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Investigation of the scale-up synthesis of nonivamide and capsaicinoids application in the complexation of cyclodextrins

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1. Introduction

I joined to the Innovative Pharmaceutical and Chirotechnological Research Group led by Dr. Erika Bálint at the Department of Organic Chemistry and Technology in September 2022. During my laboratory work, my consultants Bettina Rávai and Dóra Viktória Ujj, PhD students, helped me.

The research group is investigating the synthesis of potentially biologically active nitrogen- or oxygen-heterocycles by modern synthetic methods, such as multicomponent reactions, microwave technique or continuous flow chemistry, as well as organometallic chemistry and cyclodextrin complexations.

The aim of our research work was the investigation of the inclusion complexations of different capsaicinoids (capsaicin, dihydrocapsaicin, nonivamide) with various substituted β -cyclodextrins (CDs). In order to achieve the aim, scale-up synthesis of a famous capsaicinoid, called nonivamide was performed to obtain a guest molecule for the further complexations.

Capsaicin and capsaicinoids, such as nonivamide are well-known compounds as the main ingredients of the spicy chili pepper. Thanks to their essential biological activities, they are also used as analgesics, however, they are also promising in the treatment of prostate cancer and in plant protection as potential biopesticides. In the pharmaceutical industry, CDs as cyclic oligosaccharides are regularly applied in inclusion complexes in order to increase the solubility and bioavailability of poorly water-soluble APIs. In general, capsaicinoids have low water solubility, which can be improved by their complexations with CDs.

During the first part of our research work, the scale-up synthesis of nonivamide was studied, which consisted of two different reactions steps: the activation of the corresponding fatty acid and the *N*-acylation of the vanillyl amine compound. During this process, such reaction conditions as the role of the vanillyl amine hydrochloride salt and the free base form of vanillyl amine, the size of the reaction, the reaction time and different solvents were investigated.

In the next step, the inclusion complexations of different capsaicinoids were studied with various β -CD derivatives. First, phase solubility tests were performed in order to find the optimal capsaicinoid–cyclodextrin ratio and then the complexation reactions were carried out. During our work, the complexation of capsaicin, dihydrocapsaicin and nonivamide with two different degree of substituted (DS) hydroxypropyl- β -cyclodextrin (DS=4.5 and 7.0), sulfobutyl-ether- β -cyclodextrin (DS=6.5) and randomly methylated β -cyclodextrin (DS=12) were investigated in water.

The synthetic reactions were followed by mass spectrometer coupled high performance liquid chromatography (HPLC-MS) and gas chromatography (GC). The phase solubility studies were analysed by HPLC-UV measurements, and the complexes were studied by ^1H NMR and HSQC spectroscopy.

2. Literature background

2.1. Capsaicin and capsaicinoids in general

2.1.1. Capsaicin

Capsaicin (**1**) is a special alkaloid (and also the heaviest vanilloid in nature) that is found predominantly in the fruit of the *Capsicum* species and is responsible for its hot and spicy taste [1-6]. Capsaicinoids, the derivatives of capsaicin (such as dihydrocapsaicin (**2**), nonivamide (**3**), nordihydrocapsaicin, homodihydrocapsaicin, homocapsaicin, etc.) are a group of compounds found naturally in chili peppers. They degrade to fatty acids and vanillyl alcohol during the intake in the human body, and in some cases, they do not possess the pungency and hotness characteristics. Two particular capsaicinoids, notably capsaicin and dihydrocapsaicin, account for almost 95% of this pungent nature [7]. It is widely consumed globally, adding both flavour and spicy to food, while might also possess potential medicinal benefits [2,4].

Capsaicin (**1**) (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is a lipophilic, odourless, and colourless crystalline alkaloid. Its structure consists of an aromatic, vanillyl ring (*A*, *Figure 1*); an amide bond (*B*, *Figure 1*) and a hydrophobic side chain (*C*, *Figure 1*). It is soluble in fats, oils, and alcohols [1]. *Christian Friedrich Bucholz* was the first, who isolated this substance in an impure form in 1816 [8]. In 1876, *John Clough Thresh* isolated capsaicin in an almost pure form and gave it the name “capsaicin” [9-12]. Its molecular structure was determined by *Nelson* and *Dawson* in 1919 [6,13].

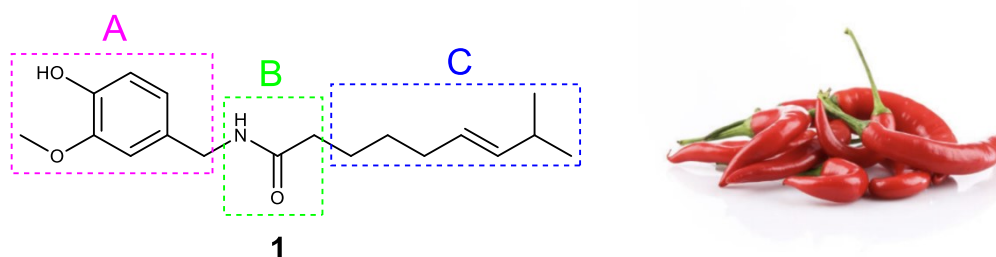


Figure 1. The molecular structure of capsaicin

Capsaicin exhibits *cis/trans* isomerism due to the double bond that hinders internal rotation. The *trans* isomer is more stable (and therefore is always found) than the *cis* isomer. It is followed from the facts that in the *trans* isomer the -CH(CH₃)₂ and the longer chain on the other side of the double bond are far apart, eliminating any steric hindrance. The opposite situation is for the *cis* form, which is less stable, because those two groups repel each other, exhibiting steric hindrance [1,14].

The spiciness level of capsaicin and other substances is quantified through assessment using the Scoville scale, which evaluates the extent to which a chili extract must be diluted to render its pungency undetectable. A higher Scoville index (SHU: Scoville heat units) corresponds to a greater degree of heat exhibited by the pepper [15-17]. Each SHU measures 1/15 ppm of capsaicin in a chili pepper [17]. The name of the scale comes from its creator, *Wilbur Lincoln Scoville*, an American pharmacist, who firstly presented his method in 1912 [19]. Capsaicin in its pure form achieves the highest Scoville scale rating, gathering 16 million units [15-18]. It is also an interesting fact that capsaicinoids play a defence mechanism against predators. They enhance the survival and reproduction of chili pepper plants by deterring herbivores such as mammals, birds, or insects and by protecting their seeds from fungal and microbial infections [20-22].

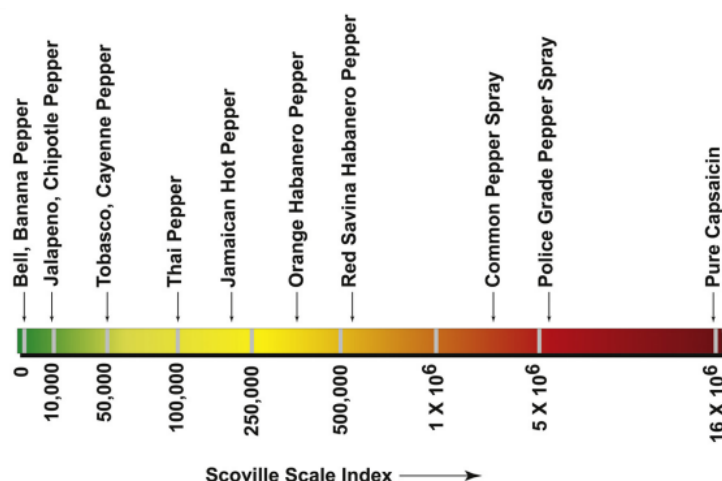


Figure 2. Scoville scale index, showing the pungency of different peppers

2.1.2. Dihydrocapsaicin

Dihydrocapsaicin (**2**) (*N*-[(4-Hydroxy-3-methoxyphenyl)methyl]-8-methylnonanamide), is one of the capsaicinoids found in the chili peppers (*Figure 3*). *Kosuge* and his co-workers conducted the first study wherein they demonstrated the isolation of natural capsaicin from Japanese *Capsicum*, resulting in a predominant mixture of capsaicin and dihydrocapsaicin in a ratio of approximately 7:3 [23,24].

Notably, capsaicin and dihydrocapsaicin elicit an equivalent degree of pungency, and they stand as the most potent pungency-inducing compounds known, with the ability to evoke a maximum pungency of 1.6×10^7 SHU.

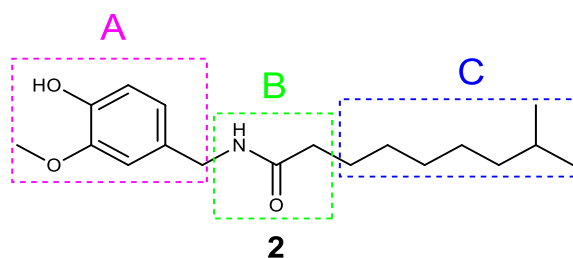


Figure 3. The molecular structure of dihydrocapsaicin

2.1.3. Nonivamide

Another important capsaicin derivative is nonivamide (**3**) [*N*-[(4-Hydroxy-3-methoxyphenyl)methyl]nonanamide], also known as pelargonic acid vanillylamide. It was found in *Capsicum* species, however, in a tiny quantity, so it is produced mostly synthetically (Figure 4). The first person synthesizing nonivamide was *E. K. Nelson* in 1919 and it was determined to have lower level of pungency and spiciness as the capsaicin [6]. Nonivamide, sometimes also called as “synthetic capsaicin,” is widely used as a cost-effective capsaicin substitute in several fields of life (food industry, agriculture, synthetic chemistry) [25,26].

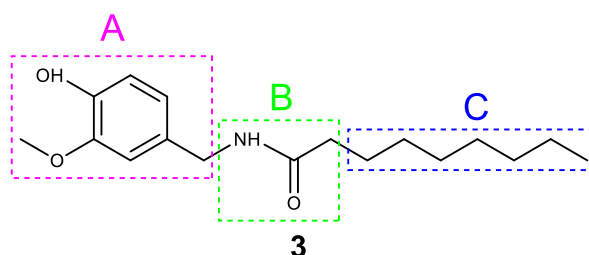


Figure 4. The molecular structure of nonivamide

2.2. Importance of capsaicinoids

Capsaicin and capsaicinoids have a variety of uses, and basically there are four major fields of application which can be distinguished.

A) Food industry

Capsicum fruits represent one of the earliest instances of food additives, frequently applied in the form of spices such as chili powder and paprika; and integrated into culinary practices as regularly as salt. The capsicum fruits are popular in most tropical and subtropical nations, and they are continuously becoming an increasingly popular cuisine addition [4,18].

B) Pepper spray and pest deterrent

Capsaicinoids also serve as the active component in pepper sprays used for riot control and self-defence by police or can be also used as chemical weapon by the army. These sprays

cause pain and respiratory distress, when they come into contact with the skin, the eyes, or mucous membranes [5].

Capsaicin is additionally used as a pest deterrent for plant protection, specifically against mammalian pests and with the nonivamide possess insecticidal properties, too [22].

C) Antifungal and antibacterial effects

Capsaicin also possesses antibacterial and antifungal properties. A research work by *Dorantes* and his group showed that *Capsicum annuum* extracts restricted the growth of a number of pathogenic microorganisms, such as *Salmonella typhimurium*, *Staphylococcus aureus*, and *Bacillus cereus* [27,28]. Also, capsaicin and dihydrocapsaicin showed to be effective against *Streptococcus mutans* bacteria, the main reason of dental caries [29].

Previous studies have also shown that capsaicin can prevent the growth of fungi like *Colletotrichum capsici*, *Aspergillus niger* and *Pythium ultimum* [30].

D) Research and pharmaceutical use

Among the clinical applications of capsaicin, the field of analgesics stands as the most prevalent one. It is simple to find without a prescription low-concentration creams, lotions, and patches containing capsaicin for daily topical usage. Furthermore, pharmaceutical formulations for oral administration of capsaicin, in the form of capsules containing chili peppers, have been developed. Capsicum nasal sprays and homeopathic preparations of *Capsicum annuum* are further pharmacological formulations that contain capsaicin; however, their effectiveness still has to be proven [22].

Numerous studies have also shown some promising results that capsaicin can be useful in the fighting of obesity. However, it is important to note that certain studies have found zero or limited effects on losing weight in human participants [22].

Furthermore, capsaicin may exert potentially beneficial effects on glucose regulation, insulin homeostasis, and diabetes. There exists compelling evidence suggest that capsaicin may confer favourable effects on the cardiovascular system. Additionally, capsaicin displays antioxidative properties and inhibits platelet aggregation.

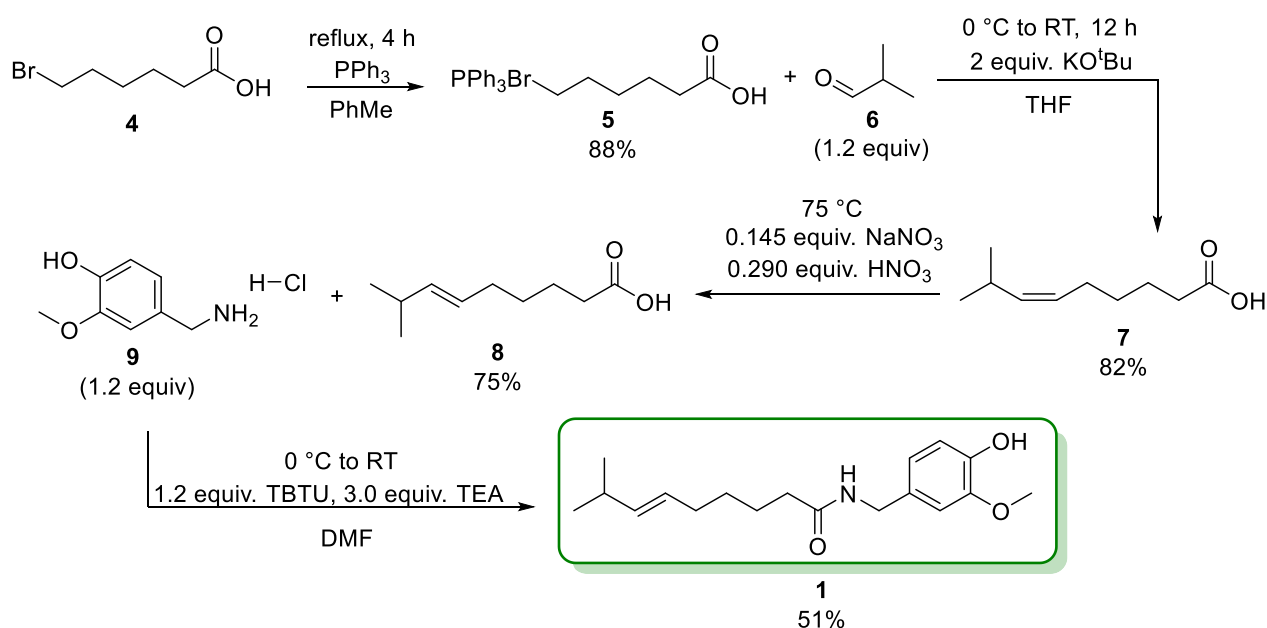
Moreover, many pieces of research are still ongoing whether capsaicin holds any effects on cancer, obesity, airway diseases, itch, and other clinical applications.

2.3. Synthetic strategies for the preparation of capsaicinoids

2.3.1. Synthesis of capsaicin

In the subsequent reaction schemes, the main methods for capsaicin synthesis are presented.

First, in a four-step synthesis (*Scheme 1*), the 6-bromohexanoic acid (**4**) is converted to a Wittig salt (**5**) with triphenyl phosphine (PPh₃) under reflux for 4 hours in toluene (PhMe). Then, the obtained Wittig salt is reacted with isobutyraldehyde (**6**) and deprotonated in dry tetrahydrofuran (THF) using potassium tert-butoxide (KO^tBu) which results the elongated (*Z*)-configured acid compound (**7**). During the next step, this acid should be isomerized to the (*E*)-configured compound (**8**) in the presence of acidic conditions. Finally, the capsaicin is obtained followed by the *N*-acylation step with vanillyl amine hydrochloride salt (**9**) in the presence of triethylamine (TEA), *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU) coupling agent in dimethylformamide (DMF) solvent with a total yield of 51% [5].



