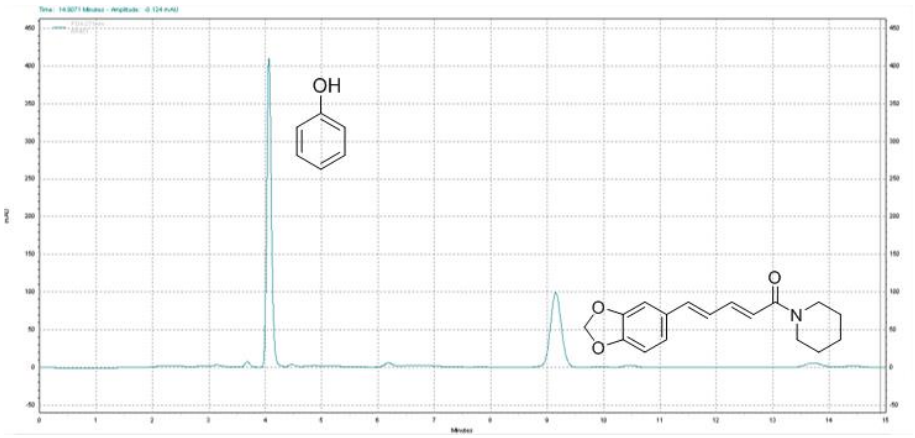


*Figure 198: Chromatogram of piperine (**58**) and standard (phenol)*

8.2.2 Eugenol (63) and isoeugenol (64)

HPLC for the detection of eugenol (**63**)

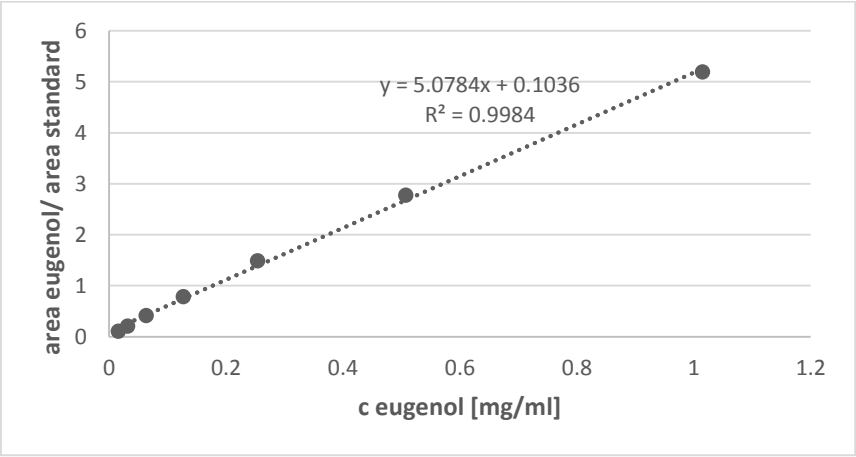


*Figure 199: Standard calibration for eugenol (**63**) for HPLC detection*

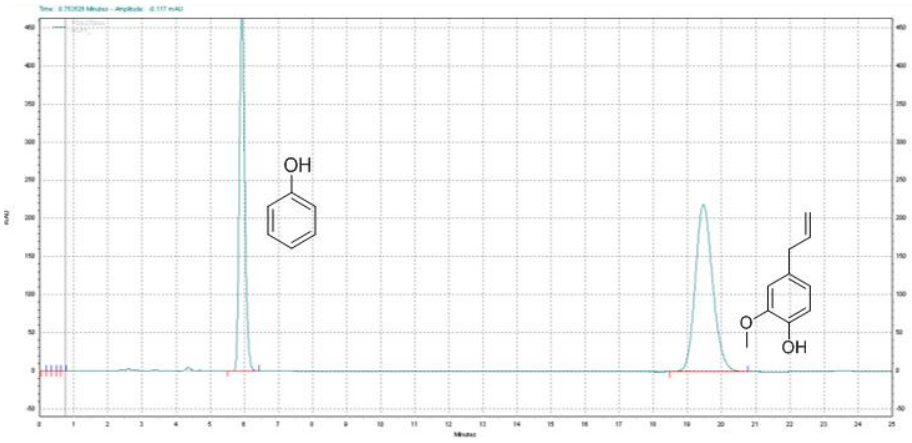


*Figure 200: Chromatogram of eugenol (**63**) and standard (phenol)*

GC for the detection of eugenol (**63**) *and isoeugenol* (**64**)

