Role of supercritical carbon dioxide in the production of enantiomers and diastereomers

In the final report major achievements of the research are summarized according to the structure of the proposal.

Racemates studied: ibuprofen (IBU), mandelic acid (MA), 2-chloro-mandelic acid (oClMA), 3-chloro-mandelic acid (mClMA), 4-chloro-mandelic acid (pClMA), metoxy-phenyl acetic acid (MPAA), cis permetric acid (cPA), cis chrysantemic acid (cCA), trans permetric acid (tPA), phenylethanol (PE)

Resolving agents: enantiopure 1-phenyl ethane 1-amine (PhEA), 1-cyclohexyl ethane 1-amine (CHEA), 2-benzylamino-1-butanol (BAB), lysine (Lys), arginine (Arg), phenylglycine (PhG), phenylglycinol (PhGl), phenylalanine (PhA)

Non-chiral reagents: sodium hydroxide, benzyl amine (BA)

1. Crystallization of diastereomers in carbon dioxide: maximizing the enantiomeric purity attainable in a single step

A large number of resolution systems were tested, and certain racemate and resolving agent pairs were selected for detailed investigation based on solubility studies and preliminary experiments. As typical strategy of the work, first high pressure solubility studies were performed in a variable volume view cell to determine the solubility of the racemate in pure carbon dioxide. If good to acceptable solubility was found the racemate became a candidate for *in situ* resolution studies (i.e. diastereomeric salt formation reaction in carbon dioxide only, see point 1.1). If negligible solubility was found, cosolvents were also tested to solubilize the racemate as this is necessary for the extraction based resolutions. Certain racemates failing to be dissolved even in a CO_2- cosolvent mixture were excluded from the studies and were not listed in this report. Solubility criteria was 0.4 mg/mL at 20 MPa and 35 °C. Racemates showing good or reasonable solubility in either CO2 or in cosolvent CO2 mixtures were candidates for gas antisolvent fractionation based resolutions. Our methodology for the latter method was presented at a conference, and is partially included into a submitted paper[1] [2] submitted manuscript, details available upon request]

All reference samples were prepared, necessary literature survey was performed also for reference single crystallographic data etc., and powder X-ray diffraction (XRD) patterns were measured.

1.1. Diastereomer salt formation in carbon dioxide with acid–base reactions

The following racemates were candidates of the in situ resolution: cCA, cPA, IBU, MA, MPAA, tPA and tests were performed to map the potentially successful resolutions. The resolutions were based on diastereomer salt crystallization in supercritical carbon dioxide. Unreacted compounds were removed by an extraction step.

The chiral resolution of two racemic cyclopropanecarboxylic acids (cCA and cPA) with the resolving agents BAB and PhEA was investigated in details. Experiments were performed in a stirred tank reactor and good enantioselectivities were obtained (optical purity >70%) in a single step. It was found that pressure, temperature and density strongly affect both the optical purity and the selectivity of these resolutions. PhEA did not prove to be a good resolving agent although diastereomers were formed, while BAB was able to resolve both acids, albeit in different pressure ranges: the optimal circumstances for resolving cCA were at 40 °C and 100 bar, yielding a selectivity value of 0.46. The resolution of cPA at 200 bar and at 35 °C resulted in a selectivity value of 0.40 in the raffinate. XRD analysis showed that the cPA–BAB diastereomer salt formed with the *in vacuo* technique (dissolving both the racemate and the resolving agent in a suitable organic solvent followed by the vacuum evaporation of the solvent and supercritical carbon dioxide extraction of the solid) had a crystal structure different from the diastereomer salts obtained in situ (the structure of which matched literature data) probably due to the role of kinetic and thermodynamic control.[3]

IBU with Lys, Arg and PhGl were tested. In case of IBU-Lys and IBU-Arg systems there was no diastereomeric salt crystallization within a week. We suppose it is because of the extremely low solubility of the resolving agents as XRD measurements did not indicate a byproduct formation either.

In case of IBU-PhGl system there was a huge amount of crystals. Pressure has limited effects on the *ee*, while temperature has an important role in the extraction step. Time requirement of the reaction is >1 days. Based on the experiences with the IBU-PhEA system[4] [5], detailed investigations were performed to understand better the correlation of the pressure and temperature effects in a certain region (<20 MPa, >50 °C).[6] Surprisingly only moderate effects of temperature or pressure were observed. On the other hand, if the carbamate of PhG is reacted with IBU in $scCO_2$, the reaction rate is much higher than in case of reacting PhG with IBU. Furthermore, initial selectivity (after 1 hour) is higher than the equilibrium selectivity (>1 days). Equilibrium selectivities and crystal structure of solid products are the same for resolutions starting from PhG and from its carbamate. Further investigations are planned with carbamates and cooperation is initiated with computational chemists to clarify the reasons.