Scheme 1. Four-step capsaicin synthesis

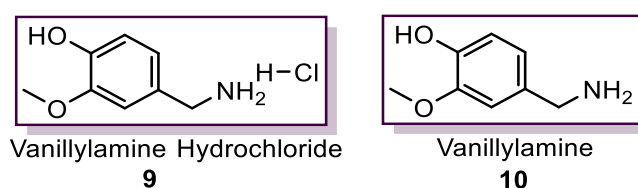
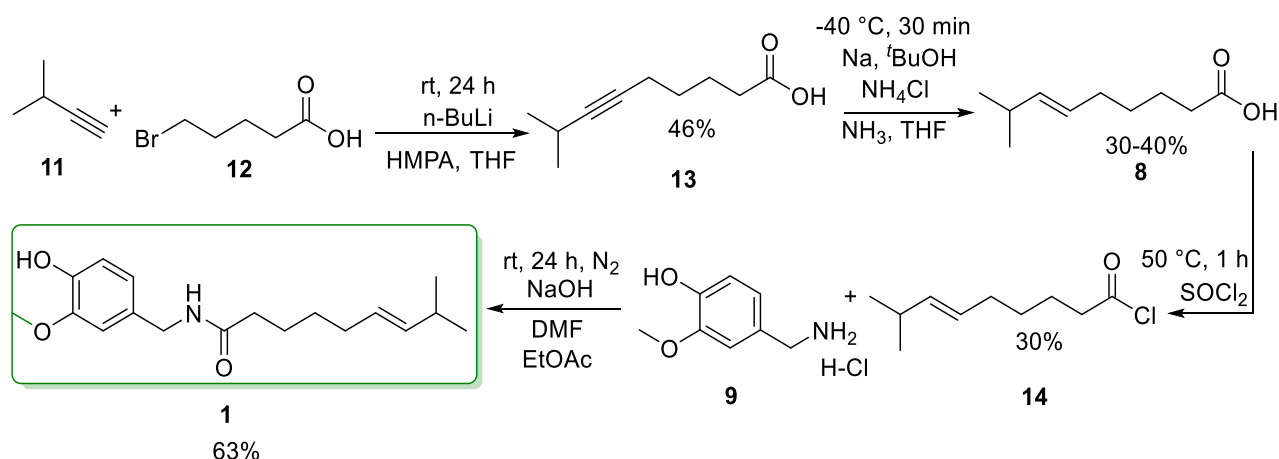


Figure 5. Most common coupled benzylamine derivatives

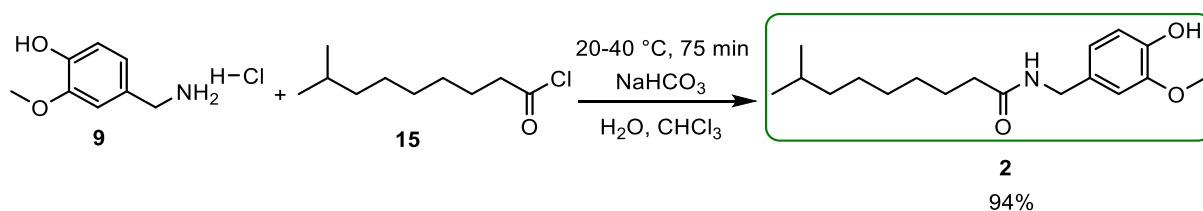
In the following reaction [31], an organometallic synthesis is applied to prepare capsaicin (Scheme 2). First, the reaction of 3-methyl-but-1-yne (**11**) and 5-bromovaleric acid (**12**) is carried out under inert conditions, with butyl lithium and hexamethylphosphor amide in dry THF. Crude product was extracted with ethyl acetate and purified using column chromatography (eluent is 2:1 hexane/ethyl acetate). The received product of the first step was light yellow oil. In the second step, the linked intermediate, 8-methyl-6-nonynoic acid, (**13**) has to be reduced in basic conditions in the presence of sodium in tert-butanol (*t*BuOH) and anhydrous THF. Ammonium chloride was added to the reaction mixture as well. The yield of the reaction mixture was determined by thin layer chromatography (TLC), and it was around 30-40%. The produced compound, 8-methyl-6-nonenoic acid (**8**), has light yellow colour and it is an oil; and this obtained trans-compound has to be activated by thionyl chloride and then *N*-acylated with the vanillyl amine (**9**) compound. Column chromatography is used for the crude product purification with the 1:1 ethyl acetate/hexane eluent. Also, recrystallization can be used in a 1:2 mixture of ether/hexane, and it gives around 74% yield.



Scheme 2. Another method for the synthesis of capsaicin

2.3.2. Synthesis of dihydrocapsaicin

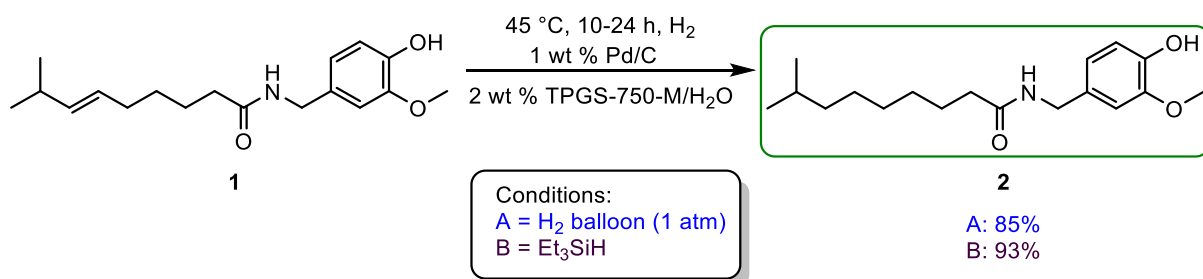
In the subsequent article the authors present one of the most convenient synthesis methods for preparation of capsaicinoids: by acylation of the vanillyl amine with acyl chloride derivative [32]. This method is highly efficient and solves problems faced earlier by other researchers such as poor solubility of vanillyl amine in anhydrous conditions, hydrolysis of acyl chloride and, as a result, poor yield.



Scheme 3. A synthetic method for the dihydrocapsaicin synthesis

This reaction is performed in the following way (*Scheme 3*): firstly, to an aqueous solution of vanillyl amine hydrochloride (**9**) sodium bicarbonate (NaHCO₃) is added in order to obtain vanillyl amine free base form (**10**). After mixture was stirred for 30 minutes under ambient temperature, chloroform was added, and the reaction mixture was stirred for 15 more minutes. Finally, 8-methylnonanoyl chloride (**15**) is added to the reaction mixture dropwise and stirring was applied for 30 minutes under 40 °C temperature. Reaction was monitored using TLC. After the reaction was complete, extraction was performed using chloroform.

Another method consists of capsaicin hydrogenation to dihydrocapsaicin, and it is described in the *Scheme 4* [33]. Pd/C-catalysed heterogeneous hydrogenation was conducted on olefin-containing natural product (**1**). 0.25 mmol of substrate should be used, and 100 ppm of 1 wt% Pd/C catalyst should be stirred in TPGS-750-M (**16**) (it is a second-generation amphiphile, which acts as surfactant, for metal-catalysed cross-couplings in water at room temperature) and water solution under conditions A = hydrogen gas balloon under 1 atmosphere pressure and conditions B = 1.2 – 1.5 equivalent of triethylsilane (Et₃SiH) per double bond. For the capsaicin reduction, 45 °C reaction temperature was applied. Using this method, it is possible to recycle catalyst and aqueous reaction medium. Also, small amount of Pd/C in ppm is used and high yield of the product is obtained, thus, these facts contribute to the “greenness” of the method.



Scheme 4. A synthetic method for the dihydrocapsaicin synthesis

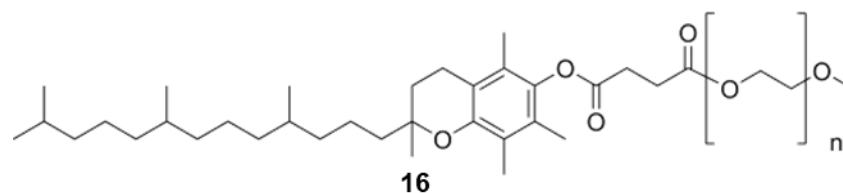
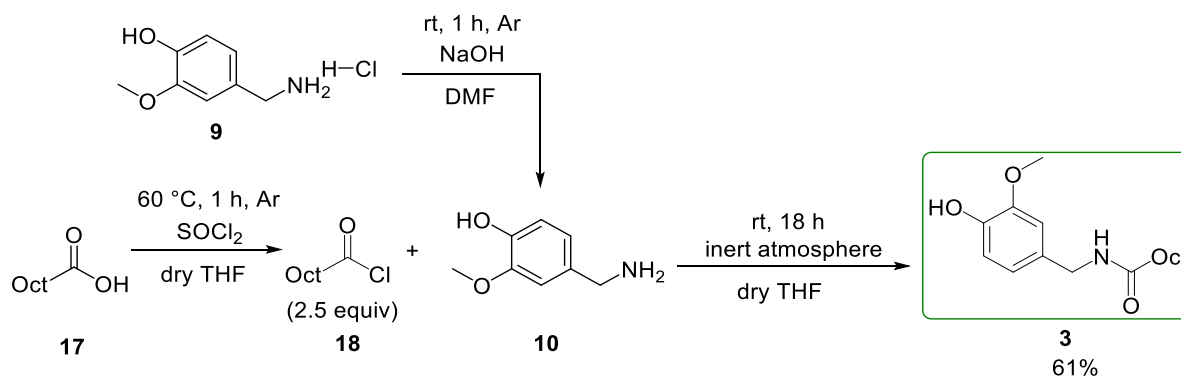


Figure 6. TPGS-750-M molecule structure

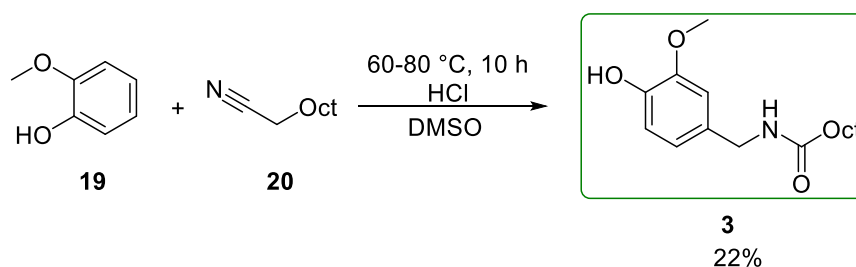
2.3.3. Synthesis of nonivamide

In the next case, the authors of the paper suggest the following synthesis method presented in the *Scheme 5* [3]: firstly, acyl chlorides (**18**) are formed from their corresponding acids (**17**) with the thionyl chloride, which is added dropwise with a syringe. Reaction mixture is heated at 60 °C for 1 h, the formed acyl nonanoyl chloride (**18**) was dissolved in dry THF. Parallely, liberation of the vanillyl amine hydrochloride (**9**) from its salt is performed in DMF under argon atmosphere. This reaction is followed by the NaOH dropwise addition in an ice bath, and after it is fully added, the mixture is left to stir at room temperature for 1 hour. After this, *N*-acylation reaction is performed: 2.5 equivalent of nonanoyl chloride (**18**) is mixed with the liberated vanillyl amine (**10**) under argon atmosphere at ambient temperature for 18 hours. In order to follow the reaction TLC was used (eluent: 1% of methanol in chloroform; chromogenic reagent: oleum). To stop the reaction, water was added.



Scheme 5. One of the nonivamide synthesis methods.

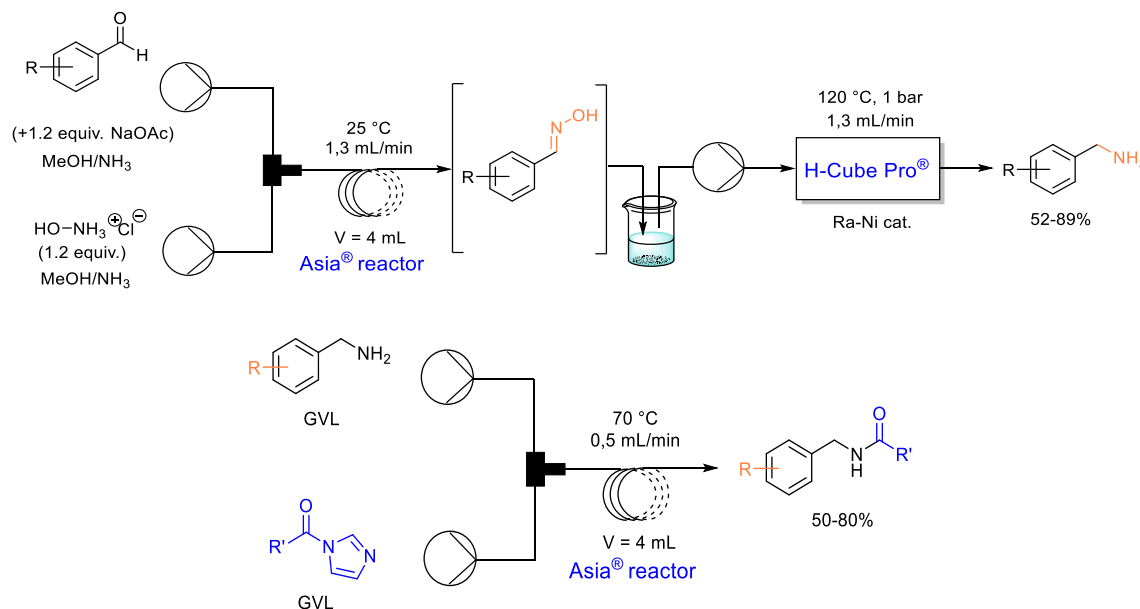
Another method for nonivamide synthesis is described in this paragraph [34]. Instead of commonly used 3-step synthesis method via oximation, reduction and amidation, starting from vanillin, authors suggest just one step method, so called amidomethylation, to produce nonivamide (*Scheme 6*). Decanenitrile (**20**) is reacted with 2-methoxyphenol (**19**) in the presence of dimethyl sulfoxide (DMSO) and HCl at 60-80 °C temperature for 10 hours.



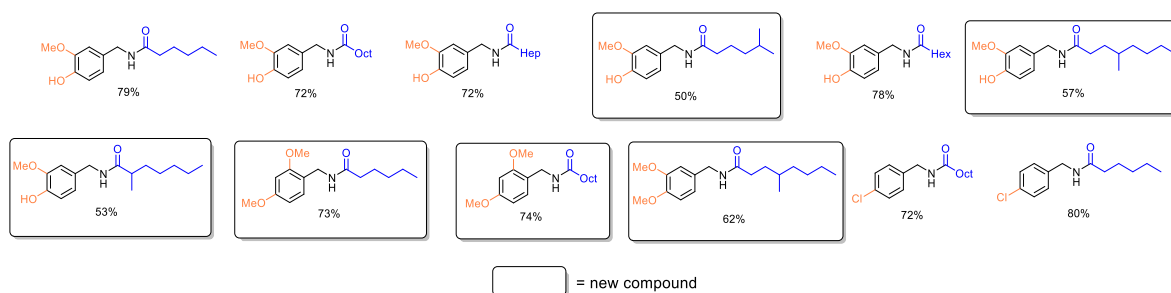
Scheme 6. One of the nonivamide synthesis methods.

2.3.4. Preliminary experiments from our research group

Concerning the flow synthesis of various capsaicinoids, there is a wide preliminary study in our research group connected to the MSc thesis work of *Máté János Orosz* [35]. In his research work, he investigated the synthesis of different capsaicinoids in continuous flow reactors. The reaction contains three, different steps: the formation of aldoximes from benzaldehyde derivatives with hydroxylamine performed in an Asia® flow system, their hydrogenation in a special H-Cube Pro® system to benzylamines and their *N*-acylation with activated carboxylic acids in the presence of a green solvent, γ -valerolactone (GVL) in a heated Asia® flow system. This procedure resulted the synthesis of 12 capsaicinoid derivatives, from which 6 are new in the literature.



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Scheme 7. The preliminary study of the synthesis of various capsaicinoids in flow chemical reactors

2.4. Cyclodextrins

2.4.1. History

The story of the cyclodextrins began when *Antoine Villiers* (1854-1932), a French chemist and pharmacist, was pursuing his investigation of the enzymes impact on different carbohydrates. Experiments centred on the degradation and reduction of carbohydrates through the use of ferments (*Clostridium butyricum*) were conducted and this led to the discovery of unwanted crystals in the residual alcohol [36]. Properties and composition of these crystals were quite evidently distinct from those of the numerous saccharides that were then known. *Antoine Villiers* named them “cellulosine.” *Franz Schardinger* (1853–1920) was first to provide comprehensive description [37] of how to prepare, separate, and purify the two cellulosines that *Villiers* first described [38,39].

Between 1936–1970, it was discovered that crystalline Schardinger-dextrins are composed solely of α -1,4-glycosidic bonds and are constructed from glucose units. In 1936, the cyclic structure of these crystalline dextrins was discovered and a first method for isolating homogenous and pure fractions was described. The γ -CD had been identified and its structure had been determined between 1948–1950. Around this time, first misinformation on toxicity of β -CD appeared, and it kept a number of scientists from creating CD-based items for human use [40,41].

From the 1970s, the number of CD-related publications demonstrated a significant increase after substantial toxicological studies demonstrated that any toxic effect attributed to CDs comes from contamination and impurities, excessive dosage, or an inadequate administration form. Price of the β -CD decreased significantly, it started to be produced in large scales and it became more available for researchers and for industrial usage.

However, the history of the CDs is not stopped here, but it continues for example with β -CD derivatives (alkylated, hydroxyalkylated, polymerized), which open new and unexpected possibilities [38-41].

2.4.2. Fundamentals

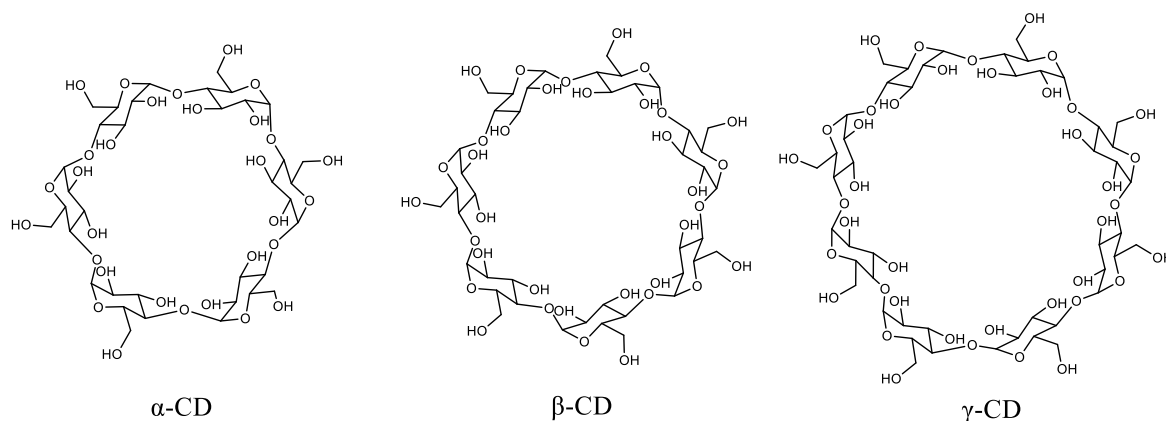


Figure 7. Structure of α -, β - and γ -cyclodextrins

Synthesis

Dextrins, as the products of the decomposition of starch, are heterogeneous, amorphous, and hygroscopic compounds. There are 2 methods for the starch enzymatic degradation using α -Amylase and CGT-enzyme (cyclodextrin glucosyl transferase enzyme) (Figure 8):

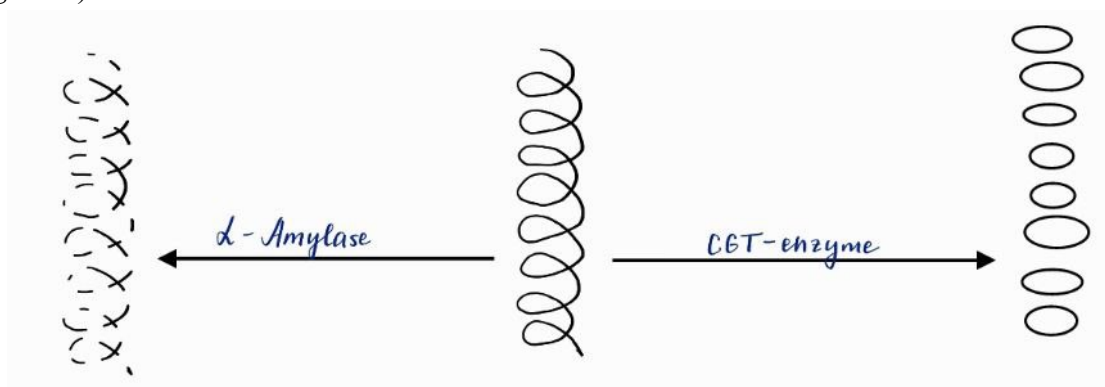


Figure 8. Formation of acyclic and cyclic dextrins from starch [42].