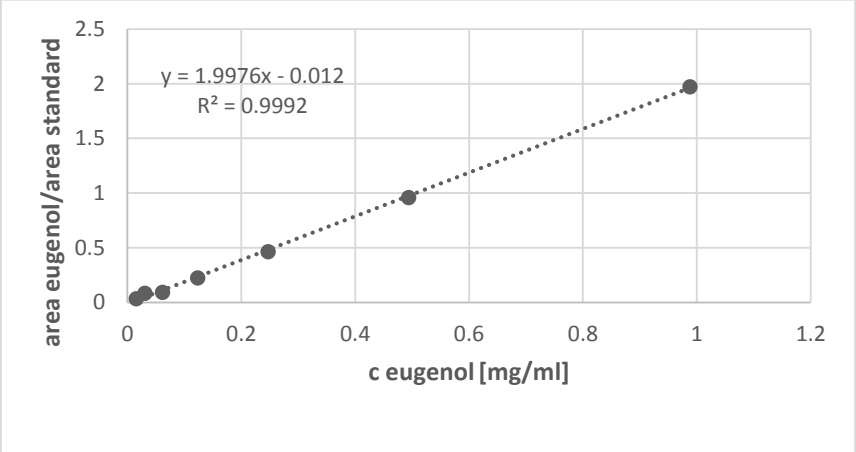


*Figure 201: Standard calibration for eugenol (**63**) for GC detection*

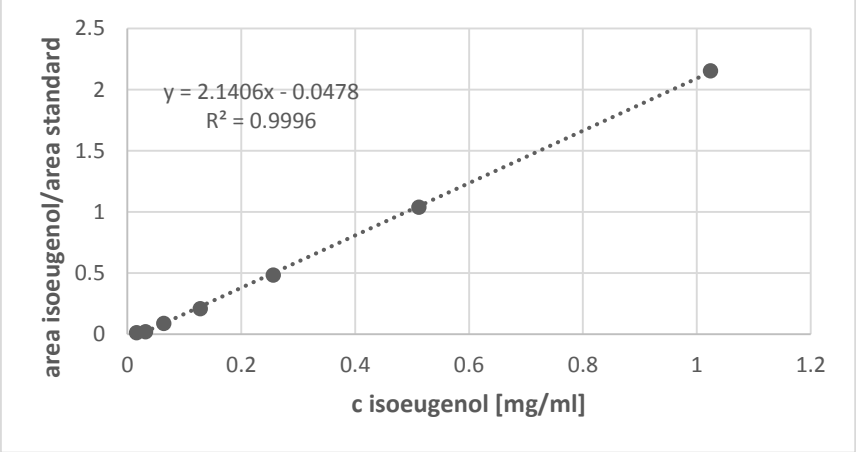


*Figure 202: Standard calibration for isoeugenol (**64**) for GC detection*

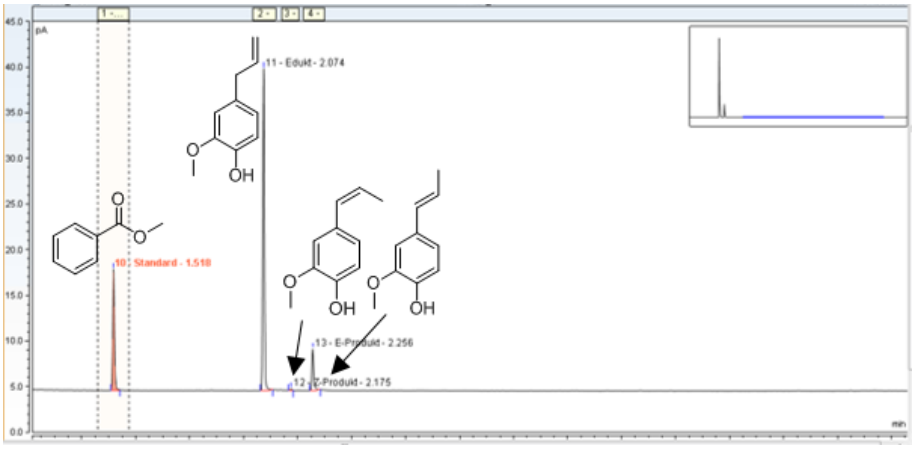


*Figure 203: Method for the detection of eugenol (**63**), isoeugenol (**64**) and standard (methyl benzoate)*