Study on the in situ resolution of MPAA with CHEA was completed also. The reaction is very slow, even after 12 days the conversion was approx. 80%. The reaction time had the major effect on the conversion, but also increasing pressure increased reaction rate especially during the initial phase. As best case approx. 50% diastereomeric excess was achieved.[7]

To summarize: in situ resolutions are only possible in a realistic time frame if both the racemate and the resolving agent are at least slightly soluble in supercritical carbon dioxide. However, a reasonable solubility of the racemate is required for the extraction step. Effects of process parameters (pressure and temperature) in principle show varied tendencies, as in cases when the reaction rate is limited by the low solubility of the racemate increasing the pressure increases reaction rate, while when not only racemate is well soluble in CO_2 but the dissociation of the diastereomeric salt is significant, the lowest possible pressure should be applied.

1.2. Antisolvent diastereomeric salt formation with acid–base reactions

Antisolvent precipitation based resolution techniques were developed for the suitable racemates. Antisolvent fractionation based diastereomeric salt formation was not possible with cCA due to the low stability (high solubility) of the formed diastereomeric salts.

We have developed a method for the crystallization and resolution of racemic cPA with PhEA as a resolving agent. The crystallized products had yields similar to those previously reported in the literature with different methods (10–40% compared to 34%), but the diastereomeric excess in a single step exceeded those reported earlier (85–95% (-)-cPA–PhEA compared to 74%). A significant pressure effect was observed which could not be explained by solubility effects alone, we assume salt structure and dissociation to be the key factors. Between 10–12 MPa, racemic cPA forms the most stable salt with PhEA, thus almost no de is observed. Between 130–170 bar, the (+)-cPA–PhEA salt dissociates while the antipode is stable, leading to excellent diastereoselectivity. Between 180–200 bar, both salts dissociate, leading to virtually no salt yield.[8]

Resolution of MPAA with ChEA is possible using gas antisolvent precipitation with carbon dioxide. Based on atmospheric solubility mapping (see point 1.3) toluene:acetonitrile 1:1 mixture was a suitable organic solvent. Half equivalent amount of resolving agent is optimal for maximizing selectivity while keeping the amount of resolving agent as low as possible. Both the temperature and pressure influence the resolution significantly, and the optimal setting was found to be 40 °C and 12 MPa. Diastereomeric excess values over 55 % along with 80 % yields were achieved at optimal conditions in a single step. The obtained diastereomeric salts show similar diastereomeric excess values as optimized atmospheric resolutions, but show higher crystallinity and yields while requiring significantly lower processing time. The results obtained by GAS antisolvent method show a good perspective for the development of a semi-continuous process.[9]

The resolution and crystallization of IBU with *R*-PhEA based on diastereomeric salt formation using the supercritical gas antisolvent (GAS) method was also successful. The diastereomeric purity of the crystalline diastereomer salt (70-80% in a single step) was not affected by either parameter (pressure, temperature, organic solvent-to-carbon dioxide ratio) in the range investigated. However, yields, and thus selectivity (a product of yield and diastereomeric purity) was strongly affected by pressure and the carbon dioxide-organic solvent ratio. The two effects were separated, and both were significant. At the optimal settings (10 MPa, 35 °C, 12.3 mol/mol carbon dioxide-methanol ratio) the selectivity (0.444) slightly exceeded previously reported values using supercritical carbon dioxide, with a significant reduction in operating time.[10]

oClMA with PhEA and pClMA with PhEA and CHEA were tested in a large number of solvents. Based on atmospheric solubility mapping (see point 1.3) methanol was selected. The solubility of the unreacted acid, in spite of the cosolvent present in the system, is still low, thus a new extraction procedure was developed for cosolvent extraction with interconnected pumps to ensure constant fluid phase composition during the extraction phase. However, the constant solvent-to-carbon dioxide extraction gave lower yields, so finally the detailed investigations were performed by pure CO2 extraction. Application of the antisolvent approach in case of pClMA and PhEA considerably reduces the time and solvent requirement compared with the classical resolution. Starting from acid:base=2:1 molar ratio, (*R*)-PhEA was found to be an efficient resolving agent for obtaining the less soluble (most stable, highest mp.) (*R*,*R*)-diastereomeric salt (yield and diastereomeric excess 80% and 64% respectively). However lower *de* was observed for (*R*)-ChEA as a resolving agent (yield and diastereomeric excess 80% and 49% respectively). Parametric studies such as the effect of different temperatures, and solvent ratios on diastereomeric salt formation reaction were studied. There was no pressure effect on *de*, but increasing the pressure the Y was decreased (12-20 MPa range). There was also no temperature effect on *de*, but by increasing the temperature the yield was increased. Only the effect of the solvent ratio was found to be significant of *de*. Applying less organic solvent, the *de* decreased, but the yield increased. 70% de was achieved in a single step as maximum. The reaction conditions were optimized to 40 °C and 16 MPa for investigating the influence of other parameters. Formed diastereomeric samples were characterized using scanning electron microscopy (SEM) and powder X-ray diffraction (XRD) Study confirmed the formation of crystalline diastereomeric salt species of *R*-*R* form. DSC study shows an improved melting behavior in comparison with literature data.[11]

