BABEŞ-BOLYAI UNIVERSITY CLUJ-NAPOCA
FACULTY OF PHYSICS

STRUCTURAL CHARACTERIZATION OF BIOACTIVE COMPOUNDS BY NMR CRYSTALLOGRAPHY

Ph.D. thesis summary

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Content

INTRODUCTION

Research and development of pharmaceutical products or dietary supplements are complex, costly and long-lasting processes. A pharmaceutical active ingredient (synthesis compound) reaches the market after 10-15 years, while, according to the legal regulations, the process of developing a dietary supplement (natural compound) takes only a few years. Consequently, controlling and understanding the solid state chemistry of a bioactive ingredient, both as pure substance and as formulated compound, is important in the development of a drug or food supplement. The essential properties of a bioactive compound (solubility, stability, bioavailability) may differ from one solid form to another (polymorphs, salts, co-crystals, hydrates/solvates) as a consequence of different packing patterns in the crystalline structure and the type of inter/intra-molecular interactions. For this reason, the screening and structural characterization of active pharmaceutical compounds and dietary supplements is an important process in developing the final product for companies in these fields. In this context, the thesis proposes to approach both the preparation of new solid forms by different crystallization methods and also their structural characterization.

Chapter 1 presents, from a theoretical perspective, key aspects related to the preparation, identification and characterization of bioactive compounds using state-of-the-art crystallization and analytical methods. For a thorough study of a solid bioactive substance, it is important to implement several crystallization methods. After obtaining new solid forms, the next steps are the identification and structural characterization by diffractometric and spectroscopic techniques. For an accurate and realistic structural characterization it is necessary to know the geometric parameters of the crystalline phase, which define the long range ordering, but also the parameters specific to the short range ordering, mainly intermolecular bonds and supramolecular synthons.

Standard methods for crystal structure determination under laboratory conditions are based on X-ray diffraction, namely: single crystal X-ray diffraction (SC) or X-ray powder diffraction (XRPD), depending on the form in which the sample can be obtained.

In recent years, traditional diffraction methods have been commonly used in combination with complementary structural analysis techniques such as solid state NMR and molecular modeling by DFT on crystalline systems, the so called *NMR crystallography*. These modern approaches have a number of advantages, all of which help to increase the accuracy level with which the final structural model is obtained, for example: removing ambiguities when XRPD data analysis leads to multiple structural solutions and obtaining

local structural details related to the positions of hydrogen atoms, usually inaccessible by diffraction methods: it often happends on single crystals, but this is especially the case of polycrystalline powders, because the number of diffraction intensities that can be collected on powders is much smaller compared to the number of intensities collected from single crystals.

Chapter 2 contains a detailed description of the implemented Research Methodology and employed infrastructure, whereas the last three chapters contain detailed description of the original results obtained in the study of the considered bioactive compounds, from the point of view of their theoretical, practical and methodological significance.

Chapters 3, 4 and 5 show the original results obtained during the PhD program. The main objective of the studies was the determination of the crystalline structure for a series of bioactive compounds. From this perspective, the focus was on selecting the most appropriate structural characterization techniques, with a special emphasis on the wide variety of situations that may arise in practice. The concrete situations treated in the thesis refer to compounds that can be obtained either as single crystals or as polycrystalline powders only. For polycrystalline compounds, the different approaches which have emerged in the process of determining the crystalline structure are due to the number of degrees of freedom of the molecule, the number of different molecules in the unit cell and the degree of conformational/dynamic disorder in the lattice.

1. SOLID FORMS OF BIOACTIVE INGREDIENTS. PREPARATION AND STRUCTURAL CHARACTERIZATION

Bioactive compounds are formed from molecules with therapeutic activity that act to treat or ameliorate a medical condition. They are divided in two categories: synthetic compounds (pharmaceutical compounds) and natural compounds (generally derived from plants). The discovery and characterization of solid forms of bioactive ingredients provides us with options in selecting the optimal solid form. By definition, the solid forms of a compound represent the totality of different structural packing modes of the same basic molecule. They can be classified as: polymorphs, salts, co-crystals, hydrates/solvates (Figure 1.1).