Degradation of starch with α -amylase yields long series or branched chain glucose. However, by CGT-enzyme, α -1,4-linked cyclic products are formed – CDs. Therefore, CDs are cyclic non-reducing oligosaccharides, which contain at least 6 ring members (formation of the CDs with less than 6 ring members is not possible for steric reasons). The three major CDs (α -, β - and γ -CD) are presented in the Figure 7. They possess properties opposite of the non-

cyclic oligomers: they are crystalline, homogenous and nonhygroscopic substances with doughnut-like shape and cavity inside, build up from the glucopyranose units in chair conformation [42].

Properties and structure

CDs are supramolecular host molecules, capable of accommodating in their cavity different types of compounds: organic, inorganic, organometallic that may be neutral, cationic, anionic, or even radical. CDs have several unique attributes that make them suitable for a wide range of applications. First and foremost, there are two sorts of numerous hydroxyl groups, which means that the possibilities of chemical alteration are nearly limitless. CDs have no specified melting point; however, they begin to decompose at temperatures exceeding 200 °C. Additional advantages include minimal toxicity, high availability, and simple biodegradability. Because of these properties CDs have received a lot of interest in the chemical research community throughout the years [42,45].

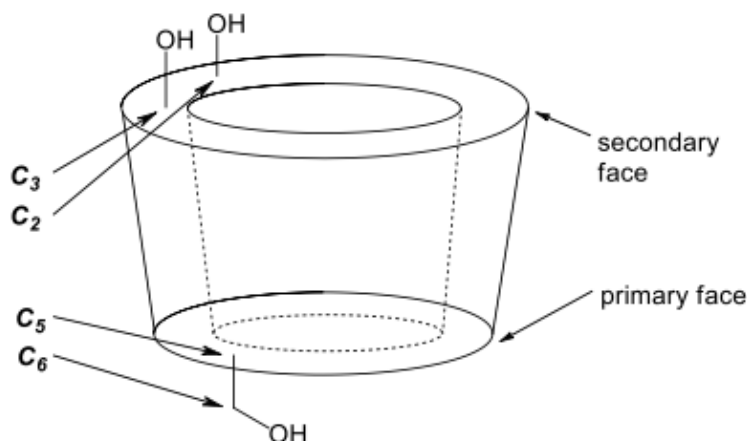


Figure 9. Schematic representation of CDs

CDs have the form of a hollow truncated cone (conical cylinder) with a moderately lipophilic (hydrophobic, low water solubility, apolar) interior chamber (presence of C-H bonds and glycosidic oxygen bridges) and a mainly hydrophilic (good water solubility, polar) surface (presence of hydroxyl groups). Primary hydroxyl group, located on C₆ carbons, are situated on one side of the torus (on the narrower one); whereas the secondary hydroxyl groups, located on C₂ and C₃ carbons, are situated in the opposite side of the torus – in the wider end. C₂ and C₃ hydroxyl groups form hydrogen bonds between each other, which stabilizes the structure, and also is the cause of the low solubility in the aqueous phase. Intramolecular hydrogen bonds form a complete ring, which prevents hydration and reduces solubility. In β -CD a complete hydrogen belt is formed, so this supramolecule has rigid structure and the lower water solubility

of all CDs. The highest solubility in water shows γ -CD: it has a flexible ring structure, and the glucose units are not coplanar [42-45].

Applications

Due to the structure of CDs and their extraordinary encapsulation abilities, they are mainly used as complexing agents. This allows to alter and/or enhance the biological, chemical, and/or physical properties of the guest molecule.

CDs are being used and tested as API: hydroxypropyl- β -CD against Niemann-Pick C disease, in autophagy process. It was reported that hydroxypropyl- β -CD can be used against atherosclerosis disease by the means of increasing cholesterol solubility. Studies on fluorophore-modified CDs showed results in the cancer and bacterial infections treatment [41].

Also, a modified γ -cyclodextrin by addition of sodium 3-(ethylthio)propanoate side chains is regularly used as a neuromuscular reversal drug. It is otherwise called sugammadex (*Figure 10*), and it acts by encapsulation of rocuronium, leading to its inactivation and formation of a stable complex [46].

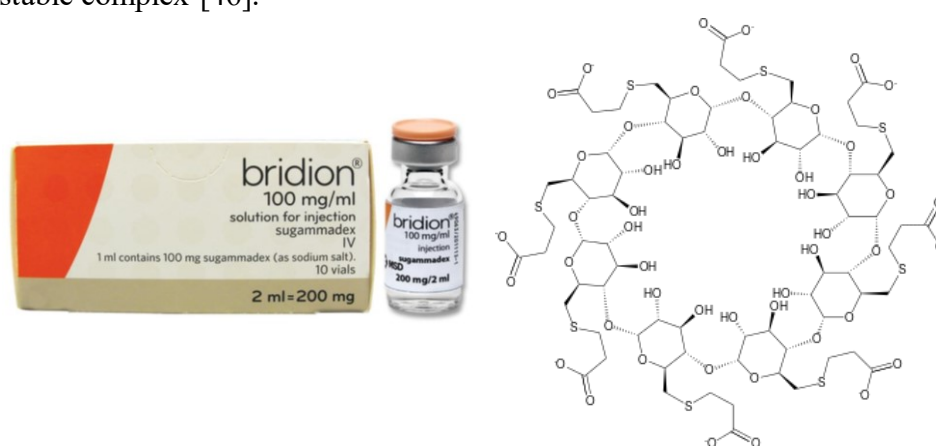


Figure 10. Sugammadex structure

Reports were made in using CDs in nanotechnology, particularly, in nanomedicine. Cyclodextrin-based nanomaterials, for example, nanosponges, have promising results in drug delivery systems [43].

Further application fields include molecular recognition, lipophilic drug solubilizers, excipients in formulation development, delivery systems and complexing agents in the food and medicine industries, and for masking of undesirable odour and taste. CDs also find applications in many industries such as cosmetics, hygiene and personal care, textiles, medicine, and biomedicine, agrochemistry, biotechnology, and catalysis. This is all possible due to the biocompatibility, low expense and simplicity of their synthesis, non-toxicity and, finally, their properties as complexing agent [43].

2.5. Inclusion complexes

2.5.1. Complexes review

A single, larger ion or molecule is created through the complexation of various groups of atoms, ions, or molecules. In various phases (liquid, solid and gas) and also at the phase boundaries it is happening between organic compounds, metals, and ligands. In contrast to both complete covalent bonds and ionic crystals, complexes are structures made up of two or more molecules or ions that are electrostatically bound together. Each complex's structural relationships are distinct and particular, however, for example, molecular complexes are typically kept together by such forces as van der Waals, hydrogen bonding, ion pairing, metal to ligand binding and others.

When a host and guest have complementary stereoelectronic arrangements of binding sites, the term "host - guest relationship" is used. The host component is an organic molecule or ion that has binding sites which converge in the complex, whereas the guest component is any molecule or ion that has binding sites which diverge in the complex [47].

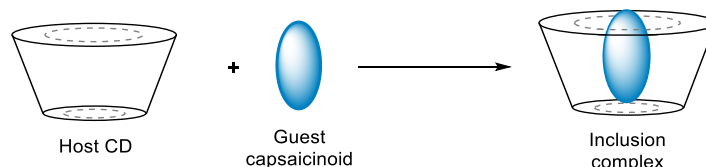
2.5.2. CD complexations

As a result of the above-mentioned structural features, CDs may form inclusion complexes with a broad variety of solid, liquid, or gaseous substances, called host-guest complexes. This unique encapsulation ability can alter and/or improve the physical, chemical, and/or biological properties of the guest molecule. Inclusion complexes can be developed in either solution or crystalline form. Although water is the most commonly applied solvent, inclusion complex formation also occurs in dimethyl sulfoxide and dimethyl formamide as well.

The cavities of CDs which undergo crystallization from aqueous solutions are not void but contain water molecules, some of which are integrated into the crystal structure as "crystal water," while others are enclosed within the cyclodextrin cavity. CD complexes usually have a CD derivative: guest ratio of 1:1. The formation of CD-inclusion complexes occurs through the replacement of included water molecules with guest molecules suitable for the desired complex. This is a commonly observed phenomenon in cyclodextrin chemistry, and is regulated by a constant known as the formation or stability constant (K_f), and stability of the inclusion complex is directly proportional to its K_f value:

$$[CD] + [guest] \rightleftharpoons [CD \dots guest]$$
$$K_f = \frac{[CD \dots guest]}{[CD][guest]}$$

CDs have the ability to create inclusion complexes with molecules that possess dimensions that are compatible with the size of the CD cavity. The type of guest molecules that are able to be incorporated into the CD cavity is primarily determined by geometrical characteristics (size, molecular shape) rather than chemical characteristics (however, it still play its role and they should be less polar than water) [42,45].



Scheme 8. Inclusion complex formation

There is ongoing debate concerning the binding force's origin. The development of inclusion complexes is accompanied by a favourable enthalpy change and an unfavourable (or slightly favourable) entropy change; thus, it cannot be the typical apolar binding. The most common attribute of apolar binding is a very favourable entropy change. Several assumptions were made regarding what kind of driving forces can influence cyclodextrin-guest complex formation: van der Waals interactions between guest and host, hydrogen bonding between the guest and the hydroxyl groups of cyclodextrin, electrostatic interactions, hydrophobic interactions [43,44].

Proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy provided the most direct evidence for the incorporation of a guest into the cyclodextrin cavity in solution. Also, changes in the absorption spectra of substrates are frequently caused by inclusion complex development. Fluorescence measurements, titration, electron spin resonance and other methods are also applied in order to verify the complexation [44].

2.5.3. Complexation methods

The major methods for producing CD inclusion complexes in solid state may be classified as follows:

1. Methods in solution, where the guest molecule and the CD are dissolved in water or organic solvent/water mixture, the conditions (temperature, pH) are regulated to ensure the best possible interaction between the substances.
2. Methods in semisolid state, for example, kneading, which is described below.
3. Methods in the solid state, for example by microwave irradiation; by “sealed-heating” method and by grinding [48].

Depending on the features of the guest and the nature of the particular CD, there are many approaches for obtaining cyclodextrin-guest complexes:

Co-precipitation method

When dealing with non-water-soluble substances, this method is advised to be applied, despite the fact that it produces low yields. It is a widely employed method known for its simplicity and efficiency. In this method, the guest molecule is dissolved in an organic solvent (e.g., chloroform, benzene, diethyl ether), followed by the addition of cyclodextrin dissolved in water and thorough homogenization. Subsequently, the solution is cooled to facilitate complex crystal formation, which is then rinsed with an organic solvent and dried at 50 °C [49,50].

Kneading method (paste method)

This method is applied when dealing with poorly water-soluble guest molecules, however, it gives higher yield. This technique is relatively simple, has high efficiency and scalable; therefore, used frequently in industry. It involves mixing the cyclodextrins with a little amount of distilled water (in mortar) in order to form paste. The guest molecule is included into the paste by using intensive stirring. The received solid is then rinsed with a little amount of solvent, and dried [49,50].

Grinding method

This technique has the benefit of providing a simple, rapid, and effective process for creating guest/CD inclusion complexes in the solid form and does not normally need the use of organic solvents, making it safe ecologically sustainable. Grinding processes result in crystal fracturing, which decreases particle size and improves the contact surface for the interaction between the guest and the CD [48].

Solvent evaporation method

The procedure involves the preparation of a guest solution combined with the host molecule, utilizing a blend of suitable solvents. Following the attainment of a uniform phase, the solution is subjected to evaporation under vacuum conditions in an evaporator until dryness is achieved. In cases where the solid residue remains incompletely dried, supplementary drying can be accomplished through the utilization of an oven [50].

Atomization, spray drying

Spray drying is an energy-intensive process where a liquid containing cyclodextrin and a guest molecule is atomized in hot gas to produce rapidly sized nano- and microparticles. It is

historically used in food and chemicals to control particle size, despite limitations such as poor yield and thermal sensitivity. This method, applicable to thermally stable compounds, has expanded to industries beyond food and chemicals, including cosmetics and pharmaceuticals, as demonstrated by recent folic acid encapsulation in cyclodextrin using spray drying [49,50].

Melting method

The melting method involves heating a mixture of the drug and carrier until they melt and then cooling and breaking them into smaller pieces. This fusion process, where the carrier molecules are highly active, facilitates thorough mixing and incorporation of the medicine. To cool and solidify the melted substance, techniques such as agitating it in a cold bath or spreading it over cold metal can be employed. This method offers an advantage over chemical-based approaches as it does not produce hazardous compounds or harm the environment, but it requires the medication to withstand elevated temperatures without damage [50].

Lyophilization (freeze drying)

Lyophilization is a crucial technique for drying biological and chemical agents, especially those that are water-soluble or thermolabile. It involves three stages: dissolution of the drug and carrier (cyclodextrin) in an aqueous solution, freezing, and subsequent elimination of the solvent through sublimation (under vacuum conditions). This technique has drawbacks such as extended processing time and unfavourable powder flow properties [49,50].

2.5.4. Phase solubility analysis

The restricted solubility of CDs and ability to undergo complexation are dependent on a number of parameters. The inclusion formation constant, present pharmaceutical excipients that affect the physicochemical properties of the cyclodextrins, and other considerations are among them. As concentration of CD in a solution becomes higher, their complexes can start to agglomerate and form self-assemblies of a large size, which may precipitate [51].

Just because an inclusion complex remains intact when it is dissolved in water does not mean it will also be the same when it is in a solid crystal form. So, it is needed to figure out if the powder that comes from making an inclusion complex is truly the complex or just a mix of the drug and cyclodextrin. When substances that do not dissolve well in water are mixed with CDs, then their solubility in aqueous media is typically increased. The strength of this inclusion complex is measured by a value called the stability constant (or equilibrium constant), represented as K_s [42,45,49].

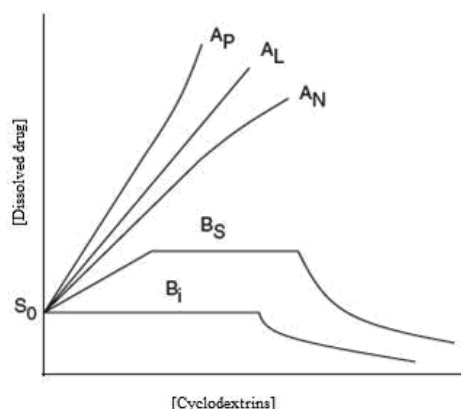


Figure 11. Types of phase-solubility diagrams according to *Higuchi and Connors*

The phase solubility analysis is a crucial investigation of the complexation capacity of the CDs with the needed API. *Higuchi and Connors* [52] theory shows the solubility profiles of the drug in the cyclodextrins. This theory helps explain the relationship between the concentration of the drug and the cyclodextrin in a solution and how it affects the solubility of the drug. Since cyclodextrins are frequently employed to make hydrophobic guests more soluble in water, a common experiment is adding an excess of guest to several kinds of cyclodextrin solutions and stirring until equilibrium is attained. Following filtering, the guest concentration is determined using any suitable analytical method, and the associated apparent solubility is displayed as a function of cyclodextrin concentration. After then, many curve types can be seen, as shown in *Figure 11* [43].

When the drug/CD complex is readily soluble in the aqueous medium, A-type diagrams are typically shown. In simpler terms, the drug dissolves well in the presence of the cyclodextrin – soluble inclusion complex is formed. Water-soluble CD derivatives are frequently linked to this kind of complexation. A_L line shows the linear proportionality, A_P and A_N represent positive and negative deviations from the linearity. When the drug/CD complex has a low solubility in the medium, B-type diagrams can be seen. In other words, the drug does not dissolve well when combined with the CD – poor soluble inclusion complex is formed. This kind of complexation is usually related to natural non-modified CDs that have limited

solubility in aqueous (water-based) environments. B_s curve shows the complex with limited solubility, B_i diagram represents the insoluble complexes establishment [49-51].

The slope of the line in these diagrams provides information about the stoichiometry of the drug-cyclodextrin complex. This theory is important in pharmaceutical research because it provides a way to predict and optimize the solubility enhancement of poorly water-soluble drugs using cyclodextrins [43,49].

3. Results and their evaluation

During my thesis research project, our aim was to study the complexation reactions of capsaicin and its derivatives, such as dihydrocapsaicin and nonivamide, with various CDs including hydroxypropyl- β -CD (HP β CD, DS=4.5 and 7.0), sulfobutylether- β -CD (SBE β CD, DS=6.5) and randomly methylated β -CD (RAMEB, DS=12).

Capsaicin and dihydrocapsaicin were available as preliminary results from our research group, thus, in order to obtain the missing capsaicinoid, the scale-up synthesis of nonivamide was studied. During the experiments, such reaction conditions as the role of the vanillyl amine hydrochloride salt and the free base vanillyl amine, the amount of the reagents, reaction time and different solvents were investigated.