Ibuprofen (IBU) with lysine (Lys) and ibuprofen with arginine (Arg) and ibuprofen with (S)-(+)-2-Phenylglycinol (PhGl), phenylalanine and phenylglycin were tested in various solvents (>6) in order to apply them for the resolution of IBU. It was difficult to find a solvent system and dissolution method, which enables a high concentration of the salts before antisolvent precipitation. In case of IBU-Lys and IBU-Arg system the diastereomeric excess values of the salts were zero, although the crystallizations were successful. The observation confirms the dominancy of kinetic effects at GAS techniques, as both amino acids are suitable resolving agents for IBU by atmospheric crystallization. Gas antisolvent precipitation of IBU-PhGl resulted in good enantiomeric excess values, around 60%, with excellent yields over 60%.

Experiments were conducted, based on the article of Martín et al.[12] , who were separating MA enantiomers using PhEA. Several set of experiments showed that the published reaction cannot be repeated in our setup. Systematic investigation showed that the reason was, that the dimethyl sulfoxide (DMSO) – ethyl-acetate solution of MA (used in the paper) does not form a single phase with CO_2 at the given pressure and temperature. They used an equipment with the filter at the bottom, while our filter is at the top. So we concluded, that due to the unexpected number of phases the same organic solvent mixture cannot be used in our setup. Using a small batch reactor we changed the organic solvent to methanol and changed the method of the experiments (setup, process conditions) resulting in even better ee values (>60%) than in the referred paper. A detailed study was conducted on the effects of the operational parameters pressure (12-20 MPa), temperature (35-55 °C) and co-solvent concentration (33-99 mg/ml) using a full fractional experimental design. At 12 MPa, 35 °C and 99 mg/ml methanol concentration, a selectivity of 0.52 and a diastereomeric excess of 62% was reached.[13,14]

It can be concluded from the short summaries of the detailed parametric optimization studies above, that the general trend of pressure and temperature effects can hardly be defined, each resolution system has its own behavior. Pressure and temperature might either effect both the yields and diastereomeric excess values of the precipitated salts, or only the yields are influenced in the studied ranges. The solvent-to-carbon dioxide ratio, which phenomenon was not known previously, plays an important role in the resolution system. Its effects were examined in ibuprofen (IBU) – PhEA, cis-permetric acid (CPA) – benzylamino-butanol (BAB) resolutions system in methanol, ethanol, and their mixture. In a wide range (at constant P and T) linear correlation was found of yield and solvent to CO2 molar ratio, values irrespective from the type of the alcohol in case of IBU-PhEA without *de* change. By a novel measurement technique we measured the dissociation of the individual, diastereopure cPA-BAB salts in methanol, which showed strong parameter dependence and a possibility to maximize the stability ratio of the two salts by optimizing parameters. By this fast *de* change found earlier (from 0 to >90% by small change in conditions) can be explained more precisely.

The effect of temperature on the selectivity of 4-chloromandelic acid with enantiopure PhEA was studied. It appeared that temperature does not have any significant effect on the enantiomeric purity or the yield of the products. Thus, it is obvious that there cannot be any interaction between the temperature and the pressure, as either of them has any significant effect at all. Mandelic acid-(*R*)-phenyl-ethyl-amine system: We carried out several experiments, changing various factors (temperature, pressure, amount of organic solvent) in order to influence the results. We reached several satisfying results, having the enantiometric excess for *R* between approximately 50% and 65%, and for *S* between approximately 30% and 45%. Then we carried out a full experiment plan, with the 3 factors mentioned above with similar results.

1.3. Carrying out the atmospheric equivalents of the resolutions in parts 1.1 and 1.2.

Atmospheric solubility measurements of each compound involved in antisolvent precipitation were performed for solvent screening. As an example the results for MPAA are detailed here.

While fair solubility of the rac-MPAA in scCO2 is required for an efficient extraction of the unreacted enantiomers, an organic solvent is also required to dissolve all components including the diastereomeric salts. Components rac-MPAA and (*R*)-CHEA are highly soluble in polar solvents and also showed comparable solubility in non-polar solvents (Table 1.). The higher solubility in polar solvents is attributed to the presence of carboxylic and amine functional groups in their chemical structure.

The solvents, which have good dissolving power for all components involved (Table 1.), were also tested for their applicability in gas antisolvent precipitation. These preliminary results are summarized in Table 2.