8.2.3 Betulin (69) and derivatives

8.2.3.1 Betulin (69)

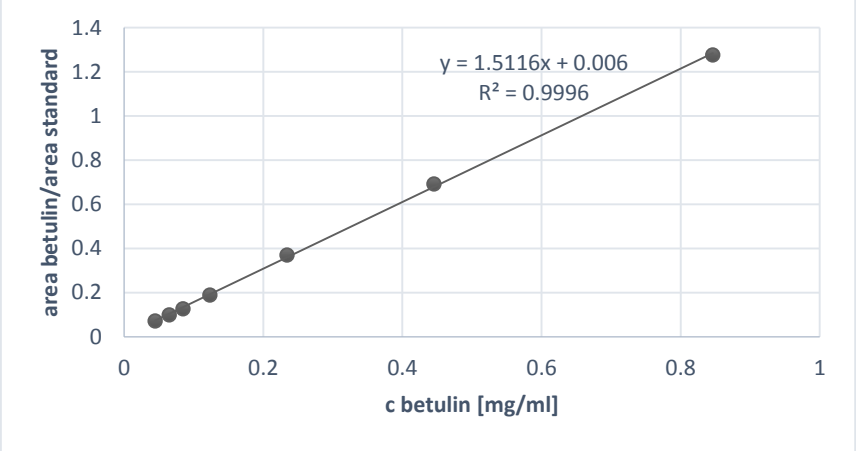


*Figure 204: Standard calibration for betulin (**69**) and standard (1-methyl-1cyclohexene)*

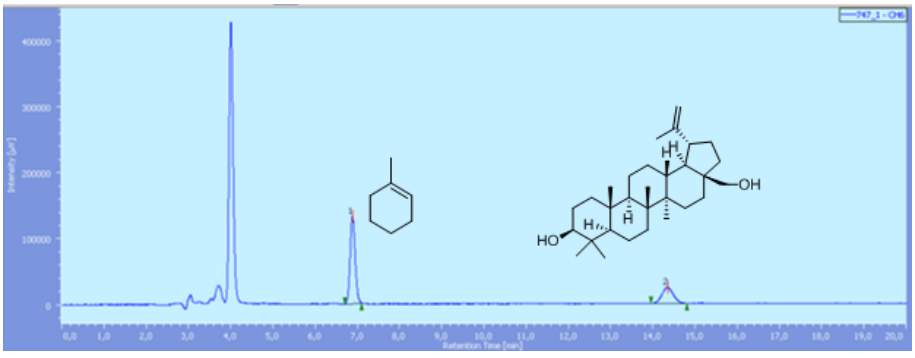


*Figure 205: Method for the detection of betulin (**69**) and standard (1-methyl-1-cyclohexene)*