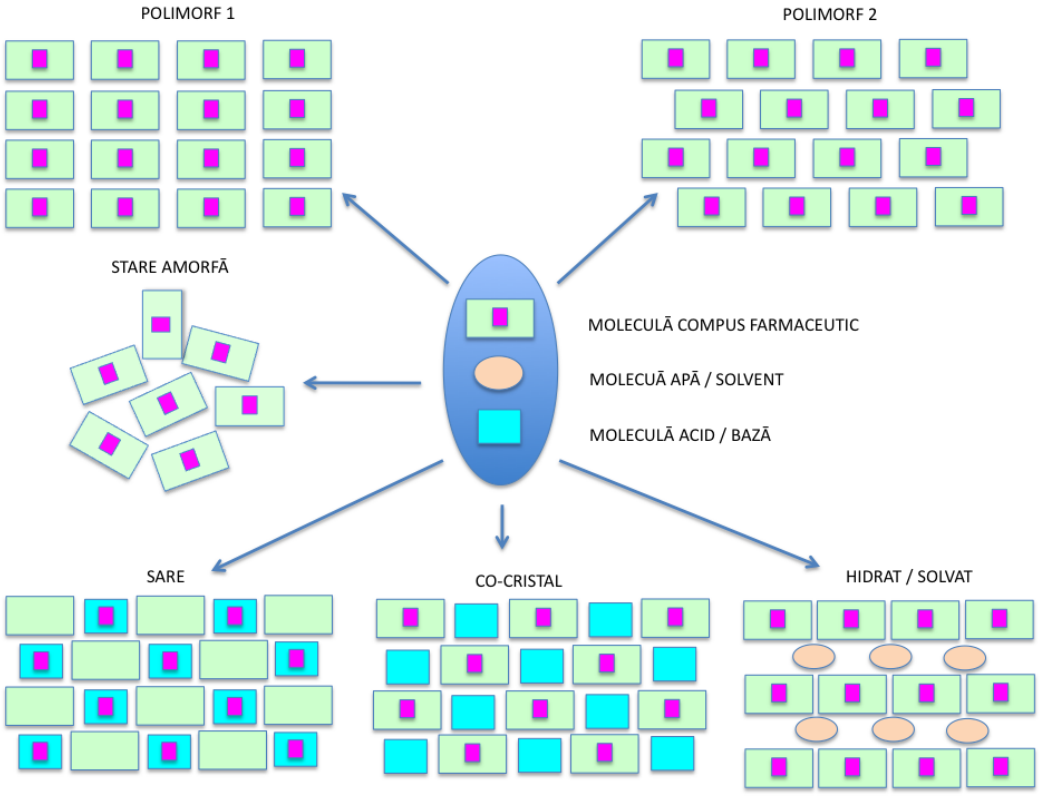

Fig. 1.1 *Classification of bioactive substances from the point of view of the molecular structure.*

The study of solid forms of bioactive compounds is extremely important because various solid forms of a compound tend to have different essential properties such as physico-chemical properties, solubility and dissolution rate [1]. For these reasons, different crystallization methods have been developed with which the exploration of solid forms is made faster [2,3]. In this thesis I mainly employed *high-throughput* crystallisation, where where parameters such as crystallization method, solvent or solvent mixture, temperature, heating/cooling rate, concentration, and molar ratio can be varied in a single experiment. The standard parallel crystallization flow contains the following crystallisation methods:

Crystallization by cooling, Slurry, Liquid Vapor Diffusion Crystallization (VDL), Solid Vapor Diffusion Crystallization (VDS), Crystallization by mechanical mixing with Solvent (solvent-drop grinding).

After a solid forms screening experiment, the next steps are the structural identification and characterization by diffractometric and spectroscopic techniques.

Solid forms identification by X-ray diffraction is a fast, non-destructive method and uses a small amount of sample [4]. Essentially, it is a comparative method. After identification by one of two X-ray diffraction methods (on single crystal or powder), we can only say if a new solid form is obtained. More complex information, for example the nature of the solid form and its crystal structure, is obtained only in the structural characterization step. In order to obtain a complete structural characterization, besides X-ray diffraction, I will also use complementary techniques in this thesis, specifically, solid state Nuclear Magnetic Resonance (ss-NMR) and molecular modeling.