In cases when crystal structure of the compound studied was not known, we aimed to grow single crystals and determine the single crystal structure in cooperation with the Hungarian Academy of Science (Petra Bombicz). For all resolution systems XRD measurements were performed and solid phases obtained by various methods were extensively compared, in some cases leading to important novel information. For example carbamate formation of ChEA and evidence of same crystalline structure with various crystallization habits (see in chapter 4.). In certain cases when further information were required melting points by DSC were measured, thermal decomposition followed by evolved gas analysis were performed, FTIR measurements were done etc.

Table 1. Solubility of MPAA, CHEA in organic solvents at ambient temperatures and pressures (solvents are listed in decreasing polarity).

Solvent	solubility of rac-MPAA (g/ml) at 26 °C	solubility of CHEA (g/ml) at 26 °C
Acetonitrile	2.29	2.70
Methanol	2.97	3.86
Ethanol	2.94	3.95
2-Propanol	2.80	3.83
EtOH : 2-PrOH (1:1)	2.85	3.83
Ethyl acetate	0.05	Insoluble
Ethanol : Ethyl acetate	1.30	0.70
Toluene	1.80	1.20
Toluene : Acetonitrile (1:1)	1.30	1.87
Methyl isobutyl ketone	0.80	Insoluble
Ether	2.10	0.35
Ether : acetonitrile (1:1)	2.50	1.35
Hexane	0.20	Insoluble

Table 2. Effect of solvent on salt formation at 12 MPa, 40 °C, 8.8 g CO2/g solvent and 0.026 g/ml MPAA and 0.013 g/ml CHEA in the reactor and at atmospheric conditions.

Solvent	Atmospheric reference, observation, results	GAS precipitation		
		precipitation observed	ee_R (%)	Y_R (%)
Methanol	No	Yes	56	23
Ethanol	No	Yes	70	21
Toluene	No	Yes	50	52
Acetonitrile	No	Yes	34	60
Toluene : Acetonitrile (1:1)	Yes, ee=42%, Y=78%	Yes	55	80
Ethanol : 2-Propanol (1:1)	No	Yes	56	71

Growing single crystals were successful for MPAA, MPAA-ChEA salts (see results below) and pClMA-ChEA salts and pClMA-triptamine salt, oClMA-ChEA salt, but the crystal structures for the latter cases are not yet resolved.

The system of intermolecular interactions is very similar in the solid state of the chiral and racemic α-methoxyphenylacetic acids (α-MPAA) indicated by both Differential Scanning Calorimetry (DSC) and FTIR-spectroscopy, and even proved by single crystal structure determination. It makes the resolution of such a racemate challenging even by supercritical fluid extraction. The chiral (S)-α-methoxyphenylacetic acid (1) crystallizes in orthorhombic crystal system. The packing arrangement in the (S)-(S) ammonium carboxylate type salt is columnar. The columns are hydrophylic inward and hydrophobic outward, that results in fibrous growth of the salt crystals melting at 163°C. The crystal habit of the higher melting diastereomeric (S)-(R) salt (mp. 187°C) is also fibrous, its structure is closely related to the (S)-(S)-salt by the similarities observed in their FTIR spectra. The DSC and powder XRD studies on the chiral and racemic acids, and on the pair of diastereomeric salts helped us to construct and calculate the binary and ternary phase diagrams of the system components, including their eutectic temperatures and compositions, as well.[15]

2. Enzyme-catalyzed resolution in carbon dioxide, with special attention given to product separation (production of enantiomers and separation of products in supercritical carbon dioxide is a new approach)

Enantioselective esterification of phenylethanol (PE) with triacetin, tributyrin and corn germ oil was studied extensively with *Candida antarctica* lypase B (CALB). Planned ring opening reaction investigations (gamma-valerolactone) were not successful because the lack of selectivity of the enzymes and also the difficult availability of the suitable candidate molecules and the enantiopure products needed for the method development. For each reaction systems detailed investigation of the phase equilibrium (or solubility of all components involved), the temperature and pressure optimization of the reaction and if there was a possibility the separation of the products were performed. Product and substrate concentrations as functions of time were measured by a newly developed sampling method and the results were described by simple models (see chapter 5).[16][17][18]

The enantioselective esterification of PE with triacetin catalyzed by CALB is efficient in CO_2. Already at 10 MPa pressure at 40 °C. Controlling the water activity is important to maximize the reaction rate and maintain the enzyme activity, however complete drying of the CO_2 phase is not favorable. The solubilities of the PE and its acetate are nearly the same. The reaction has a high enantioselectivity towards the (*R*)-ester (E>1000 in all cases). 23 different immobilized enzyme preparations (non-commercial ones were prepared by the group of Prof. Poppe, BME) were evaluated. Sol-gel immobilized enzyme preparations were more active in $scCO_2$ than in organic solvents, while the performance of covalently bonded and adsorbed enzymes were comparable in the various non-polar solvents. Neat reactions were also efficient.