8.2.3.2 TPAP/NMO-oxidation - betulonic acid (80)

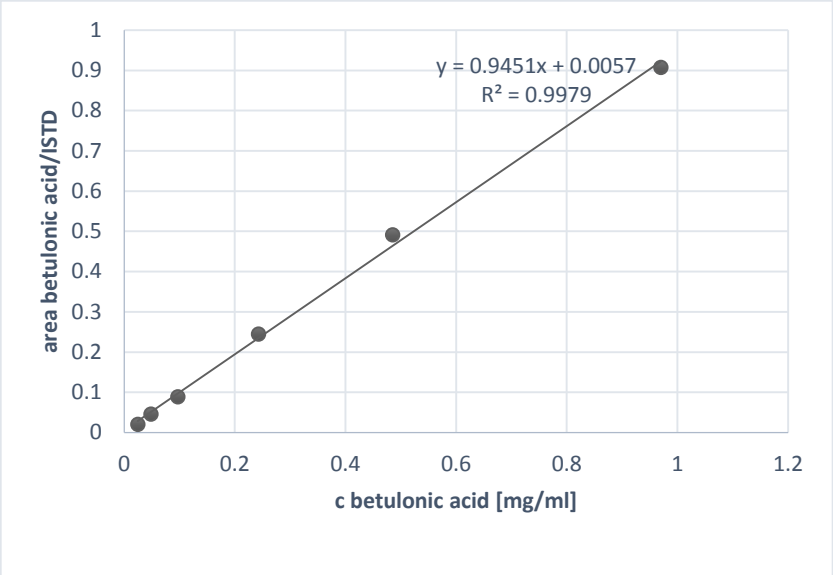


*Figure 206: Standard calibration for betulonic acid (**80**)*

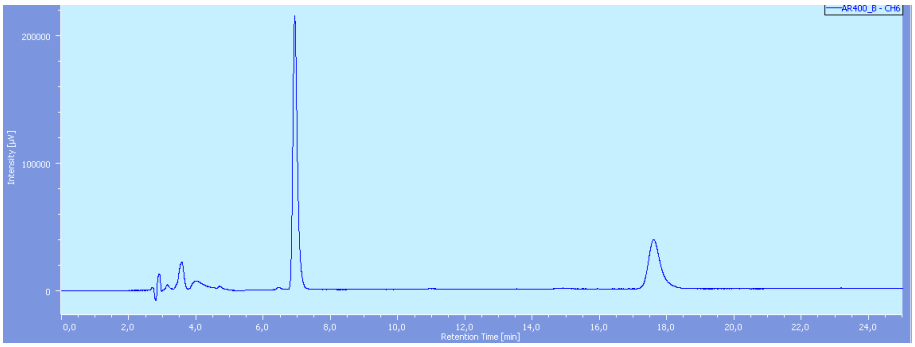


*Figure 207: HPLC chromatogram of internal standard and betulonic acid (**80**)*

8.2.3.3 BAIB-oxidation - betulinic acid (70) and betulinic aldehyde (78)

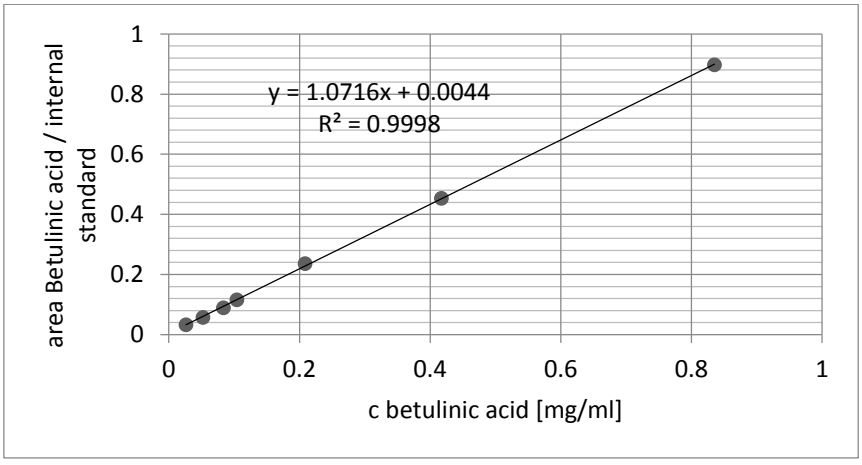


Figure 208: Calibration curve betulinic acid

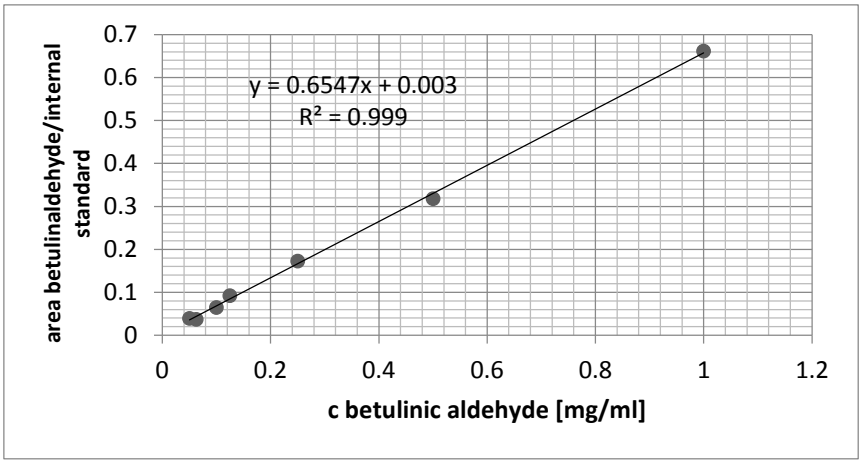