Traditionally, the structural characterization of crystalline compounds is based on X-ray diffraction. Whether it is single crystal or microcrystalline powder, the analysis of diffraction data actually provides information on the electronic charge distribution and not directly the positions of the atoms in the lattice [5]. So, in the following sections of Chapter 1, the general principles of X-ray diffraction are described first: methods to produce X-rays, Bragg's Law, definition the reciprocal space, the relation between the structural factor and the electronic density. Further on in this section are presented in detail the methodologies for obtaining crystal structure by X-ray diffraction on single crystals and powders. Also, the most important limitations of the X-ray techniques, which motivate the use of complementary analytical methods, are presente [6]: (i) the crystal structure obtained from single crystal has the highest degree of precision, but often organic systems do not provide single crystals of sufficient size and quality; (ii) the use of polycrystalline samples in these cases leads to a significant reduction of the confidence level and accuracy degree with which the structural solution is obtained; (iii) the presence of lattice defects, structural or dynamical disorder diminishes the quality of the crystalline structure determined from single crystals or powders; (iv) X-ray patterns are dominated by the effect of heavy atoms, making it very difficult to locate hydrogen atoms, which on the other hand are very important in defining the crystalline packaging patterns of molecules in organic compounds. Consequently, it is necessary to develop new approaches combining the information obtained from the analysis of X-ray data with other structural details, preferably of a local character, extracted by using complementary techniques and capable of removing or diminishing as many of those limitations.

One of the alternative approaches developed over the last two decades is called *NMR crystallography* [7]. Despite of what its name suggests, *NMR crystallography* does not assume crystal structure determination of a compound exclusively by specific NMR spectroscopy methods. Although the NMR crystallography approaches reported so far on organic compounds are quite divers, we can still envisage a general implementation scheme (Figure 1.2), regardless the characteristics of the investigated compound.