At the enantioselective esterification of PE with tributyrin, a strong correlation was found with the phase equilibrium of the system. At certain setups (lower P and/or higher T) the reaction is highly heterogeneous (fluid phase enriched in esters; liquid phase enriched in reagents and mono-, diglycerides and glycerol; immobilized enzyme). At intermediate parameters the reaction is initially running in a homogenous fluid phase (+ immobilized enzyme) but later the precipitation of mono- and diglycerides and glycerol starts at certain conversion. At the lowest pressure the system is continuously biphasic (fluid phase + enzyme phase). Selectivity is independent from the number of phases, while the reaction rate is significantly higher at lower pressure and higher temperature (3 phases).

For the tributyrin - phenylethanol reaction system, although the phase equilibrium measurements showed some differences in the solubility of the ester product and the alcohol, this difference was not high enough for an efficient preparative scale separation. As a consequence we decided to investigate a novel system (not planned originally): how can we apply the corn germ oil as esterification reagent for the kinetic resolution of 1-phenylethanol. Instead of further trials to do ring opening reaction and optimize those, we modified the idea to use natural oils. Further advantages, that the suitable raw materials for novel ring opening reactions are hardly available, while the pure natural oils (corn germ, pumpkin oil) were prepared in our laboratories by supercritical fluid extraction. The major problem was to develop the suitable analytical methods, which took more than a year. The reaction was performed with a great selectivity in an oil-CO_2 mixture. At lower pressures only the alcohol is extracted from the reaction mixtures, while at higher pressures (>13 MPa) the longer fatty acid - alcohol extras can also be extracted, while the oil phase is practically insoluble at pressures <25 MPa.

Resolution of racemic 1-phenylethanol with corn germ oil in $scCO_2$ was successfully done with ≥45% equilibrium conversion concerning the racemic alcohol, and ≥99.5% enantiomeric excess of the ester products was obtained. Corn germ oil forms biphasic system with scCO2 up to 20 MPa, therefore the ester products and the remained alcohol after the resolution process were extracted with scCO2. The separation process of alcohol and esters was based on sequential pressure drop (10 MPa and atmospheric). Owing to the specified extraction - separation system, the ester products and the remaining alcohol could be separated with the purity over 90% and with the mass balance error under 10%. Further aim is to design a semi-continuous system based on the presented results, for the resolution of continuous racemic alcohol feed and direct separation of enantiopure alcohol and ester by $scCO_2$.[19]

3. Further purification of enantiomer mixtures, production of very high purity enantiomers in an upscalable process

The semi-continuous crystallization equipment was set up (see 4.) in order to produce larger quantity of the equimolar diastereomeric salt mixture of IBU-PhEA. Some of the measurements were performed additionally starting form calculated mixtures of enantiomer and racemate. Although when the original research plan was constructed we had concerns if the enantiomeric or diastereomeric further purification by supercritical fluid techniques will be successful, the reality is, that not only the repeated resolution, the recrystallization of the diastereomeric salts, but also a completely novel field, the antisolvent further purification of enantiomeric mixtures are all success stories. Detailed investigations IBU - PhEA, pClMA – PhEA system were done compared to the melting point phase diagram. MPAA, oClMA were also tested, melting point vs. composition diagrams were determined as well as the best way to produce pure enantiomer out of those. This novel area raised several interesting questions regarding planning the reactions, equipment setup, evaluation methods to compare most fairly to literature data etc.

3.1. Recrystallization of the diastereomers of enantiomer mixtures with antisolvent processes

The resolution will be carried out by applying a method optimized in Part 1, after preparing but without decomposing the diastereomer salts, which is equivalent to the well-known diastereomer salt recrystallization techniques.

Achieving diastereomeric or enantiomeric purities (de or ee) over 0.99 is a critical part of any optical resolution techniques. Most of the methods (optical resolution of a racemate, asymmetric synthesis, kinetic resolution) give only enantiomeric or diastereomeric mixtures of less than sufficient purities in a single step. The first detailed study on the application possibilities of antisolvent precipitation with supercritical carbon dioxide to achieve high purity diastereomeric salts was submitted to the Journal Chemical Engineering and Technology. Detailed investigation of the effect of the molar ratio of the chiral amine to the racemic acid led to the conclusion, that although salt are known not to be soluble in supercritical carbon dioxide, the nascent salts' stabilities are detectable. Salts' solubilities were different and both salts' decomposition in carbon dioxide – organic solvent mixtures were proved. The further purification was investigated as repeated resolution (0.5 molar ratio of amine to acid) and recrystallization (1 molar ratio) using both batch and semi-continuous antisolvent methods. Significant changes for the yields in function of starting ee could not been observed neither in the 1 nor in the 0.5 molar ratio experiments. A clearly visible tendency can be observed plotting the raffinate and extract ee in function of the starting ee. With the increasing starting ee the raffinate ee values are continuously increasing over ee>0.99 (pure enantiomer according to the resolution of the analytics). No eutectic behavior was detected. The effect of racemic compound and resolving agent molar ratio between 0.3-1.26 were investigated in details. Based on the results we can say in each cases that the resulting crystalline phase reaches de>0.99 in three consecutive steps.[20]