*Figure 209: Calibration curve betulinic aldehyde (**78**)*

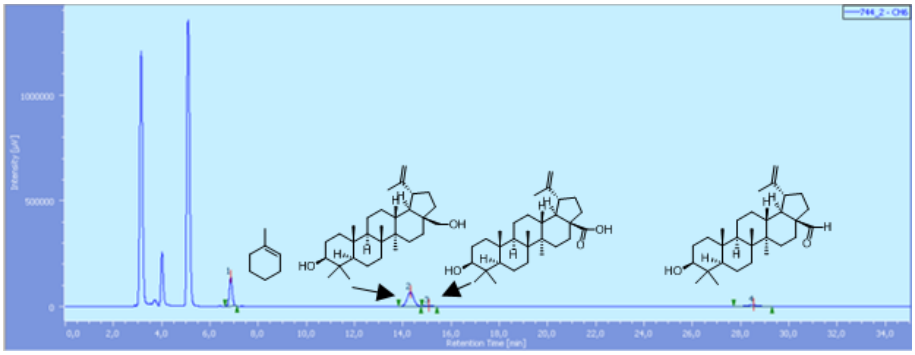


Figure 210: Chromatogram of a BAIB oxidation

8.2.3.4 Bevirimat (71) and regioisomer iso-(71)

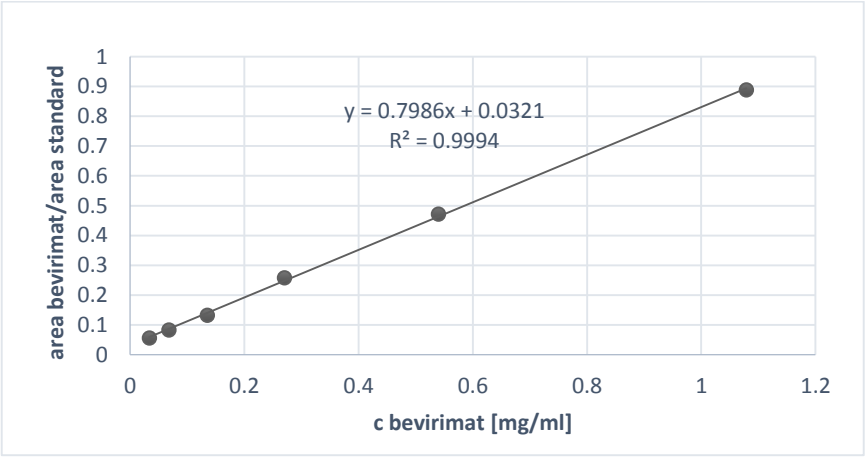


*Figure 211: Standard calibration of bevirimat (**71**)*

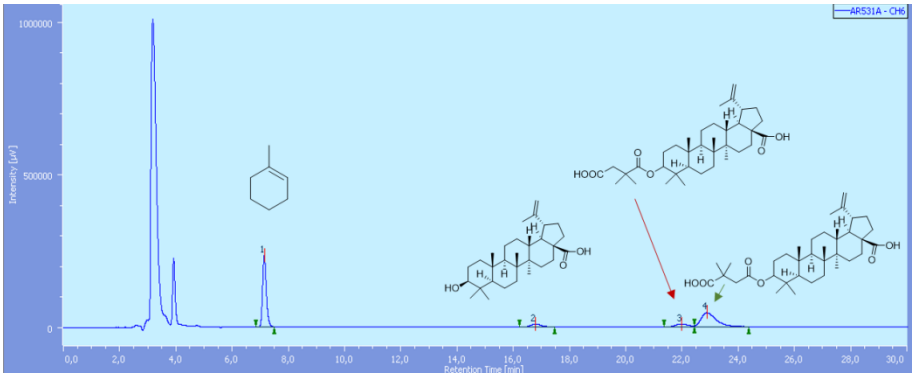


*Figure 212: Chromatogram of betulinic acid (**70**), bevirimat (**71**), regioisomer iso-(**71**) and standard*