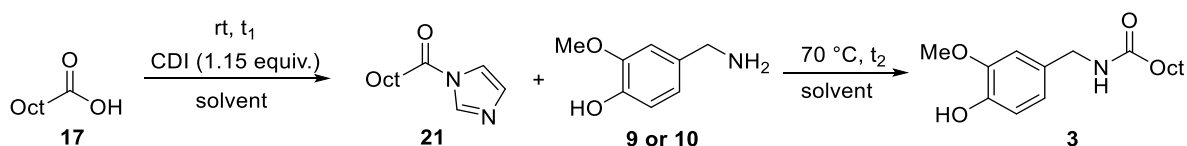
After that, the phase solubility studies of capsaicinoids and CDs were performed in order to determine the optimal concentration for the complexation reactions. Phase solubility studies were followed by HPLC measurements. After the determination of the optimal conditions, the complexation reactions were performed.

The products of the synthesis reactions were purified by column chromatography and characterized by GC and HPLC. The complexation reactions were analysed by ^1H NMR and HSQC spectroscopy.

3.1. Investigation of the scale-up synthesis of nonivamide

During our work, the synthesis was performed according to the work of *Máté János Orosz* [35], however we wanted to scale-up his former optimal reaction.

In the first part of our research work, we studied the reaction of the vanillyl amine and the corresponding fatty acid, nonanoic acid for the preparation of nonivamide (*Scheme 9 and Table 1*). The reactions were carried out in respect of the molar ratios of the starting materials, usage of vanillyl amine free base (**10**) and hydrochloride salt (**9**), solvent and reaction time. In all of the cases, two main steps resulted the final product of nonivamide: the activation of nonanoic acid and *N*-acylation of the vanillyl amine compound. In all of the reactions, 1.15 equivalent of carbonyldiimidazole (CDI) was used for the activation of the nonanoic acid (**17**), yielding its activated form (**21**), and also the temperatures used were the same: 25 °C for the acid activation and 70 °C for the second step of the reaction. The composition of the reaction mixtures was determined by GC and HPLC-MS.



Scheme 9. Synthesis of nonivamide

Table 1.: Synthesis reactions of nonivamide

Entry	Reagent	n [mmol]	Solv.	Solv. [ml]	t ₁ [min]	t ₂ [min]	Conversion [%] ^a
1	vanillyl amine	1	GVL	10	30	5	63
2	vanillylamine- HCl	1	GVL	10	30	5	68
3	vanillylamine- HCl	5	GVL	30	60	60	91
4	vanillylamine- HCl	5	2-Me- THF	50	90	60	83 ^b
5	vanillylamine- HCl	10	GVL	100	60	120	99 ^c

^a Based on the ratio of nonanoic acid: nonivamide, according to GC measurements.

^b Decomposition occurred (11%)

^c Yield: 89% after column chromatography.

First, the reaction was carried out with 1 mmol of reagents in GVL (γ -valerolactone) solvent (*Table 1/Entry 1*), which is a natural compound, received from cellulosic biomasses, and it is often used as a green solvent. During both steps of the reaction, bubbles formation was observed, probably, due to the CO₂ gas evolution, which is likely indicating the activated acid formation (**21**). However, GC results (Conversion: 63%; *Table 1/Entry 1*) showed that there is still a lot of starting nonanoic acid (**17**) in the mixture.

After that, the effect of the salt form of the vanillyl amine was studied, while the reaction conditions coincide with the previous entry (*Table 1/Entry 2*). The GC results were similar to the previous entry, meaning that the salt form of the vanillyl amine can also be used successfully.

As the conversion was not complete, the reaction time of both reaction steps was modified. The next reaction was the first attempt to perform scale-up of the synthesis, thus 5 mmol of the reactants were used, and the reaction was run in total for 2 hours (*Table 1/Entry 3*). Bubbles formation as in the previous entries was also observed during the nonanoic acid activation. According to the GC measurements, a significantly higher conversion of

nonivamide was observed, and there was much less of the original nonanoic acid present in the reaction mixture (9:91). These results showed the necessity of the increased reaction time.



Figure 12. From left to right: reaction mixtures of entries 1-3

Another attempt to study the scale-up synthesis of nonivamide was the usage of the 2-methyl-tetrahydrofuran (2-Me-THF) solvent instead of GVL (*Table 1/Entry 4*). The first step of the reaction was performed for 90 minutes, because the continuous bubble formation was observed. During the second step of the reaction, it was noted that vanillyl amine hydrochloride (**9**) does not dissolve well in the solvent. The GC measurements presented 83% conversion of nonivamide; however, some decomposition peaks were also observed on the chromatogram, indicating that 2-Me-THF is not the appropriate solvent for this reaction.

Finally, another attempt was made to scale-up the reaction, when 10 mmol of reactants were used. Initially, one hour reaction time for each step was used (*Table 1/Entry 3*), however the reaction time was further increased for the second step (*Table 1/Entry 5*). The reaction was followed by GC and TLC, and the sample taken after one hour of acid activation with CDI showed that nonanoic acid (**17**) is no longer present in the reaction mixture; thus, there was no need to increase the reaction time of the first step. However, it was required for the *N*-acylation reaction – GC samples after 30, 60 and 90 minutes showed the presence of the activated acid (**21**); thus, the reaction was run for two hours until it was no longer present. The obtained crude product was purified by column chromatography, yielding 89% of the nonivamide.

It can be concluded that the optimized parameters for the scale-up synthesis of nonivamide (**3**) were the following: 10 mmol of reactants, GVL solvent and reaction time $t_1 = 60$ minutes and $t_2 = 120$ minutes.

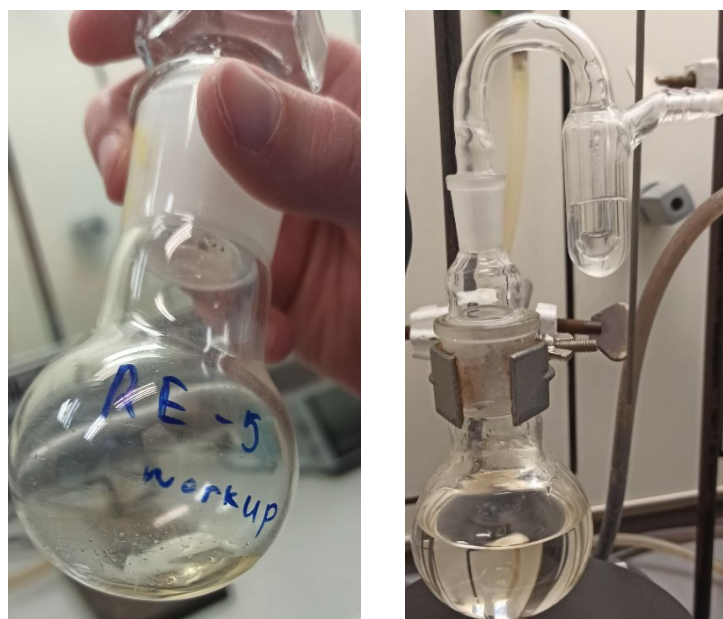


Figure 13. From left to right: entries 4 and 5 (during the reaction)

3.2. Investigation of the inclusion complexes of different capsaicinoids with various β -CD derivatives

In the next part of our research work, the capsaicinoid-CD inclusion complexes were investigated, firstly, by phase solubility tests (*Table 2*), and then by NMR spectroscopy (^1H and HSQC) in order to check the solubility of the capsaicinoids in various cyclodextrins solutions and to examine the complex formation (*Figure 14*).

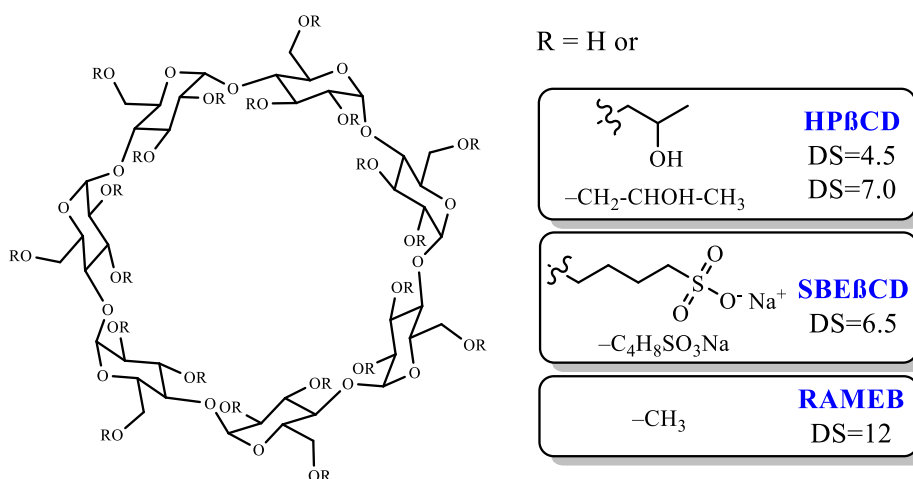


Figure 14. General structure of used β -CD derivatives

Table 2.: Performed phase solubility tests

Guest molecule	CD	DS
Capsaicin	HP β CD	4.5
Capsaicin		7
Nonivamide		4.5
Nonivamide		7
Dihydrocapsaicin		4.5
Dihydrocapsaicin		7
Capsaicin	SBE β CD	6.5
Dihydrocapsaicin		
Nonivamide		
Capsaicin	RAMEB	12
Dihydrocapsaicin		
Nonivamide		

¹H-NMR:

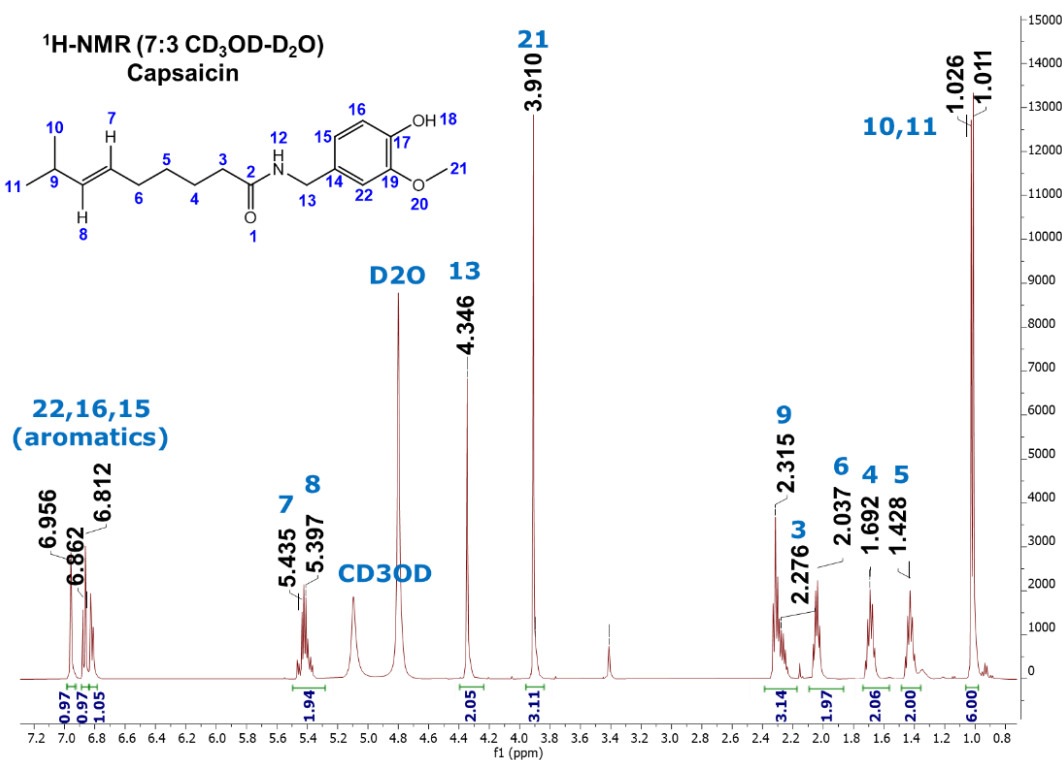


Figure 15. ¹H-NMR spectrum of capsaicin

Not only phase solubility tests results would be presented in this section, but also ^1H NMR and HSQC spectra. ^1H NMR displays the difference of the chemical shift of pure capsaicinoids and their complexes with various CDs; this can indicate whether the complexation reaction was successful or not. In *Figures 15-17* pure capsaicinoids spectra are presented, however each complex and its spectra would be discussed separately in the according section. ^1H -NMR was performed in 3:7 $\text{D}_2\text{O}/\text{CD}_3\text{OD}$ solutions.

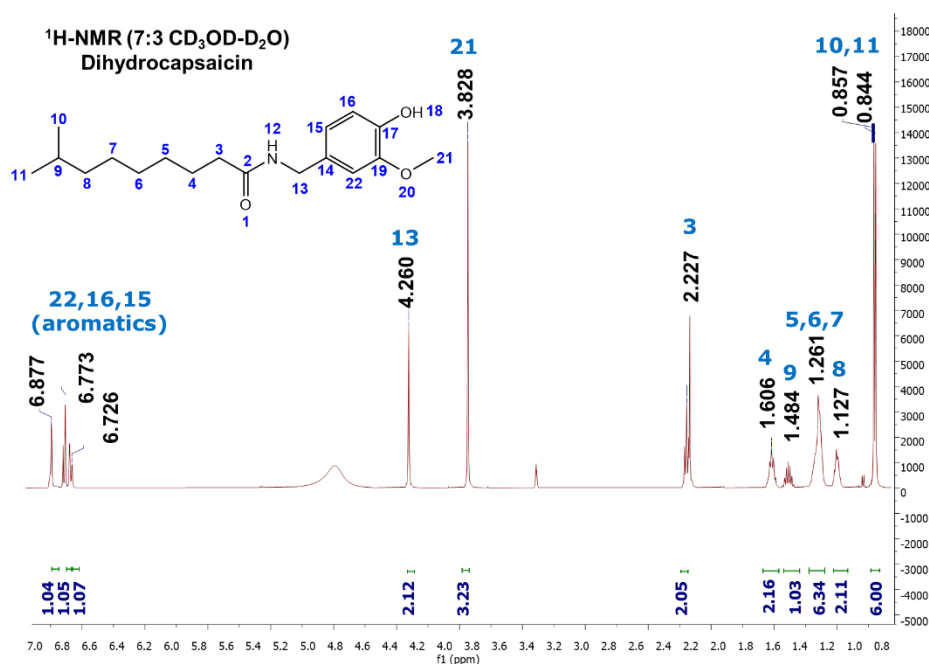


Figure 16. ^1H -NMR spectrum of dihydrocapsaicin

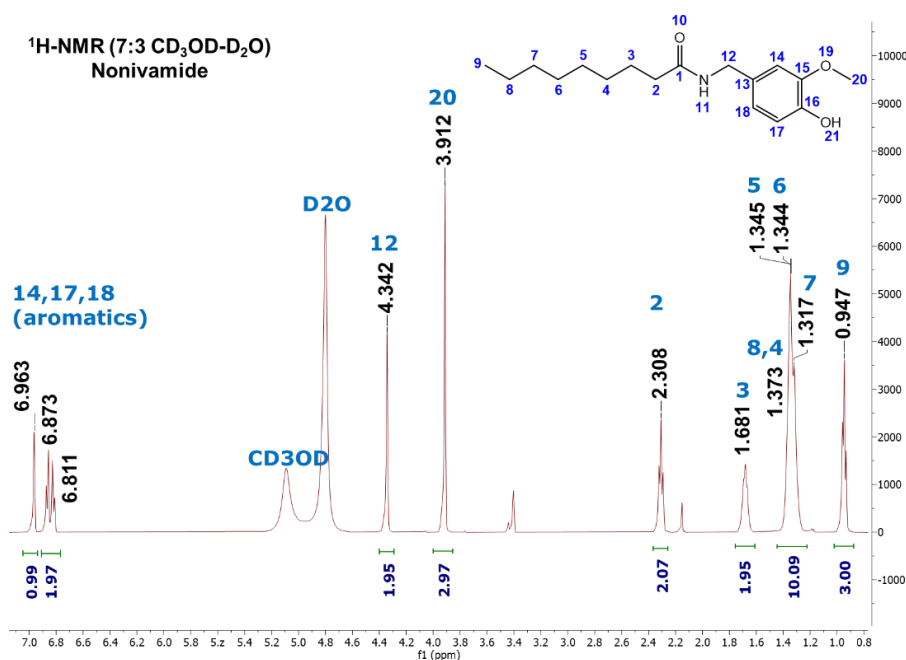


Figure 17. ^1H -NMR spectrum of nonivamide

HSQC NMR:

HSQC analysis was performed in order to have an idea whether there is an interaction between the inner hydrogens (H_3 and H_5) of the HP β CD or RAMEB with the encapsulated molecule (*Figure 18*).

In order to determine, whether the encapsulation took place or not, HSQC NMR analysis of pure CDs was performed using deuterated water (D_2O) and the resulting spectra are presented in *Figures 19-21*. In this section each capsaicinoid would be discussed separately and some of the capsaicinoids- β CD complex systems would be investigated using HSQC spectroscopy. Comparison of the chemical shifts difference would be done between pure CDs and capsaicinoids- β CD complexes.