A novel, green possibility of the further purification of the diastereomeric salt of 4-chloromandelic acid and 1-phenylethylamine has been also developed. Gas antisolvent method using supercritical carbon dioxide was applied for the first time to precipitate the diastereomeric salts with increased purity followed by the supercritical fluid extraction of the dissolved diastereomers. The *RR*-salt can be purified to >99%, while fractionation based purification of the *SR*-salt is limited to approx. 80%. The limiting initial diastereomeric excess correlates strongly to the atmospheric melting eutectic composition of the same salts which suggests that despite the fast precipitation, the diastereomeric excess of the solid product is not kinetically determined. The efficiency of the diastereomeric enrichment is in the same range as that of the atmospheric reference experiments, however, technological advantages provided by the antisolvent precipitation method such as fast processing and dry product obtained, suggest that this novel procedure is a promising alternative of the atmospheric methods.[21] Further studies with varied solvent-to-carbon dioxide ratio showed that the eutectic composition was not influenced in a detectable measure by the varied solvent polarity, but the de final – de initial curve was shifted and also yields were modified. This means, that number of the further purification steps are highly influenced by the composition of the fluid phase, while limits of diastereomeric excess are not influenced. As result the enhancement of diastereomeric excess (de*yield) values were at certain initial de-s independent from solvent-to-carbon dioxide ratio.

Recrystallization-based further purification of the mandelic acid (MA) – (*R*)-phenylethane-1-amine (PhEA) diastereomeric salts, applying the resolving agent in molar equivalent quantity to a non-racemic mixture of mandelic acid. It has been found that the raffinate de – initial de diagram correlates strongly to the diastereomeric salts' atmospheric melting point phase diagram, and that two additional recrystallization steps are sufficient two exceed 98% de.[13]

3.2. Recrystallization of the salts of enantiomer mixtures formed with achiral reagents with antisolvent processes

Non-chiral salt precipitation based diastereomeric enrichment turned out to be less efficient and more problematic with antisolvent method than by the *in vacuo* technique. When solutions of the studied enantiomeric mixture with a strong base (NaOH or CaOH) was contacted with CO_2, the inorganic salt of the base and carbon dioxide precipitated instantly instead of a purified enantiomeric mixture. Thus as achiral reagent weak bases can be selected. Enantiomeric enrichment of IBU with BA can be performed, but selectivity is low, lower than with the recrystallization of the diastereomeric salts (see previous point). Crystallization of the oClMA - triptamine salt did not lead to any enrichment.

As crystallization of achiral salts of enantiomeric mixtures were not really successful, although some purifications could be achieved, instead of forcing the original idea we did something completely novel: developed a process to enrich enantiomeric mixtures in supercritical carbon dioxide – solvent mixtures by the self-disproportionation of the enantiomers.

Gas antisolvent precipitation is a particle formation technique, when typically pressurized carbon dioxide is added to an organic solution resulting in immediate and high oversaturation and precipitation of fine particles. Provided that a reasonable share of the originally dissolved material remains dissolved in the carbon dioxide – organic mixed solvent, these components can be extracted during the washing phase. The method has been applied for the first time for enantiomeric enrichment of non-racemic mixtures, demonstrated on the example of chlorinated mandelic acid derivatives. Due to self-disproportionation of enantiomers, the precipitated solid and the extracted fractions have different enantiomeric excesses if GASF is done on a non-racemic mixture. However, there is a limit in the enantiomeric excess (*ee*) that can be achieved correlating strongly with the atmospheric melting eutectic behavior of the compounds. Thus, if initial enantiomeric mixtures have a higher than eutectic *ee*, a >99% *ee* can be reached in the crystalline product. The strong correlation between the high-pressure experiments and the atmospheric melting eutectic behavior suggests that despite the very large oversaturation during the antisolvent precipitation, the composition of the products (i.e. the crystalline and the extracted phases) is thermodynamically determined. Technological advantages such as short operational time, or the possibility of controlling the crystal morphology suggest that the development of an efficient technique of enantiomeric purification is possible based on gas antisolvent fractionation.[22]

3.3. Atmospheric reference experiments for the further purifications of enantiomer mixtures

When atmospheric reference experiments were well published in the literature, only some repetitions were done to confirm the result. (IBU – PhEA)

Atmospheric experiments using a molar equivalent quantity of PhEA were carried out investigating the 4-chloromandelic acid–PhEA system. Although there is a literature reference about this resolution system, the possibilities of the further purification were not studied in details. Due to the higher raffinate yields, the selectivity of the single step resolution starting from a racemic acid is higher in the case of the GAS experiments (roughly 55%) than in the case of the atmospheric experiments (42%) and is comparable to the literature reference (51%). Furthermore, atmospheric self-disproportionation based separations of pClMA and oClMA were not possible from hexane.