8.2.4 Limonene (83)

GC

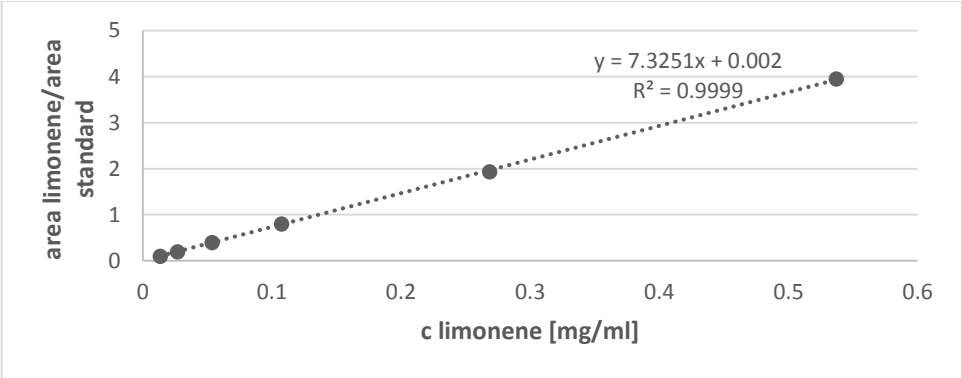


*Figure 213: Standard calibration for limonene (**83**)*

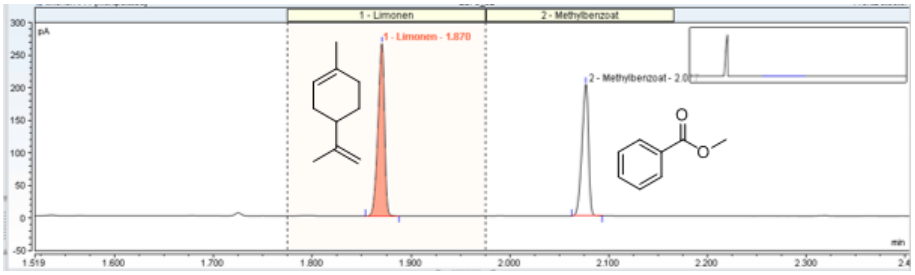


*Figure 214: GC chromatogram for the detection of limonene (**83**)*

HPLC

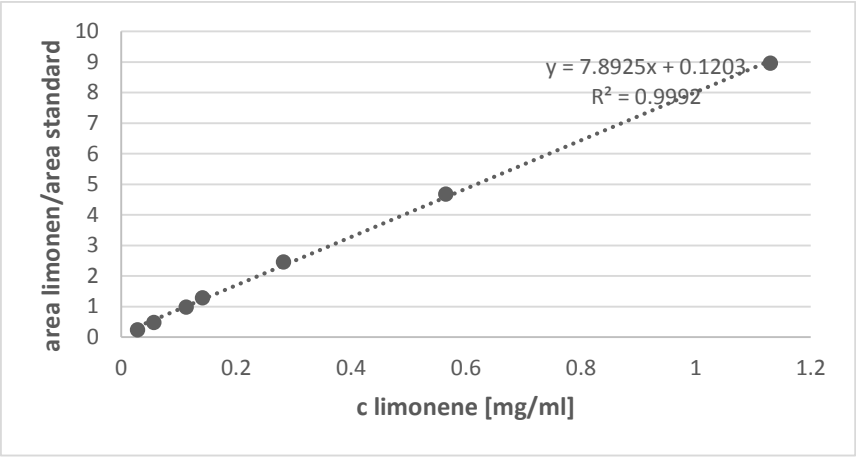


*Figure 215: Standard calibration for limonene (**83**)*

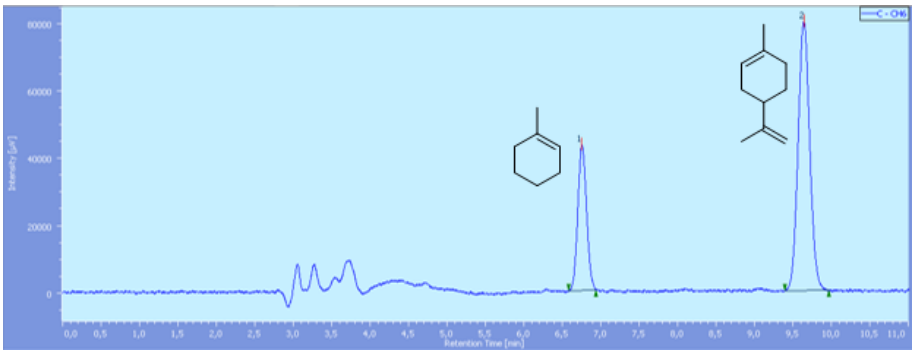


Figure 216: HPLC chromatogram for the detection of limonene

8.2.5 10-Deacetyl-baccatin III (91)