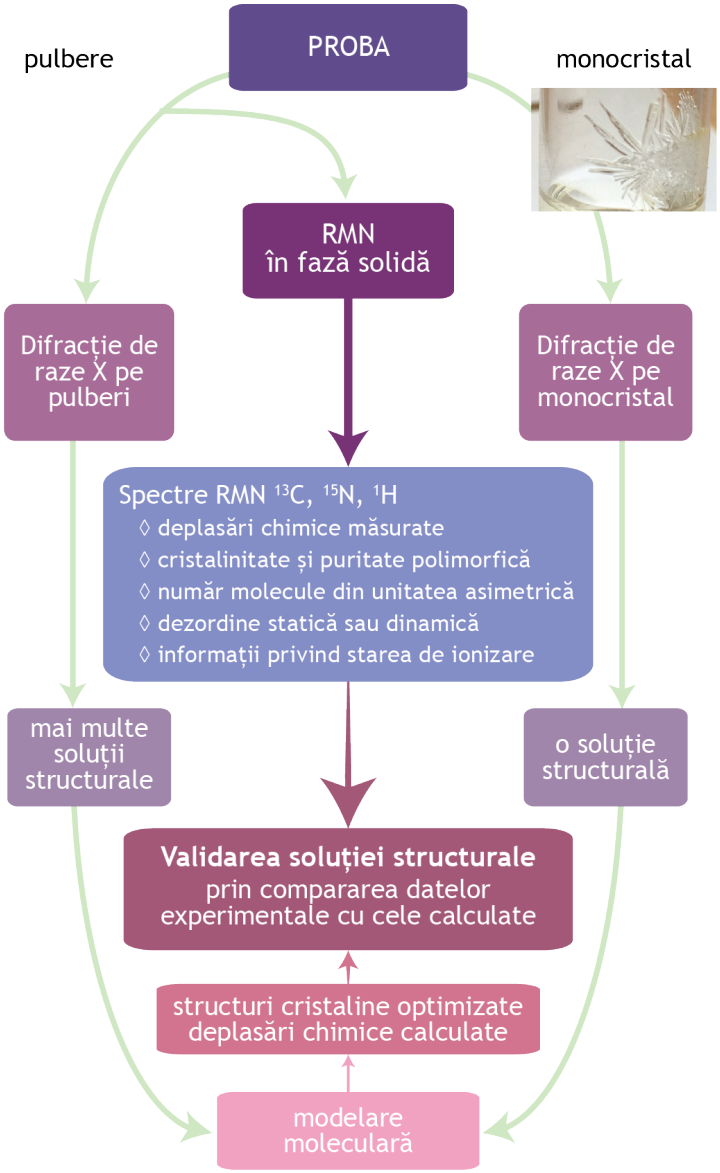

Fig. 1.2. *Principle scheme of MNR crystallograph*

According to this scheme, the XR structural solutions are first subjected to a DFT optimization of the hydrogen atom positions. In organic solids it is important to know these positions [8], especially in cases where strong hydrogen bonding networks, which provide crystal stability, or proton transfer systems are formed. This step is followed by geometry optimization using the same methods, but this time considering the positions of all atoms,

including the heavy atoms. This step is mandatory in the case of structural solutions obtained from powders, but it is also often used for the single crystal structures [9]. The difference between the lattice energy of the non-optimized DFT (or hydrogen-only optimized) model and the fully optimized one provides a first indication of the accuracy degree with which the structural solution could be determined by the analysis of the diffraction data. However, the result is not to be considered absolutely quantitative, especially due to the approximations included in the computational algorithms and also due to the fact that the DFT optimizations are performed at T = 0 K, while the experimental patterns are recorded at higher temperatures. However, a difference in energy values greater than 10-20 kcal/mol is already a clear indication that the structural solution obtained only from the analysis of X-ray data can be further refined [10].

In the final stage, the results of theoretical modeling are subjected to practical validation based on experimental data obtained by NMR methods. The parameters to be analyzed are selected according to their relative relevance to the problem under study and divided into two categories: (i) those which indirectly encode local structural information such as isotropic chemical shifts, quadrupole interaction constants, or J coupling constants and (ii) parameters that directly encode structural information, e.g., internuclear distances or torsion angles.

The most frequently reported are the parameters in the first category, especially the isotropic chemical shifts of nuclei such as ^{13}C, ^{15}N and ^{1}H, because they are easy to obtain experimentally from simple one-dimensional (1D) spectra on natural abundance samples. A more particular situation is that of the ^{1}H nuclei (protons), because of the limited spectral resolution. In this case, however, proton chemical shifts can be obtained entirely by means of extra information extracted from two-dimensional (2D) ^{13}C-^{1}H HETCOR (Heteronuclear Correlation) spectra. Thus, information on the most abundant chemical elements in organic crystalline systems (hydrogen, carbon, nitrogen) is accessible through fast and inexpensive NMR methods, which is consistent with the practical requirements of applications in the pharmaceutical industry. There is also some disadvantages of this approach: the measured values of chemical shifts (as well as the quadrupolar interaction and J coupling constants) can not be directly transformed into spatial details. They can only be taken as control parameters in the validation of crystal structure solutions based on a comparative analysis between the experimentally measured and the theoretically simulated values on the proposed structural model. For example, in the case of isotropic chemical displacements, the root mean square deviation (RMSD) between the measured and calculated values for all distinct chemical

positions in the molecule is a global indicator of the extent to which the tested structural solution approaches the actual structure of the crystal: the lower the RMSD values, the better the accuracy with which was obtained that particular solution. If we further analyze the differences between the experimental and the calculated values for each individual nucleus, we can also obtain local information around that particular chemical position, for example one can identify those regions of the molecule that deviate more or less from the real crystal structure.

The majority of NMR crystallography studies reported so far in the literature are based on the experimental determination of isotropic chemical shifts for the nuclei of interest, which are then used to validate and further refine the structural models resulting from the analysis of X-ray data and from DFT optimization [11,12]. This approach is already well established in the common practice, particularly in pharmaceutical applications, due to the continuous improvement of DFT computational methods, extended to the entire crystalline lattice. They allow now the analysis of systems with a complexity level and at an accuracy degree inaccessible 10-20 years ago. Basically, there is a major progress in the sensitivity with which the effects produced on the NMR parameters can be traced back by minor variations in the crystal structure parameters.

2. RESEARCH METHODOLOGY

The major objective of the studies undertaken in the thesis was to determine the crystal structure for a series of bioactive compounds. From this perspective, the focus was on selecting the most appropriate structural characterization techniques that take into account the wide variety of situations that may arise in practice.