4. Development of antisolvent crystallization to control particle size and morphology

In the literature, there are various crystallization methods with abbreviated names that utilize supercritical solvents. In all studied crystallization based resolution systems the XRD patterns of the obtained solid phases were checked and relevant shapes and sizes of crystals were observed by SEM. In most of the cases we did not observe any unusual tendency, mean particle sizes were influenced by the oversaturation (the higher the solvent-to-carbon dioxide ratio the larger the mean particle size) and morphology deviations were only found for pClMA – ChEA salt.[11] Samples were highly crystalline and mean particle size differed largely at the same process conditions and varied salt system.

We have developed a method for the crystallization and resolution of racemic cPA with PhEA as a resolving agent and the batch GAS process was successfully realized as a semi-continuous SAS process, which involved a 10-fold scale-up with respect to the mass of cPA. The crystallized salt had excellent diastereomeric excess (up to 95% (-)-cPA–PhEA) and a uniform fibrous structure with very high length-to-diameter ratios (500–700 nm diameter versus several tens of μm length).[8]

Antisolvent crystallization development for controlling particle size and morphology was performed by developing an automated semi-continuous (SAS) equipment. Similarity of results both *de* and crystallization habits were proven of the novel SAS and the GAS methods. At IBU-PhEA system using methanol and ethanol, yield curves as a function of the molar carbon dioxide-organic solvent ratio are the same for both solvents. By selecting appropriate parameters, the crystal habit (average crystal size and crystal size distribution) can be influenced. Some crystals formed thin fibers with diameters in the micrometer range (1–3 μm), but certain parameters yielded shorter and thicker bladed crystals.[10]

5. Modeling and analytical developments

For all studied racemates a suitable routine analytical method was developed either at BME or in cooperation with Cylcolab Ltd. to determine the enantiomeric purity of the obtained large number of samples. Methods are presented in the relevant publications and are also available upon request. [2324]

In case of kinetic resolutions (presented in point 2.) it was also important to determine the concentration of the remaining substrates and products. This was especially challenging when corn germ oil was used as a reagent. [19, 25,]

When it was needed 1H-NMR spectroscopic studies, FTIR (Fourier-transform infrared spectroscopy) analyses were performed. Solid products were analyzed with the most suitable analytical methods, selected from thermoanalytical methods (simultaneous thermogravimetry and differential thermal analysis: TG/DTA; differential scanning calorimetry: DSC; evolved gas analysis by mass spectroscopy or Fourier-transform infrared spectroscopy: EGA-MS and EGA-FTIR), Fourier-transform infrared spectroscopy (FT-IR), powder X-ray diffraction (XRD) and scanning electron microscopy (SEM). The goal of the analytical measurements is the comparison of the crystal structure and size of the crystalline phases obtained in different processes. Crystal structures will be compared against available reference structures, and we will attempt to identify unknown structures using complex analytical methods.

All analytical methods were carefully tested and their accuracy is taken into account when evaluation is done.

Development of suitable modeling strategies was a novel area where we made important progress.

At various *in situ* resolutions studied (point 1.1) the enantiomers are involved in competitive reactions for the resolving agent applied to less than molar equivalent amount, and this is the explanation of the observed long equilibration time compared to high conversions also at short times in many cases. As the equilibrium composition is pressure and temperature dependent, we proposed that the equilibrium is shifted in the $scCO_2$ environment significantly compared to analogous atmospheric resolutions. Pressure effects on reaction rates are probably due to the negative activation volume of the reaction, thus higher pressure is preferred. But careful selection of process parameters must ensure that the stability of the salts is not affected meanwhile.

Our original idea was at the submission of the proposal, that at diastereomeric salt precipitation techniques (antisolvent predominantly) the negative activation volume of the salt forming reaction might be the reason of the increased selectivity observed in some cases as well. However, as increasing pressure also influences solubility, we came to the idea, that solvent strength and pressure and temperature individual effects must be separated somehow to enable a good understanding of the phenomenon. Experimental results are summed up in point 1.