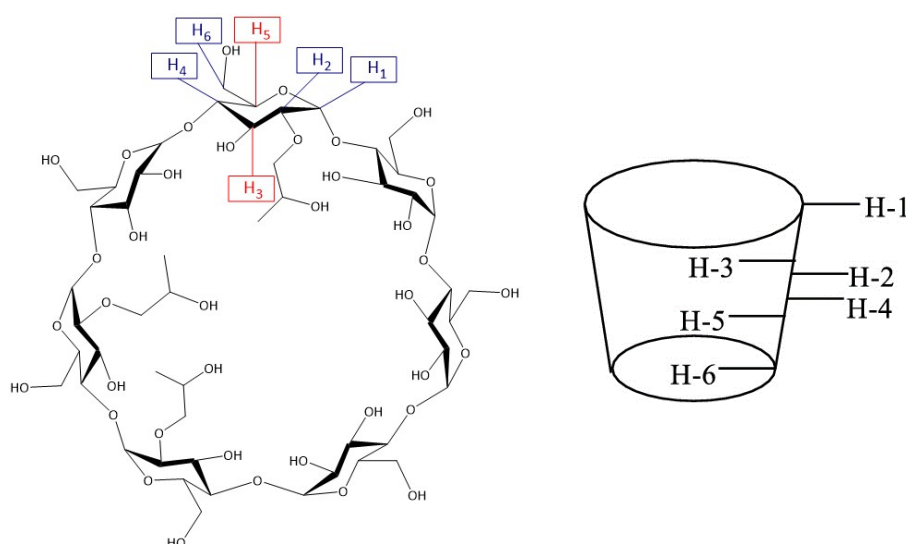


Figure 18. Atomic numbering of β -CD

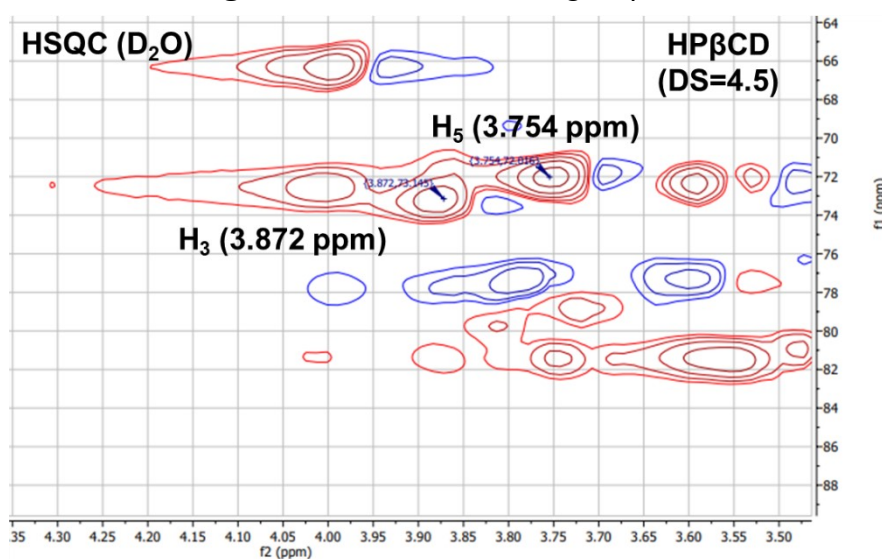


Figure 19. HSQC spectrum of HP β CD with DS=4.5

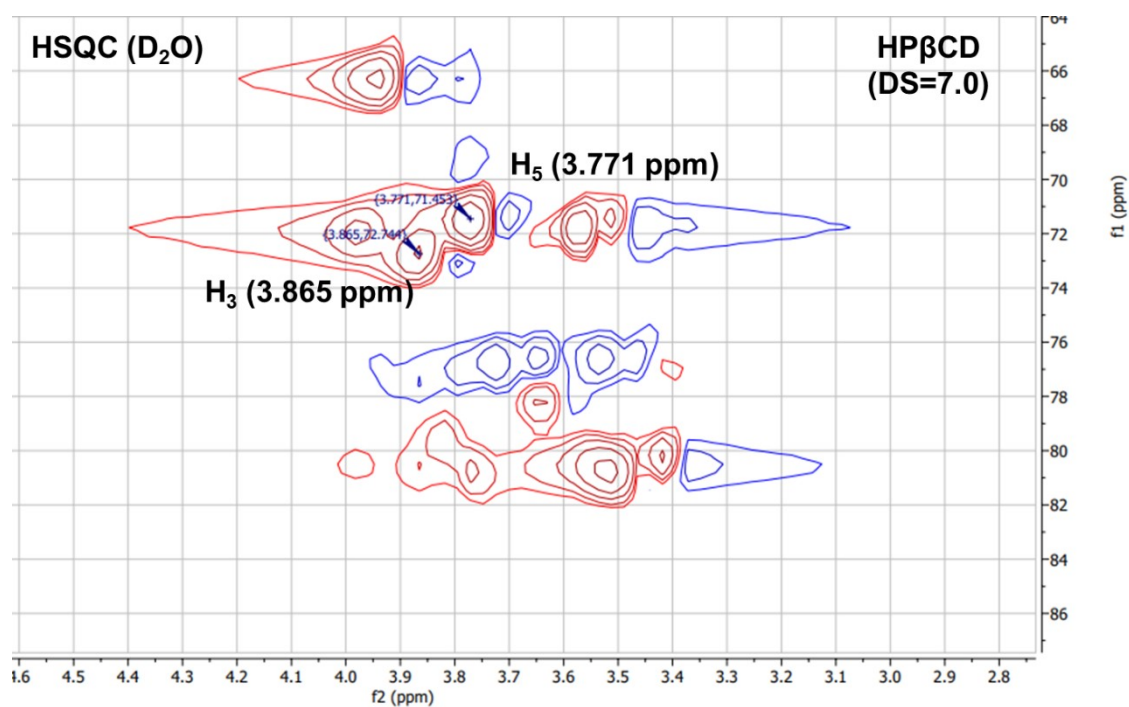


Figure 20. HSQC spectrum of HP β CD with DS=7.0

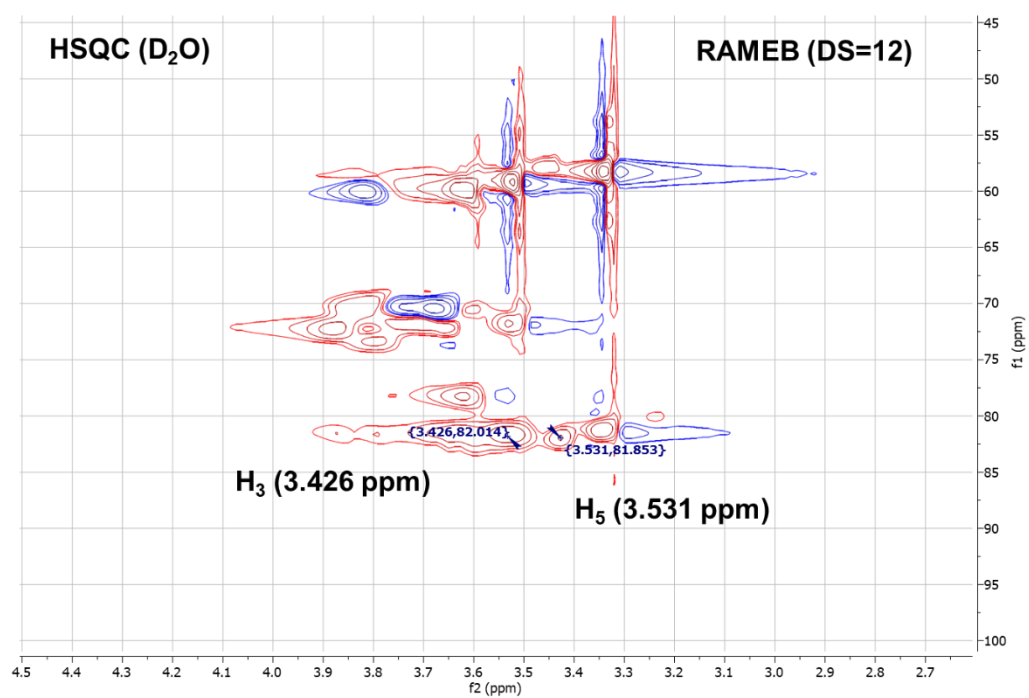


Figure 21. HSQC spectrum of RAMEB with DS=12

3.2.1. The phase solubility studies of capsaicin with β -CD derivatives

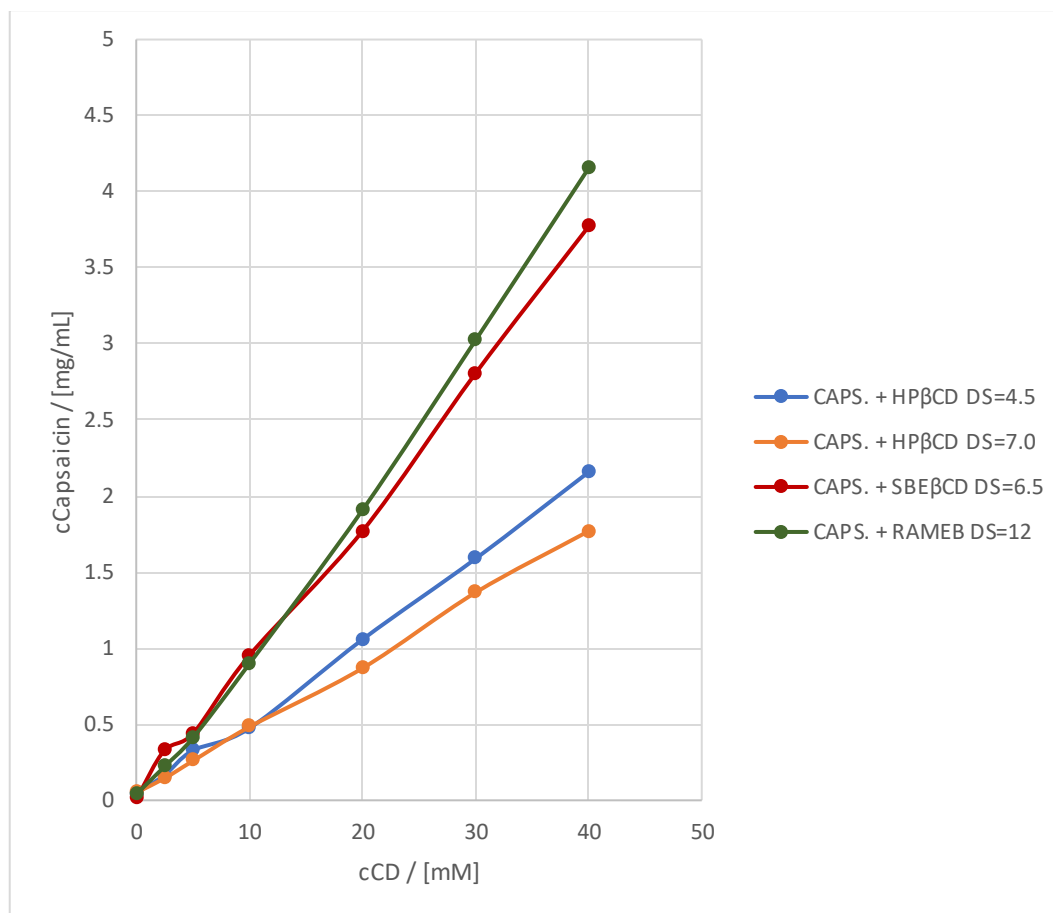


Figure 22. Phase solubility diagram of capsaicin- β CD complexes

Figure 22 demonstrates four different curves of similar behaviour, which possess a linear relationship with the concentration of host molecule. Water solubilisation of capsaicin was successful with all four CDs; however, it can be concluded from the graph that the best complexation agent for the capsaicin is RAMEB. It can possibly indicate that the cavity of RAMEB is optimal for the capsaicin molecule due to its structural features – probably, the substituted phenyl group is more likely to be encapsulated by this CD derivative. SBE β CD also presents successful results in dissolution property, and both of the HP β CDs have significantly smaller complexation potential than the other CDs. Probably, hydroxy-propyl group presents a steric hindrance or has some properties that hinder its encapsulating activity.

Table 3.: Optimal concentrations for capsaicin complexation reactions:

Host molecule		Guest molecule	
Type	Concentration	[mg/ml]	[mM]
RAMEB DS=12	40 mM	4.154	13.6
SBE β CD DS=6.5		3.771	12.3
HP β CD DS=4.5		2.157	7.1
HP β CD DS=7.0		1.769	5.8

3.2.2. Evaluation of the capsaicin complexes by NMR spectroscopy

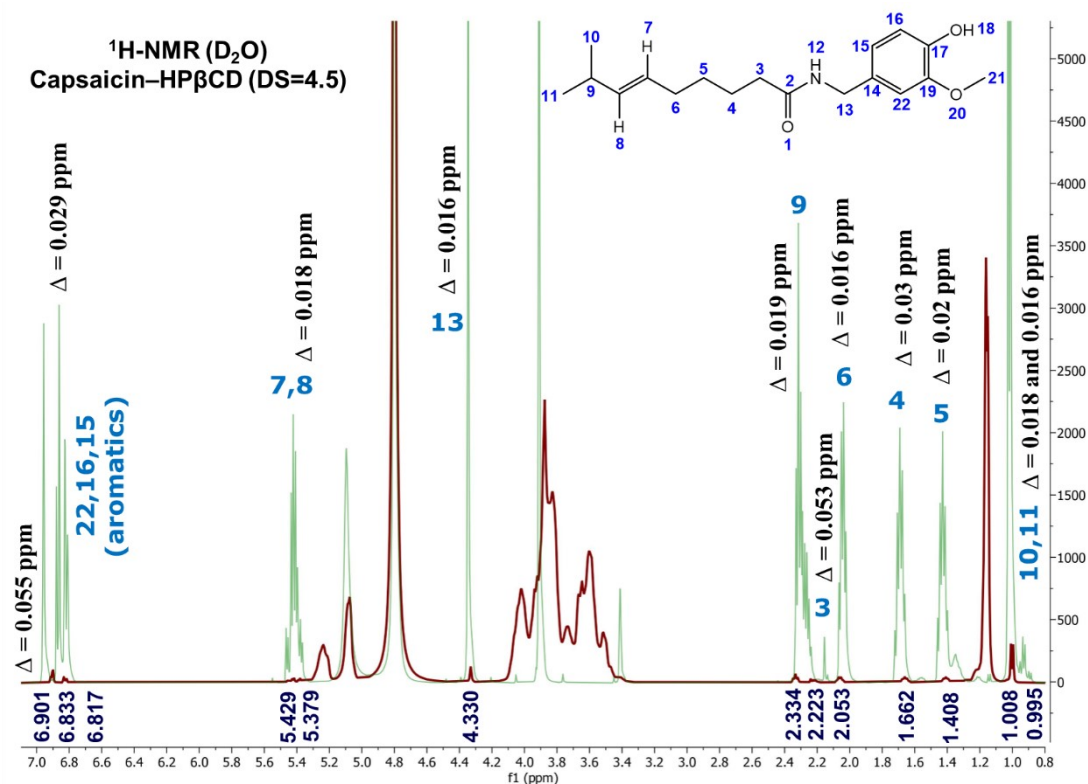


Figure 23. Overlaid ^1H NMR spectra of capsaicin (green) and capsaicin-HP β CD (DS=4.5) complex (red)

According to the *Figures 23 and 24*, where green line represents capsaicin peak and red line represents capsaicin-CD peak, there is a difference in chemical shifts, which indicates that the chemical environment is different. This can lead to conclusion that the complex formation was probably successful in both cases; however, HSQC NMR spectroscopy analysis was also performed.

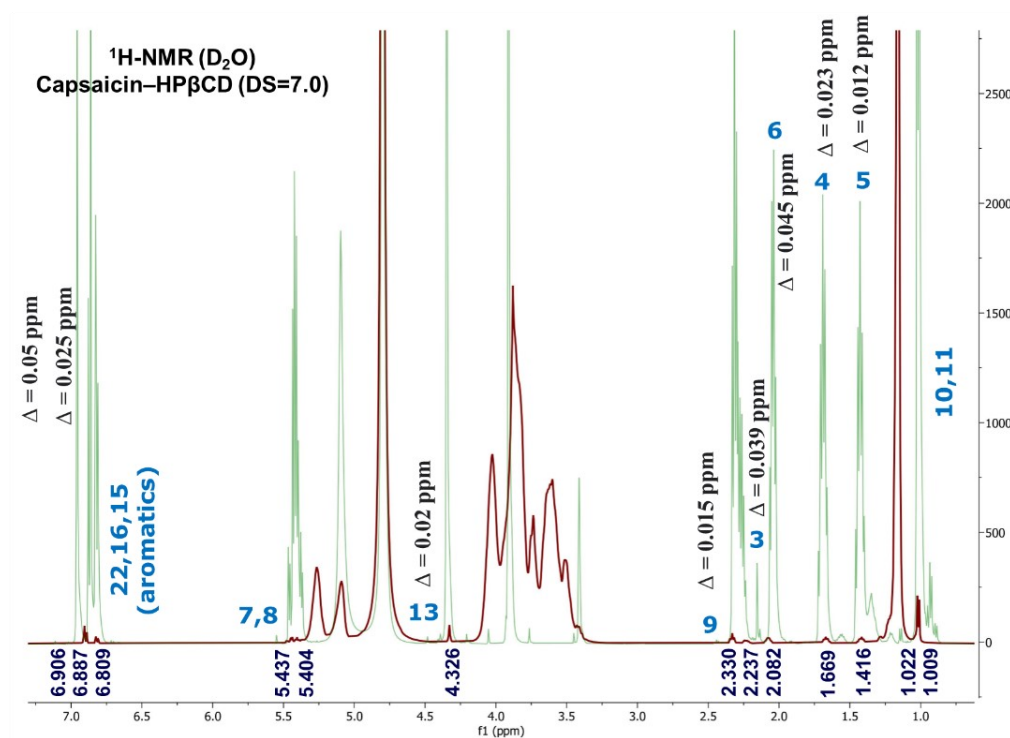


Figure 24. Overlaid ^1H NMR spectra of capsaicin (green) and capsaicin-HP β CD (DS=7.0) complex (red)

According to the HSQC NMR spectroscopy analysis of capsaicin-HP β CD DS=4.5 (Figure 25), there is a difference in chemical shift of the H_3 atom, and no difference for H_5 . In case of capsaicin-HP β CD DS=7.0 (Figure 26) a shift difference is observed for both protons.

This indicates, that H_3 atom is in a different chemical environment, and interacts with the guest (capsaicin) molecule. It can be concluded that the complexation was successful, and the guest molecule is likely to form a complex from the secondary side of the CD's cavity.