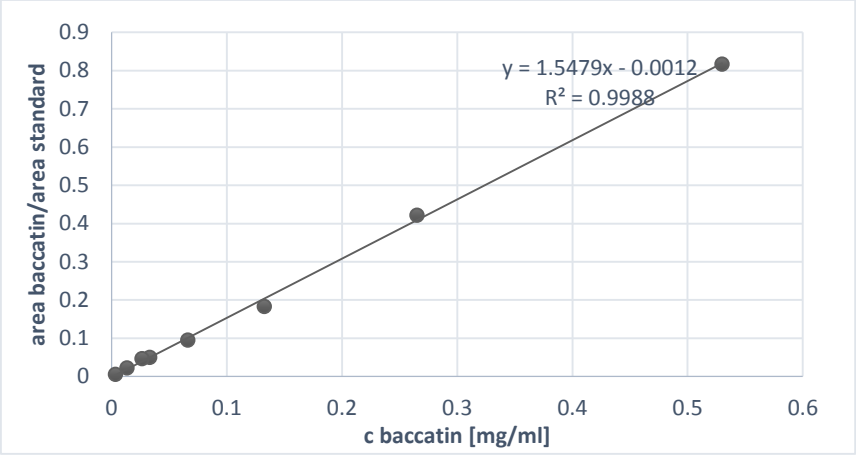


*Figure 217: Standard calibration for 10-deacetyl baccatin III (**91**)*

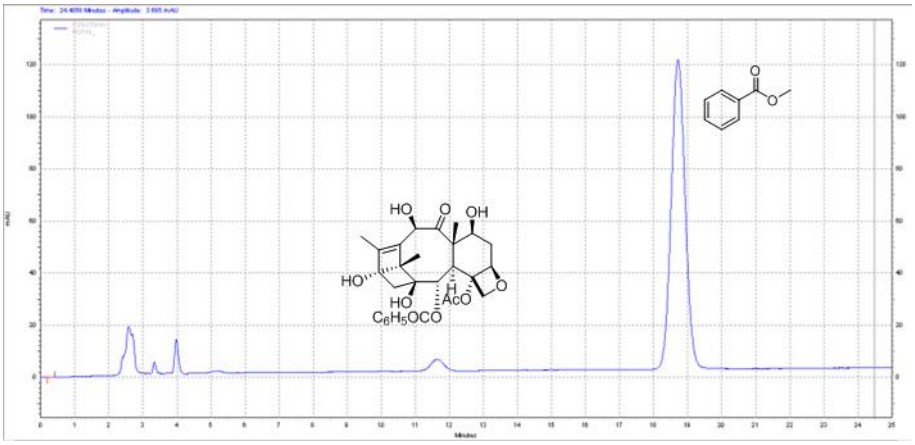


*Figure 218: Chromatogram of 10-deacetyl-baccatin III (**91**) and methylbenzoat*

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8.4 Curriculum vitae

PERSONAL DATA

Name:	Anna Ressmann
Date and place of Birth:	20.09.1986
Nationality:	Austrian

EDUCATION

04/2012 -	PhD thesis at the Institute of Applied Synthetic Chemistry, Vienna University of Technology: *Ionic liquid technologies for valuable ingredient isolation from biomass* under the supervision of Prof. Gärtner and Dr. Schröder (née Bica)
07/2010 – 05/2011	Diploma thesis at the Institute of Applied Synthetic Chemistry, Vienna University of Technology: *From plant to drug: Ionic liquids for the isolation of active ingredients* under the supervision of Prof. Gärtner and Dr. Schröder (née Bica)
10/2005 – 06/2011	Studies of Technical Chemistry at the Vienna University of Technology; Specialisation: Technical Chemistry – Synthesis (graduation with distincition)
09/1997 – 06/2005	Grammar school „Theresianum“ in Vienna (graduation with distincition)
09/1993 – 06/1997	Primary school „St. Ursula“ in Vienna

WORK EXPERIENCE

04/2012 –	Project assistant in the group of Prof. Gärtner, involved in teaching in student labs and supervision of several bachelor theses.
07/2015	Co-worker at the Biotrans 2015
2012-2015	Trainer at “FIT Tage”
07/2011 – 03/2012	Co-worker at project “Micellar catalysis in aqueous ionic liquid systems”, Vienna University of Technology under the supervision of Prof. Gärtner and Dr. Schröder (née Bica)
07/2010 – 05/2011	Diploma student in the group of Prof. Gärtner
09/2010 – 09/2011	Tutor at the Institute of Applied Synthetic Chemistry, Vienna University of Technology
08/2010	Co-worker at the European Colloquium on Heterocyclic Chemistry (ECHC) 2011
12/09 – 08/10	Internship at Affiris AG, Vienna, Austria (www.affiris.at)
08/2005, 2007, 2008	Co-worker at the theatre “Alma”, (http://alma-mahler.com/)