For several antisolvent resolution systems we proved, that in the diastereomeric salt formation reactions in $scCO_2$ or in antisolvent systems the dissociation of the salt is the most important factor. It is however difficult to measure the individual solubility products, although we developed suitable methodology, but uncertainty of the measurements was high due to the low concentration of the dissociated salts in the fluid phase (the value of solubility products were calculated to be in the order of magnitude of 10^{-5} $(mmol/mL)^2$ for CCA-BAB and 10^{-7}-10^{-8} $(mmol/mL)^2$ for IBU-PhEA, CPA-PhEA and MPAA-ChEA).[26]. As the solubility product was independent from the molar ratio of the reagents, we concluded that our hypothesis was in line with the result. We developed a novel model assuming, that the better the resolution the higher the difference in the dissociation of the individual diastereomeric salts, which can be well in accordance with the results for CCA-BAB system, but due to the measurement difficulties it cannot be proved for less soluble salts. In some cases the model fits very well to the results of solubility and resolution efficiency measurements as function of pressure, temperature and solvent compositions, while in other cases the agreement is less straightforward.

Recently we decided, that the method of using solubility parameters for modeling is applicable for separating the solubility and independent pressure and temperature effects. The efforts to use the

(Hansen) solubility parameters to improve our model were successful and led to a completely novel idea to describe antisolvent precipitation based chiral resolutions.[27]

While CALB catalyzes reactions by the Ping-Pong bee-bee mechanism, we've proved that the neat reaction with tributyrin can be described by the simple Michaelis-Menten kinetics. Furthermore, while it is well known, that catalytic activity and often selectivity of enzymes are strongly influenced by temperature, dependence of Michaelis-Menten constants on the temperature is rarely even mentioned. We showed, that incorporating the Arrhenius and Sitzer equations into the Michaelis-Menten model, it is suitable to describe the temperature dependence of Michaelis-Menten constants, and activation energy of 30985.4 J/mol and free enthalpy difference of 15220 J/mol were determined from the experimental data. Using this model the time frame, the concentration profile over time and the time requirement to achieve a given yield can be calculated with good accuracy at any temperatures between 30-60 °C.[22]

Conclusions

The fundamental research supported by NKFIH led to important new achievements in the field of enantioseparation supported by supercritical fluid technology. Besides the developed resolution routes the major generalizable outcomes are:

- Diastereomeric salt crystallization in carbon dioxide (*in situ* method) is possible, but in most of the cases the solubility limitations result in long reaction times. When racemate has a good solubility and the resolving agent also at least moderate solubility in CO_2 the reaction rate is accelerated. The method is useful for fundamental studies on the reaction kinetics and reaction equilibria under pressure.

- Gas antisolvent fractionation (GASF) is a widely applicable tool for diastereomeric salt precipitation with short contact times and highly crystalline micronized products are obtained, although amorphization is also possible. Scale up is straightforward via a semi-continuous setup. When kinetic controlled resolution is preferred, after parameter optimization, the GASF result in comparable or higher purities and yields than the atmospheric crystallization.

- Limits of GASF based enantiomeric enrichment via diastereomeric recrystallization correlate to the atmospheric melting eutectic composition of the diastereomeric salts.

- GASF technique was first applied for self-disproportionation based enantiomeric enrichment. Suitable methodology was developed for the method development and correlation was found between the limits of GASF based direct enantiomeric enrichment and melting eutectic compositions.

- Parameter effects of GASF based enantioseparation are the results of pressure, temperature and solvent composition affecting solubilities, reaction rates and equilibrium compositions at the same time. These observations are supposed to be suitable for the development of a generalizable model for the GASF technique using solubility parameter based modelling.

- Natural oils were first applied as esterification agent in enantioselective enzyme catalyzed reaction in supercritical CO_2. As supercritical carbon dioxide acts as a plasticized in the oil phase, the reaction rate is increase compared to atmospheric neat alternatives. However, distribution of the substrates between the oil and fluid phases have to be taken into account.

- Combination of highly selective enzymatic catalysis with supercritical carbon dioxide based separation of the products and remaining substrates is an efficient tool, but reagents have to be carefully selected to ensure the large solubility difference between the substrate and the product at optimized pressure and temperature.

One PhD thesis was defended (Bánsághi Gy.) , and two further PhD theses (Lőrincz L., Kőrösi M.) are going to be submitted based on the results of the project. One PhD thesis (Kmecz I.) was submitted based partly on the results the project and two additional PhD fellows (Mihalovits M., Zodge A.) also participated in the research. 10 master theses, 6 BSc theses, 3 scientific student conference works (3 prizes) were also the product of the support, besides papers already published (12) and manuscripts still in progress (4, manuscripts or drafts are available upon request). Besides international journal publications the results were presented at international and national conferences as well. Theses based on the research are listed in Appendix 1.

Furthermore, the good cooperation between participating research groups of BME became even stronger and also external collaborations were established (e.g. Cyclolab, Ruhr-University of Bochum) which are also beneficial consequences of the project.

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