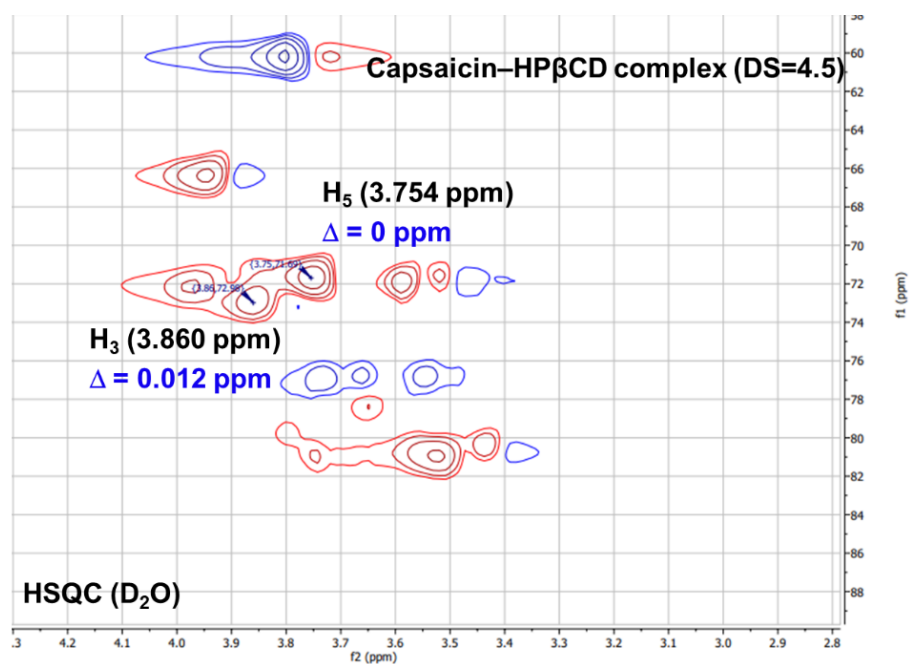


Figure 25. HSQC spectrum of capsaicin-HPβCD (DS=4.5)

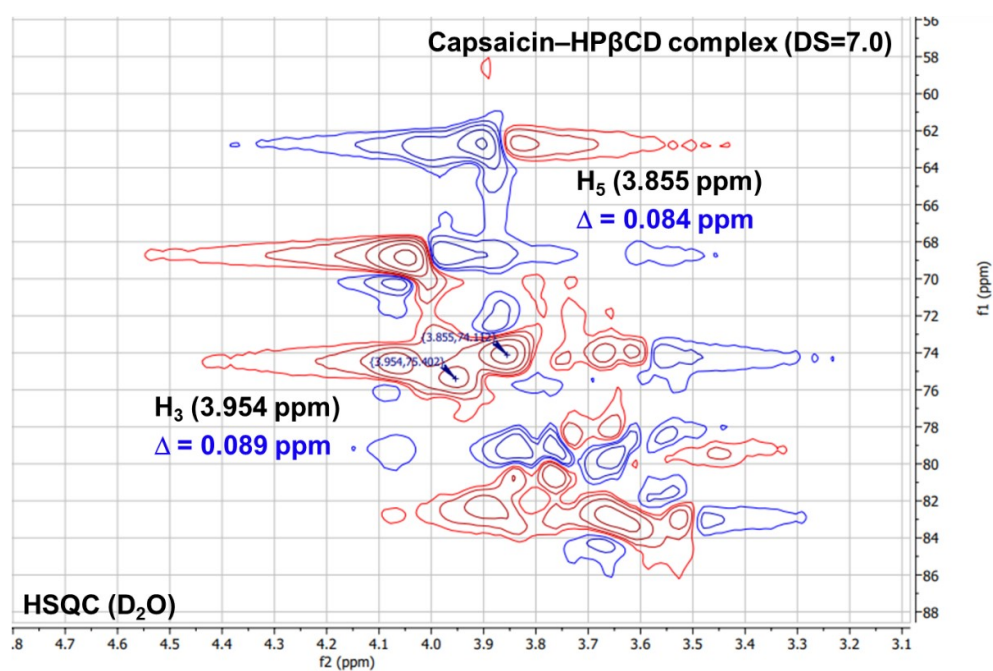


Figure 26. HSQC spectrum of capsaicin-HPβCD (DS=7.0)

3.2.3. The phase solubility studies of dihydrocapsaicin with β -CD derivatives

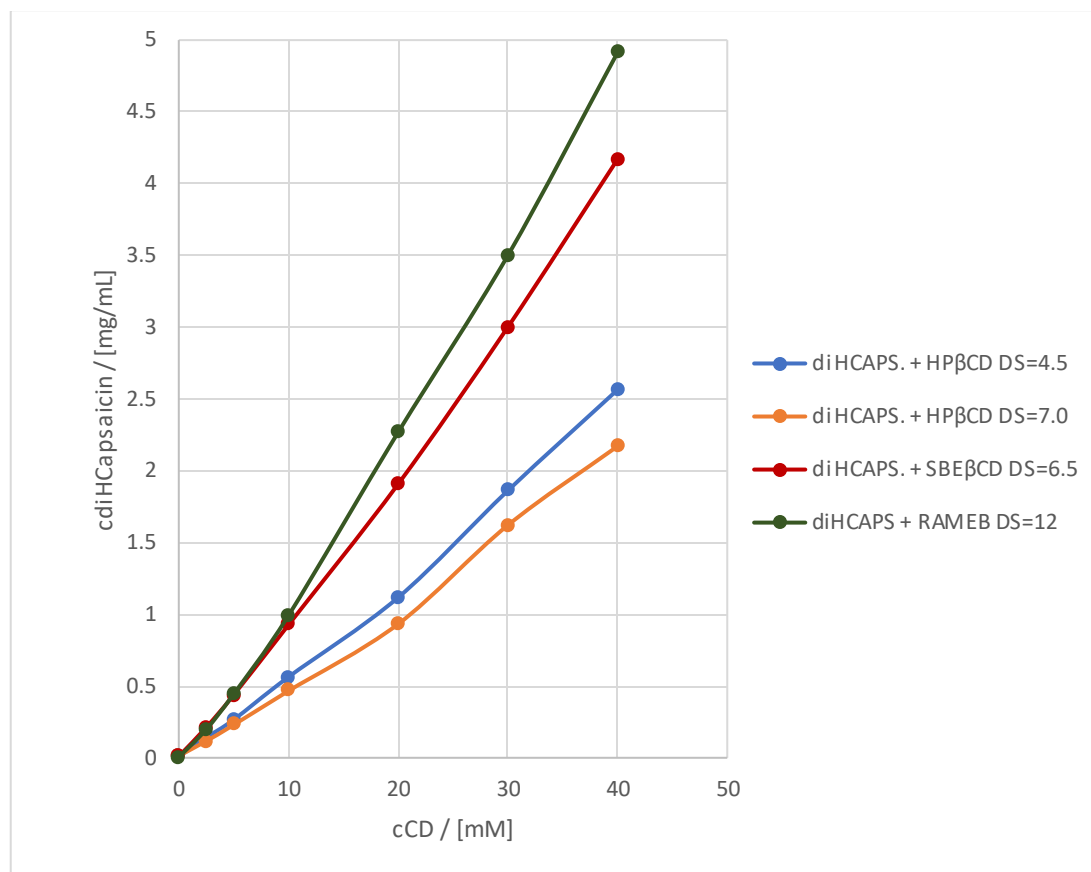


Figure 27. Phase solubility diagram of dihydrocapsaicin– β CD complexes

Figure 27 presents four different curves of similar behaviour: all the dihydrocapsaicin–CD complexes dissolve well in water and have linear relationship with the concentration of host molecule. Again, the RAMEB shows the best encapsulating activity among all other CDs, and it is the highest solubility reached in phase solubility tests in this work. It can possibly indicate that the cavity of RAMEB is optimal for the dihydrocapsaicin molecule due to its structural features. Probably, the substituted phenyl group is encapsulated by the cavity of the CD, although this cannot be clearly established from NMR measurements, further 2D ROESY NMR measurements are needed to determine the exact structure. Similarly, to the capsaicin results, the second-best complex formation agent is SBE β CD, and the worst are HP β CDs.

Table 4.: Optimal concentrations for dihydrocapsaicin complexation reactions:

Host molecule		Guest molecule	
Type	Concentration	[mg/ml]	[mM]
RAMEB DS=12	40 mM	4.914	16.0
SBE β CD DS=6.5		4.17	13.6
HP β CD DS=4.5		2.564	8.3
HP β CD DS=7.0		2.171	7.1

3.2.4. Evaluation of the dihydrocapsaicin complexes by NMR spectroscopy

According to the ^1H NMR spectroscopy results (Figures 28-30), all of the complexations were performed successfully, since there are differences in the chemical shifts, which are extremely high in the case of HP β CD with DS=7 (Figure 29). Probably, there is a strong interaction between the host and guest molecule, which gives such result. It can indicate that the structure of the dihydrocapsaicin is suitable for the inclusion complexes formation with HP β CD.

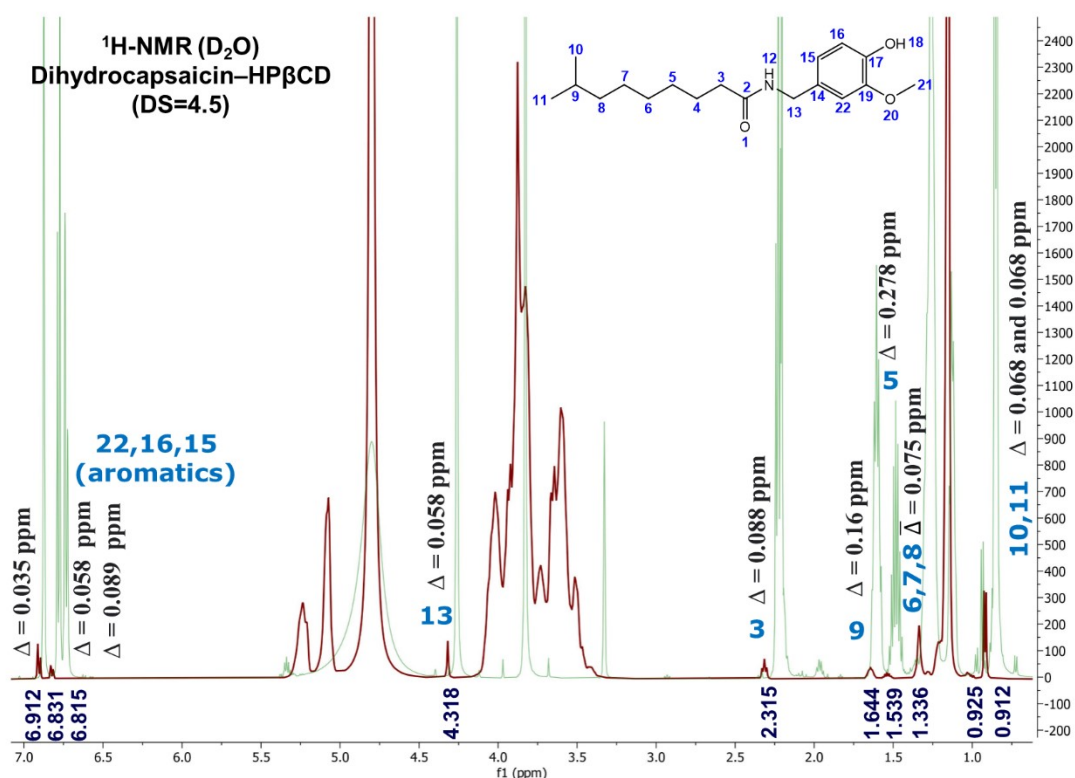


Figure 28. Overlaid ^1H NMR spectra of dihydrocapsaicin (green) and dihydrocapsaicin–HP β CD (DS=4.5) complex (red)

In the case of dihydrocapsaicin-RAMEB complexation (Figure 30), only some of the peaks were identified, since there are too big chemical shifts in the further peaks. This is, likely, indicating, that the complexation was successful, and the chemical environment of the guest molecule significantly differs, as it can be concluded from the phase solubility test.

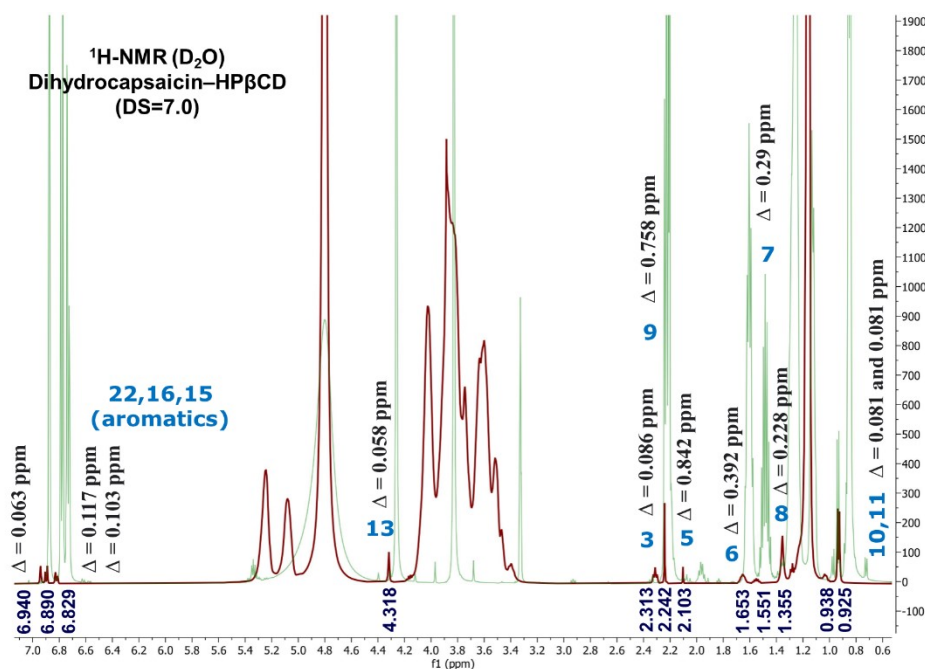


Figure 29. Overlaid ¹H NMR spectra of dihydrocapsaicin (green) and dihydrocapsaicin-HPβCD (DS=7.0) complex (red)

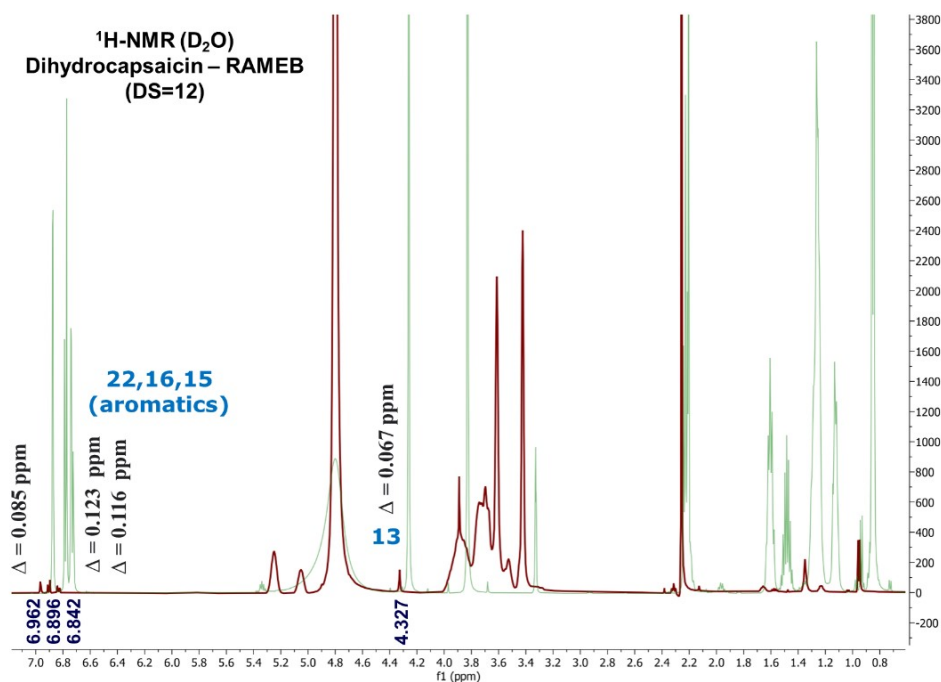


Figure 30. Overlaid ¹H NMR spectra of dihydrocapsaicin (green) and dihydrocapsaicin-RAMEB (DS=12) complex (red)

According to the HSQC spectroscopy results (Figures 31-33), both of the complexations were performed successfully, since there are differences in the chemical shifts. It can indicate that there is an interaction between the dihydrocapsaicin and the following H-atom and that the structure of the dihydrocapsaicin is suitable for the inclusion complexes formation not only with HP β CD, but also with RAMEB (Figure 33).