AWARDS AND SCHOLARSHIPS

- DOC-fFORTE scholarship for PhD thesis for 3 years (2012 – 2015)
- Scientific scholarship TU Wien for 1 year (2011/2012)
- Best poster award, Biotrans 2015, Vienna, Austria

LANGUAGES

- German (native)
- English (fluent)
- French (fluent)
- Russian (good knowledge), 2nd place at the Viennese Russian language contest 2005
- Spanish (basics)

ACTIVITIES AND HOBBIES

- Player (since 2006) and voluntary secretary (since 2008) in a football club
- Voluntary member of the Viennese football association (since 2015)
- Outdoor activities: Snowboarding and white water kayaking

LIST OF PUBLICATIONS

1. Ressmann, A. K.; Gaertner, P.; Bica, K. *Green Chem.* **2011**, *13*, 1442
2. Ressmann, A. K.; Strassl, K.; Gaertner, P.; Zhao, B.; Greiner, L.; Bica, K. *Green Chem.* **2012**, *14*, 940
3. Bica, K.; Gaertner, P.; Gritsch, P. J.; Ressmann, A. K.; Schroeder, C.; Zirbs, R. *Chem. Commun.* **2012**, *48*, 5013
4. Ressmann, A. K.; Zirbs, R.; Pressler, M.; Gaertner, P.; Bica, K. *Zeitschrift fuer Naturforschung, B: A Journal of Chemical Sciences* **2013**, *68*, 1129.
5. Gonzalez Garcia[#], E.; Ressmann, A. K.[#]; Gaertner, P.; Zirbs, R.; Mach, R. L.; Krska, R.; Bica, K.; Brunner, K. *Anal. Bioanal. Chem.* **2014**, *406*, 7773; (Forefront paper) [#]equal contributors
6. Ressmann, A. K.[#]; Gonzalez Garcia, E[#]. G.; Khlan, D.; Gaertner, P.; Mach, R. L.; Krska, R.; Brunner, K.; Bica, K. *New Journal of Chemistry* **2015**, *39*, 4994-5002; [#]equal contributors
7. Ressmann, A. K.; Bica, K. Leaching of active ingredients from plants with ionic liquids. In "Ionic Liquids for Better Separation Processes". Book series Green Chemistry and Sustainable Technology, H. Rodriguez ed, Springer, **2015**, accepted.

LIST OF POSTER PRESENTATIONS

1. Ressmann, A.K., Gärtner P., Bica, K.:
   "Active Ingredient Isolation with Ionic Liquids";
   14th Blue Danube Symposium on Heterocyclic Chemistry, Podbanské, SK; 26.11.2011 - 29.11.2011.
2. Ressmann, A.K., Gärtner P., Bica, K.:
   "Biomass Dissolution with Ionic Liquids: Improved Isolation of the Pharmaceutically Active Steroid Betulin";
   EUCHEM 2012 Conference on Molten Salts and Ionic Liquids, Newport, Wales; UK; 05.08.2012 - 10.08.2012.
3. Ressmann, A.K., Gärtner P., Bica, K.:
   "Ionic Liquids for active ingredient isolation from biomass: a greener approach for drug synthesis";
   5th Congress on Ionic Liquids, Algarve, Portugal; 21.05.2013 - 25.05.2013; in: "5th Congress on Ionic Liquids", (2013).
4. Ressmann, A.K., Gonzales Garcia, E., Gaertner, P., Mach, R.L., Brunner, K., Bica, K.:
   "An ionic liquid-buffer system for the enhancement of DNA extraction from maize";
   15th EUCHEM on Ionic Liquids and Molten Salts 2014, Tallinn, Estonia; 06.07.2014 - 11.07.2014.
5. Ressmann, A.K., Pressler, M., Zirbs, R., Gaertner, P., Bica, K.:
   "Surface-active ionic liquids for micellar extraction of piperine from black pepper";
   15th EUCHEM on Ionic Liquids and Molten Salts 2014, Tallinn, Estonia; 06.07.2014 - 11.07.2014.
6. Oberleitner, N., Ressmann, A. K., Bica, K., Gaertner, P., Mihovilovic, M.D., Bornscheuer, U.T., Rudroff, F.:
   „From waste to value – ionic liquid based extraction meets whole cell multistep biocatalysis";
   BIOTRANS 2015, Vienna, Austria; 26.-30. 7. 2015.