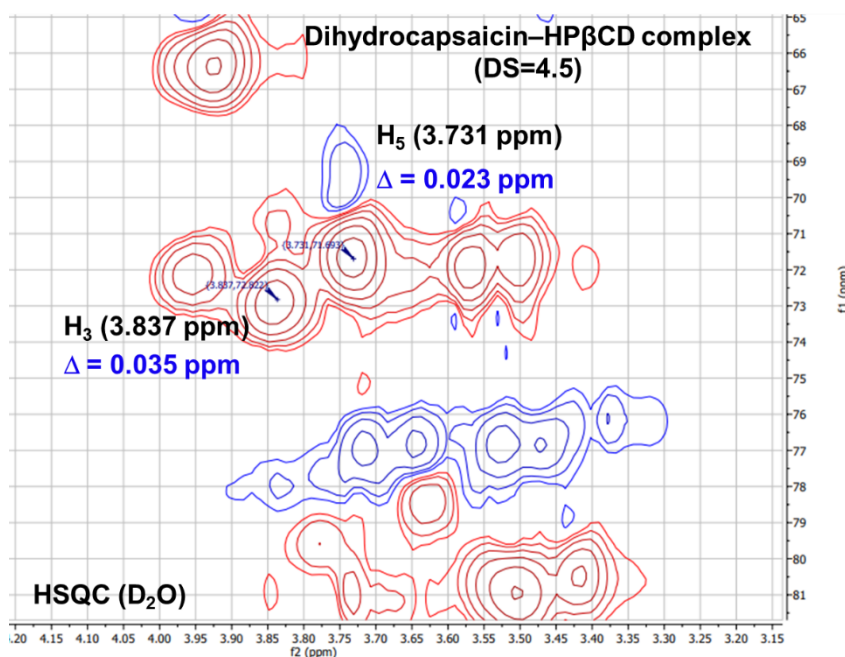


Figure 31. HSQC spectrum of dihydrocapsaicin-HP β CD (DS=4.5)

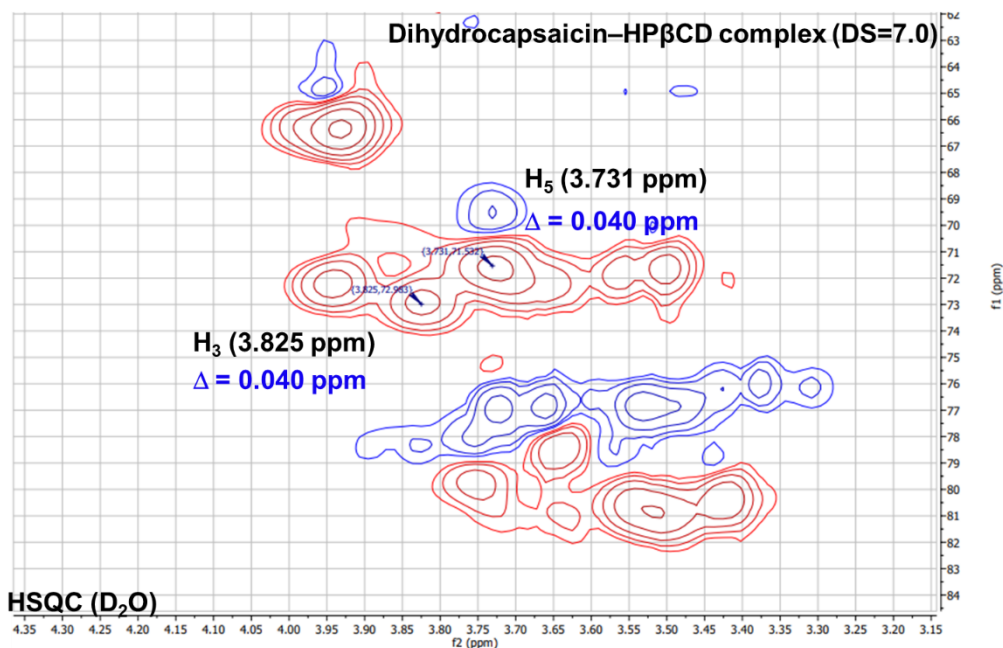


Figure 32. HSQC spectrum of dihydrocapsaicin-HP β CD (DS=7.0)

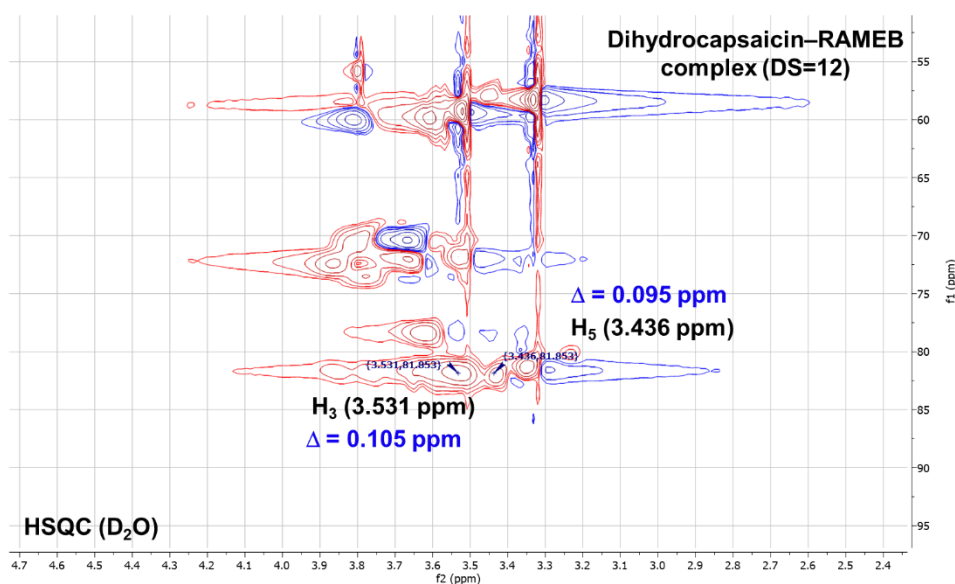


Figure 33. HSQC spectrum of dihydrocapsaicin–RAMEB (DS=12)

3.2.5. The phase solubility studies of nonivamide with β -CD derivatives

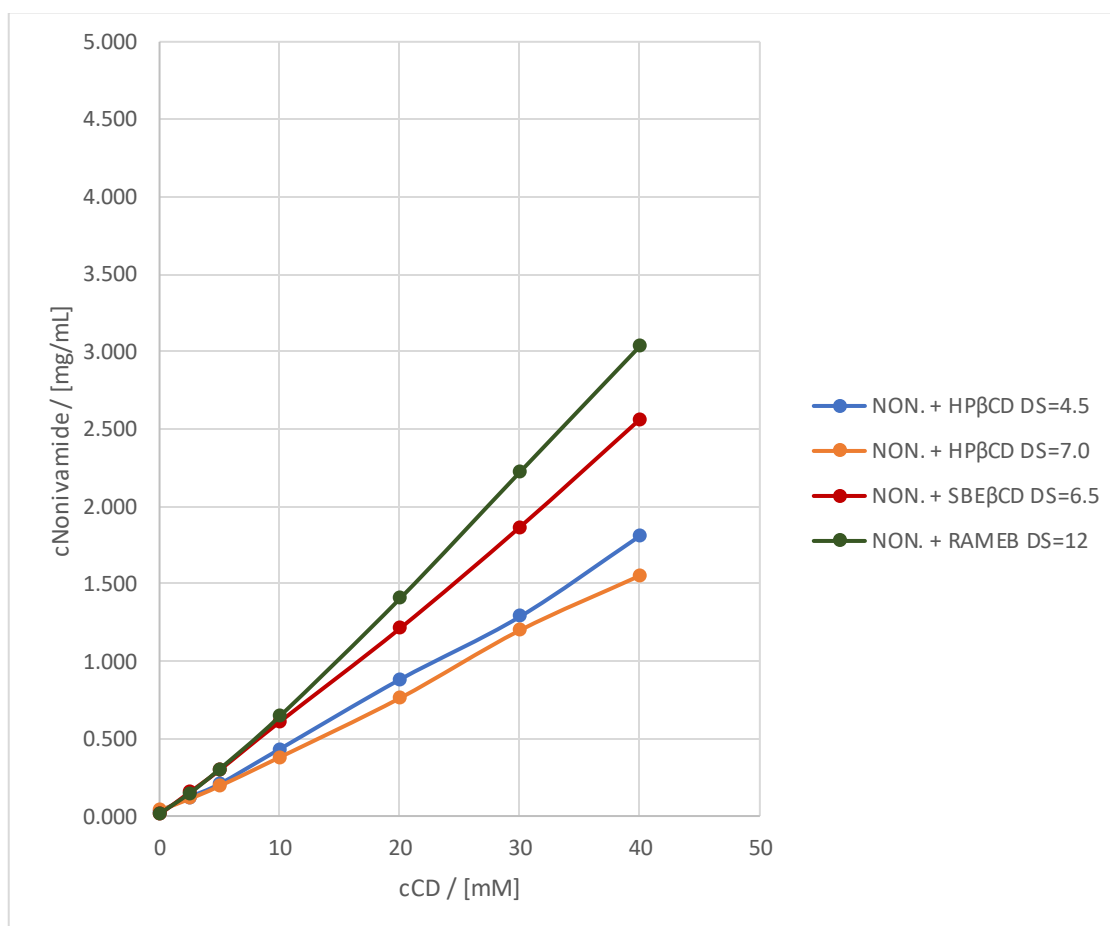


Figure 34. Phase solubility diagram of nonivamide– β CD complexes

Figure 34 presents the phase solubility diagrams of nonivamide with different β CD derivatives. The curve represents the A_L type diagram according to *Higuchi and Connors*, which means that the dissolved nonivamide is linearly proportional to the concentration of the CDs. Similarly with the results of previous capsaicinoids, RAMEB in all three cases showed the best encapsulation ability, after it came SBE β CD, and HP β CDs displayed the worst complex formation potential. Nonivamide showed to be the least soluble API in β CDs, probably, due to the linear chain at the end of the molecule. Based on the characteristic property of β CD, in case of nonivamide–CD complex we can also assume that the phenyl group of guest molecule is complexed in the CD's cavity. Further NMR measurements are needed to determine the exact 3D structure, but from the present results we can clearly infer the formation of the complexes.

Table 5.: Optimal concentrations for nonivamide complexation reactions:

Host molecule		Guest molecule	
Type	Concentration	[mg/ml]	[mM]
RAMEB DS=12	40 mM	3.033	10.3
SBE β CD DS=6.5		2.561	8.7
HP β CD DS=4.5		1.808	6.2
HP β CD DS=7.0		1.551	5.3

3.2.6. Evaluation of the nonivamide complexes by NMR spectroscopy

As it can be seen in *Figures 35 and 36*, there is a small, but not negligible, chemical shift difference between nonivamide (green) and its complex with HP β CD DS=4.7 and 7.0 HP β CD (red). Also, it can be noted from both of the HSQC spectra (*Figures 37 and 38*) that there are differences in the chemical shifts as well.

This difference of the chemical shift in both ^1H and HSQC spectra (*Figures 35-38*) indicates that nonivamide molecule is in a different chemical environment and shows interactions with the CD molecules.

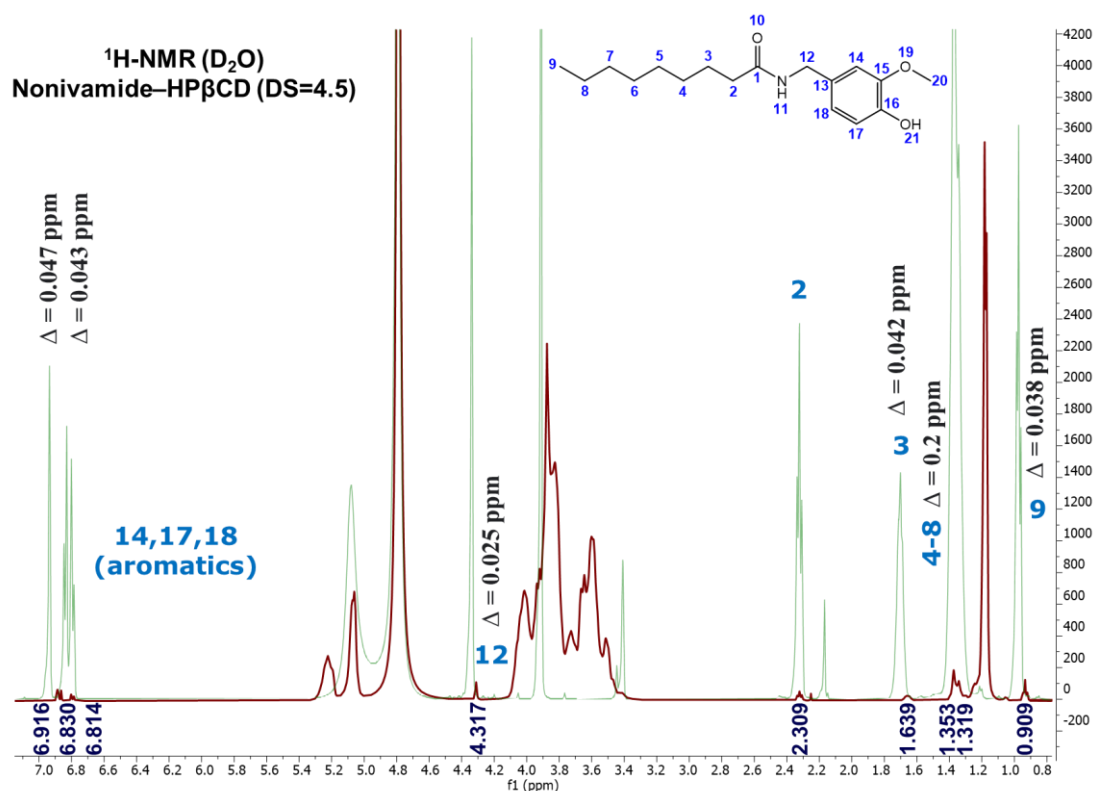


Figure 35. Overlaid ¹H NMR spectra of nonivamide (green) and nonivamide-HPβCD (DS=4.5) complex (red)

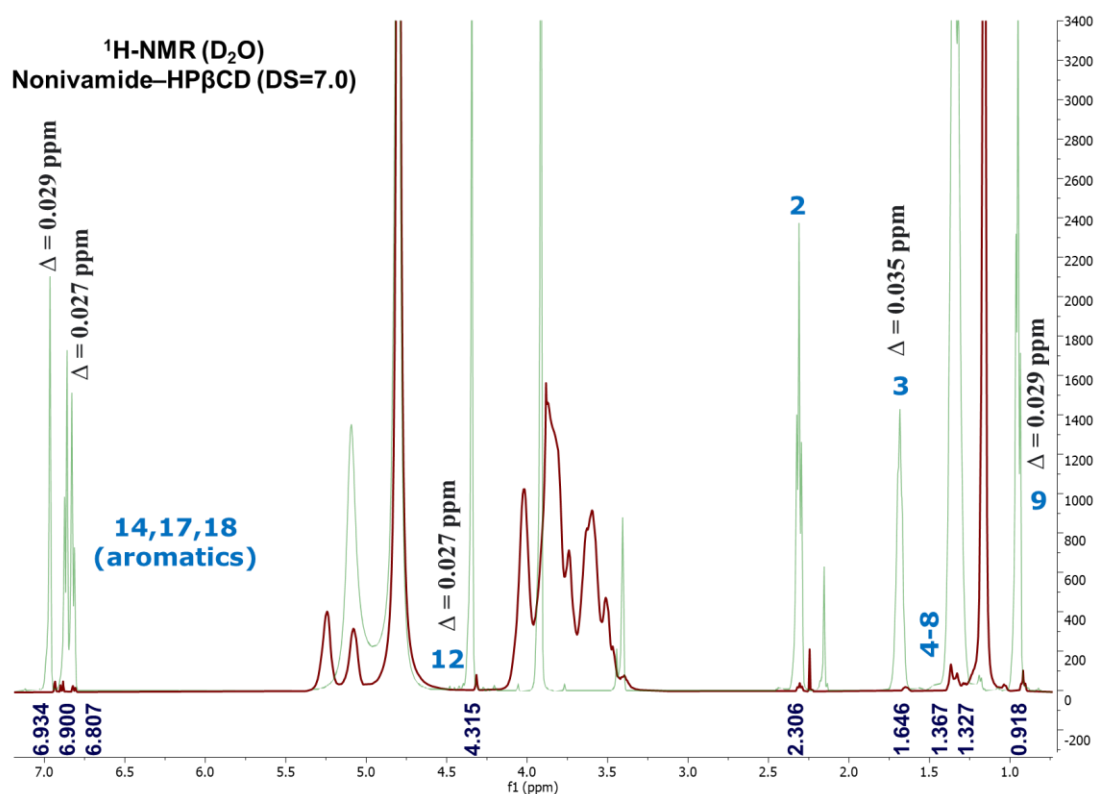


Figure 36. Overlaid ¹H NMR spectra of nonivamide (green) and nonivamide-HPβCD (DS=7.0) complex (red)

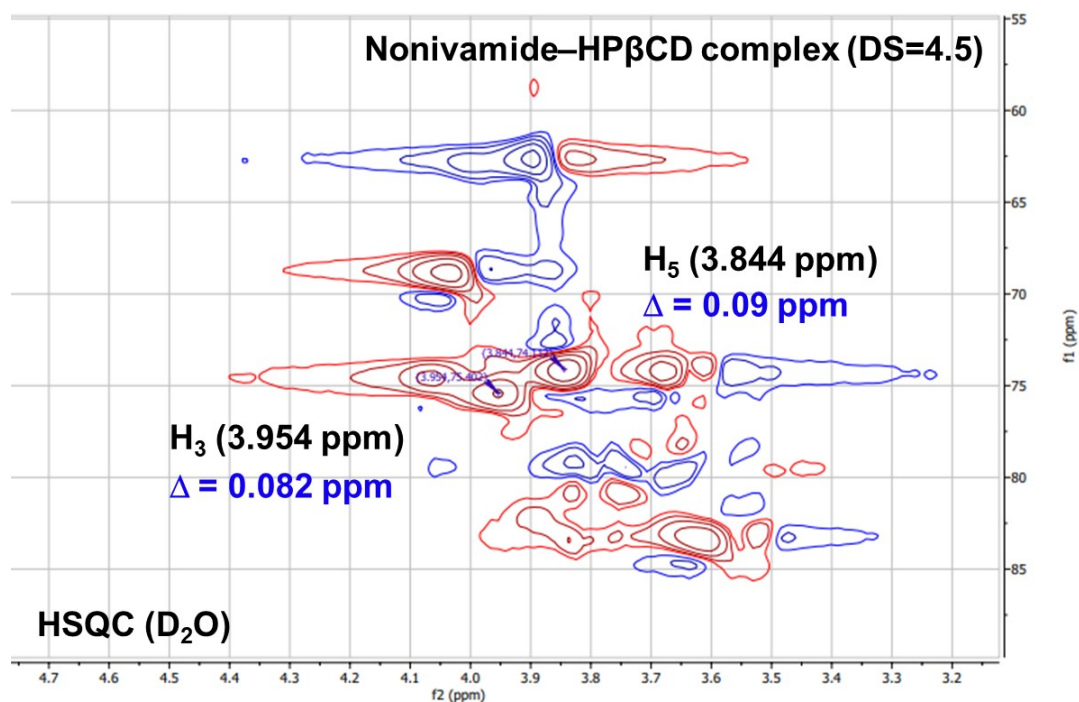


Figure 37. HSQC spectrum of nonivamide-HP β CD (DS=4.5) complex

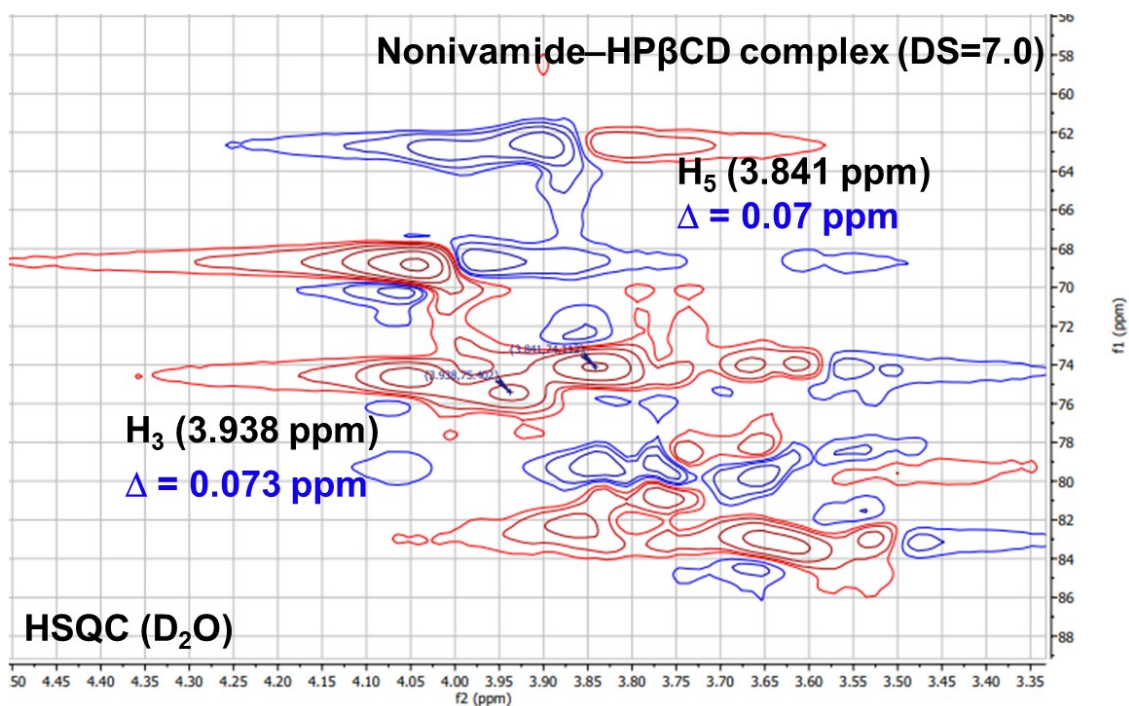


Figure 38. HSQC spectrum of nonivamide-HP β CD (DS=7.0) complex

4. Experimental part

4.1. Analytical equipment

High-performance liquid chromatography-mass spectrometry (HPLC-MS) measurements were performed with an Agilent 1200 liquid chromatography system coupled with a 6130 quadrupole mass spectrometer equipped with an ESI ion source (Agilent Technologies, Palo Alto, CA, USA). Analysis was performed at 40 °C on a Gemini C18 column (50 mm × 4.6 mm, 3 µm; Phenomenex, Torrance, CA, USA) with a mobile phase flow rate of 0.5 mL/min. Composition of eluent A was 0.1% (NH₄)(HCOO) in water; eluent B was 0.1% (NH₄)(HCOO) and 8% water in acetonitrile, 0–3 min isocratic at 20% B%, 3–13 min gradient to 100% B%, 13–20 min 100% B. The injection volume was 2 µL. The chromatographic profile was registered at 254 nm. The MSD operating parameters were as follows: positive ionization mode, scan spectra from m/z 120 to 1000, drying gas temperature 300 °C, nitrogen flow rate 12 L/min, nebulizer pressure 60 psi, capillary voltage 4000 V, fragmentor voltage 70 V.

The gas chromatographic measurements were performed using an HP 5890 type 2 device with an FID detector. The gas chromatograph contains a 15 m × 0.18 mm Restek Rtx-5 column with a film thickness of 0.20 µm. The temperature of the column is 40 °C for 1 minute, then the temperature program increases by 25 °C per minute until it reaches 300 °C and keeps this temperature at a constant value for 10 minutes. The injector temperature is 290 °C, and that of the FID detector is 300 °C. The carrier gas is N₂.

¹H NMR and HSQC spectra were recorded with a Bruker DRX-500 spectrometer at 125.7 and 500.1 MHz, TMS was used as a reference material.

4.2. General procedure for the scale-up synthesis of *N*-[(4-Hydroxy-3-methoxyphenyl)methyl]nonanamide (3)

In the entry **1** (*Table 6*) the mixture of 1.0 mmol of nonanoic acid (**17**) (0.174 ml), 1.15 mmol of carbonyldiimidazole (CDI) (0.1865 g) was stirred in 10 ml of gamma-valerolactone (GVL) at 25 °C for 30 minutes. After this, to the reaction mixture 1.0 mmol of vanillyl amine free base (**10**) (0.1532 g) were added and stirred at 70 °C for 5 minutes. The reaction mixture was analysed by HPLC and GC.

In the entry **2** (*Table 6*) the mixture of 1.0 mmol of nonanoic acid (**17**) (0.174 ml), 1.15 mmol of carbonyldiimidazole (0.1865 g) was stirred in 10 ml of gamma-valerolactone at 25 °C for 30 minutes. After this, to the reaction mixture 1.0 mmol of vanillyl amine hydrochloride

salt (**9**) (0.1896 g) were added and stirred at 70 °C for 5 minutes. The reaction mixture was analysed by HPLC and GC.

In the entry **3** (Table 6) the mixture of 5.0 mmol of nonanoic acid (**17**) (0.8695 ml), 5.75 mmol of carbonyldiimidazole (0.9324 g) was stirred in 30 ml of gamma-valerolactone at 25 °C for 1 hour. After this, to the reaction mixture 5.0 mmol of vanillyl amine hydrochloride salt (**9**) (0.9482 g) were added and stirred at 70 °C for 1 hour. The reaction mixture was analysed by HPLC and GC.

In the entry **4** (Table 6) the mixture of 5.0 mmol of nonanoic acid (**17**) (0.8695 ml), 5.75 mmol of carbonyldiimidazole (0.9324 g) was stirred in 50 ml of 2-methyltetrahydrofuran (2-Me-THF) at 25 °C for 90 minutes. After this, to the reaction mixture 5.0 mmol of vanillyl amine hydrochloride salt (**9**) (0.9482 g) were added and stirred at 70 °C for 1 hour. The reaction mixture was analysed by HPLC and GC. The product was purified by extraction with hydrochloric acid/distilled water solution and ethyl acetate.

In the entry **5** (Table 6) the mixture of 10.0 mmol of nonanoic acid (**17**) (1.734 ml), 11.5 mmol of carbonyldiimidazole (1.865 g) was stirred in 100 ml of gamma-valerolactone at 25 °C for 1 hour. After this, to the reaction mixture 10.0 mmol of vanillyl amine hydrochloride salt (**9**) (1.8964 g) were added and stirred at 70 °C for 2 hours. The reaction mixture was analysed by HPLC and GC. The product was purified by normal phase column chromatography, using silica gel as a stationary phase and 1:1 ethyl acetate-hexane eluent with an 89% yield.

Table 6.: Amount and mass of the used reagents

Entry	17		CDI		9 or 10		Solvent	
	n	V	n	mass	n	mass	name	V
	[mmol]	[ml]	[mmol]	[g]	[mmol]	[g]		[ml]
1	1	0.174	1.15	0.1865	1	0.1532	GVL	10
2	1	0.174	1.15	0.1865	1	0.1896	GVL	10
3	5	0.8695	5.75	0.9324	5	0.9482	GVL	30
4	5	0.8695	5.75	0.9324	5	0.9482	2-Me-THF	50
5	10	1.734	11.5	1.865	10	1.8964	GVL	100

4.3. General procedure for the phase solubility test and complexation reactions

In order to perform phase solubility test, a 40 mM stock solution of the required β -CD derivative was prepared. To the seven different sample holders, around 6-10 mg of the capsaicinoid was added, and 1 ml of the β -CD solution diluted with the distilled water to the following concentrations was added: 0 mM (pure distilled water), 2.5 mM, 5 mM, 10 mM, 20 mM, 30 mM, and 40 mM (pure stock solution). Magnetic stirrer bar was added to all of the sample holders, and they were left to stir for 24 hours. After this, filtration was performed, and the filtrate was analysed by HPLC in order to determine capsaicinoid concentration.

After the optimal concentration of the capsaicinoid-CD was found out, the inclusion complexes reactions were performed and summarized in *Table 7*. In order to execute such reaction, firstly, the received optimal concentration data from the phase solubility test were taken and used. 1.5 equivalent of the optimal CD concentration was used (60 mM) and added to the 25 ml volumetric flask. After CD was dissolved in the distilled water, capsaicinoid amount calculated from its optimal concentration was added to the same volumetric flask and stirred under ultrasonic water bath for 30-60 minutes. Then, the solution was placed in rotary evaporator to dissolve water (80 °C, 50-100 mbar), and the obtained white crystals was sent to NMR analysis.

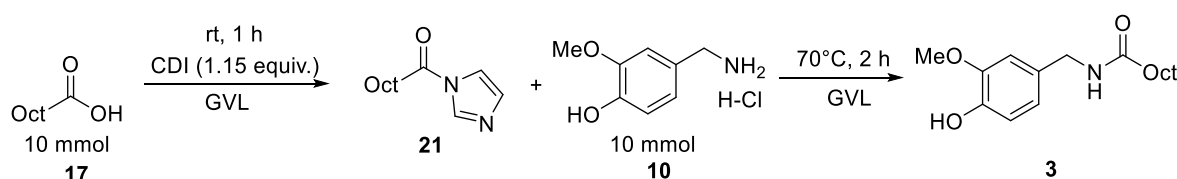
Table 7.: Summary of the performed phase solubility tests and complexation reactions

Reaction	Host molecule				Guest molecule		
	Type	DS	Concentration	Mass [g]	Type	C [mM]	Mass [g]
Complex	HPβCD	4.5	60 mM	2.095	Capsaicin	7.1	0.053
		7		2.3125	Capsaicin	5.8	0.0443
		4.5		2.095	Nonivamide	6.2	0.0455
		7		2.3125	Nonivamide	5.3	0.0389
		4.5		2.095	Dihydrocapsaicin	8.3	0.0645
		7		2.3125	Dihydrocapsaicin	7.1	0.0545
Phase solubility test	SBEβCD	6.5	0-40 mM	0.8653	Capsaicin	-	0.01
					Dihydrocapsaicin		
					Nonivamide		
	RAMEB	12		0.5212	Capsaicin		
					Dihydrocapsaicin		
					Nonivamide		
Complex			60 mM	1.9545	Dihydrocapsaicin	16	0.1229

5. Conclusions

During our work, the complexation reactions of capsaicinoids (capsaicin, dihydrocapsaicin, nonivamide) with various β -CDs (HP β CD, SBE β CD, RAMEB) were investigated through phase solubility studies and ^1H and HSQC NMR spectroscopy. In order to achieve the aim, scale-up synthesis of nonivamide was performed to obtain a guest molecule for the further complexations.

First, the model reaction of nonivamide scale-up synthesis was studied and optimized in respect of the molar ratio of the starting materials, usage of the vanillyl amine free base or salt form, solvent, and reaction time. The reaction was successfully scaled up (Conversion 99%; *Table 1/Entry 5*) with the following optimal conditions: 10 mmol of reagents, vanillyl amine hydrochloride salt, in GVL solvent and room temperature and 1 h for the first step (activation of the nonanoic acid) and 70 °C and 2 h for the second step (*N*-acylation of vanillyl amine hydrochloride). After the reaction, the product was purified with 89% yield through column chromatography.



Scheme 10. Scaled up nonivamide batch synthesis reaction (optimized)

After the scale-up reaction, the complexation reactions of nonivamide and other capsaicinoids (capsaicin and dihydrocapsaicin) were carried out with various β -CD derivatives. Altogether, 12 phase solubility tests as well as 7 inclusion complexes reactions were performed and studied (*Table 7* and *Table 9*).

According to the phase solubility results, all of the curves showed A_L type diagram, which indicated the linear relationship of capsaicinoid and the β -CD derivatives dissolution concentration and was a sign of good solubility of capsaicinoid CD solutions.

Capsaicinoids naturally are poor water-soluble compounds, however, the solution for this problem lies in complexation with β -CDs. Even the worst complexing agent HP β CD can increase solubility of capsaicinoids at least to 10-12 times. However, HP β CD has such low water solubility, probably, due to the sterical hindrance of the 2-hydroxy-propyl group.

Table 8.: Solubilities of used capsaicinoids and β -CD derivatives

Compound	Solubility in water at 25 °C
Capsaicin	0.14 g/L ^a
Dihydrocapsaicin	0.083 g/L ^a
Nonivamide	0.15 g/L ^a
RAMEB DS=12	~800g/L ^b
SBE β CD DS=6.5	~700 g/L ^c
HP β CD DS=4.5	450 g/L ^d
HP β CD DS=7.0	Unlimited ^e
β -cyclodextrin	18.5 g/L

^a Prediction using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2023 ACD/Labs)

^b According to Fischer Scientific

^c According to Jain A. S., Date A. A., Pissurlenka, R. R. S. et al., *AAPS PharmSciTech*, **2011**, 12, 1163–1175

^d According to Sigma Aldrich

^e It is stored and sold as an aqueous solution

Table 9.: Optimal concentrations results summary from highest to lowest

Host molecule		Guest molecule		
Type	Concentration	Type	[mg/ml]	[mM]
RAMEB DS=12	40 mM	dihydrocapsaicin	4.914	16
RAMEB DS=12		capsaicin	4.154	13.6
SBE β CD DS=6.5		dihydrocapsaicin	4.17	13.6
SBE β CD DS=6.5		capsaicin	3.771	12.3
RAMEB DS=12		nonivamide	3.033	10.3
SBE β CD DS=6.5		nonivamide	2.561	8.7
HP β CD DS=4.5		dihydrocapsaicin	2.564	8.3
HP β CD DS=4.5		capsaicin	2.157	7.1
HP β CD DS=7.0		dihydrocapsaicin	2.171	7.1
HP β CD DS=4.5		nonivamide	1.808	6.2
HP β CD DS=7.0		capsaicin	1.769	5.8
HP β CD DS=7.0		nonivamide	1.551	5.3

The best inclusion complex formation host molecule for capsaicinoids guest molecules showed to be RAMEB, and its complexes have the best solubility in water (*Table 8* and *Table 9*): dihydrocapsaicin, which has the lowest solubility, became 60 times more water-soluble. Probably, structural features of the RAMEB due to small size of the substitution group (and small sterical hindrance) allow it “take” molecules inside of its cavity easily. The second-best solubility enhancer is SBE β CD, while HP β CDs proved to be the worst for the enhancing for the water solubility of capsaicinoids.

Dihydrocapsaicin displayed the best solubilizing properties as a CD complex, while nonivamide, on the contrary, showed the worst solubility improvements. Regarding the structure of the complexes, it is assumed that the guest molecule is coordinated in the CD cavity with the phenol group, but further 2D ROESY NMR measurements are needed to investigate the exact the proper structure of the complex. The determination of the complex association constant can also help us to understand more deeply the stability of the complex. Furthermore, ^1H NMR measurements are needed to determine the association constant. The CD complexation of the capsaicinoids can be remarkably interesting with α CD derivatives as well, because the different cavity size can cause different complex structure. The α CD derivatives can create a complex with the alkyl chain of the capsaicinoids. One of our future goals is the investigation and in-depth analysis of this. Another further aim is to study the biological activity, such as *in vitro* antibacterial, antifungal, and anticancer activity of the complexes synthesized.

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Statement of the supervisor

I, Dr. Erika Bálint as supervisor, hereby declare that the thesis written by Ecaterina Revenco (Neptune code: JZ7PCG) titled Investigation of the scale-up synthesis of nonivamide and capsaicinoids application in the complexation of cyclodextrins is her own writing prepared under my supervision. I also declare that the thesis meets the formal and professional requirements of the Budapest University of Technology and Economics and those of the Faculty of Chemical Technology and Biotechnology, thus I support its submission.

14.12.2023, Budapest

Bálint Erika

Dr. Erika Bálint

Statement of the advisor

I, Bettina Rávai as thesis advisor, hereby declare that the thesis written by Ecaterina Revenco (Neptune code: JZ7PCG) titled Investigation of the scale-up synthesis of nonivamide and capsaicinoids application in the complexation of cyclodextrins is the work of the student supported by consultation. Based on its professional merits, I support its submission.

14.12.2023, Budapest

Rávai Bettina

Bettina Rávai

I, Dóra Viktória Ujj as thesis advisor, hereby declare that the thesis written by Ecaterina Revenco (Neptune code: JZ7PCG) titled Investigation of the scale-up synthesis of nonivamide and capsaicinoids application in the complexation of cyclodextrins is the work of the student supported by consultation. Based on its professional merits, I support its submission.

14.12.2023, Budapest

Ujj Dóra Viktória

Dóra Viktória Ujj

Statement of the student

I, Ecaterina Revenco, (Neptun code: JZ7PCG) as author of the thesis hereby declare that my thesis titled Investigation of the scale-up synthesis of nonivamide and capsaicinoids application in the complexation of cyclodextrins is my original writing and I have not plagiarised any other work. All third-party materials including published and unpublished sources were referenced.

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Ecaterina Revenco