The structural characterization of all the analyzed compounds was done by employing the most appropriate techniques, many of them based on advanced *NMR crystallography* approaches. Apart from the determination of actual crystal structures, the thesis also contains elements of methodological development, since each compound required a customized approach, depending on the structural parameters that had to be additionaly refined relative to the X-ray diffraction model.

In order to obtain new solid forms of the active ingredients with improved solubility, the investigated compounds were subjected to polymorphism and co-crystallization experiments. The procedure we followed consists of:

(i) estimation of solubility

(ii) selection of crystallization solvents

(iii) screening of polymorphs and

(iv) crystallisation experiments.

The resulting solids (single crystals, polycrystalline or amorphous powders) were then subjected to routine analysis by single crystal X-ray diffraction (SC) or powder X-ray diffraction (XRPD) to verify if new solid forms were obtained. Routine analysis for SC consists in choosing a single crystal, mount it on a goniometer, and perform a pre-experiment – this gives us information about the unit cell parameters (a, b, c, α, β, γ), which were then compared with the parameters reported in the literature. For identifying new solid forms through XRPD, a short measurement is performed (range 2θ: 3-30^o, step of 0.02^o and time per step of 0.4 s/step) and the experimental pattern is compared with the simulated or experimental pattern of the solid forms found in literature.

The complete structural analysis on single crystals consists of: choosing the single crystal, mounting it and conducting the pre-experiment, then record complete diffraction data, whereas in the last step data processing is performed with the purpose of obtaining the crystal structure of the investigated compound.

In the case of XRPD, measurements were performed in the 2θ range of 3-40^o, with a 0.01^o step at 3 s/step. This is next analyzed in order to determine the crystal structure. The

main steps in crystal structure determination by PXRD are: (i) recording the pattern; (ii) indexing the pattern, which provides the approximate values of the unit cell parameters (a, b, c, α, β, γ); (iii) searching for a structural model; (iv) Rietveld refinement and derive the final structural solution.

Finally, the newly identified solid forms have been structurally analyzed by NMR spectroscopy on solids and molecular modeling, in the framework of *NMR crystallography* approaches.

For structural analysis by NMR, the following experimental techniques were used:

i. $^{13}C(^{15}N)$ CP-MAS *(Cross-Polarization under Magic Angle Spinning)*: generează spectre ^{13}C RMN-s 1D de înaltă rezoluţie, care permit determinarea experimentală a deplasărilor chimice $^{13}C(^{15}N)$ pentru toate poziţiile distincte de carbon, respectiv azot, din moleculele aflate în reţea.

ii. ^{13}C CPPI (Cross-Polarization Polarization-Inversion): also generates 1D ^{13}C NMR spectra, but includes a polarization inversion period that allows to differentiate various chemical groups according to the corresponding proton multiplicity.

iii. One-pulse ^{13}C experiments: provides ^{13}C NMR spectra for all the carbon atoms in the system, not only for those who may be involved in the polarization transfer process (i.e., those belonging to the "rigid" molecules in the crystal lattice).

iv. One-pulse ^{1}H experiments: provides 1D ^{1}H NMR spectra under ultra-fast MAS conditions (sample rotation frequencies up to 65 kHz).

v. Double-Quantum under Magic Angle Spinning 2D experiments: during the mixing period, correlations are established between nuclei which are closely spaced (up to ~ 5-6 Å).

In the case of molecular modeling, the steps of a regular chemical shielding parametres calculation using the GIPAW method are:

- for an initial crystalline structure obtained by X-ray diffraction, the energy of the fundamental state and the forces acting on the atoms are calculated.

- if the forces in the system are too higher, a geometry optimization is performed until these forces are minimized.

- for the obtained optimal geometry, the GIPAW calculation will give the NMR chemical shielding tensor.

3. STRUCTURAL CHARACTERIZATION OF TADALAFIL MONOSOLVATES

The first biologically active compound studied in this PhD thesis is called Tadalafil. The main reasons for choosing this compound are: (i) TDF was introduced on the market quite recently, used as an active substance in Cialis® 2003 (treating erectile disfunction), in 2011 for benign hyperplasia prostate and from 2009 for the treatment of pulmonary arterial hypertension; (ii) TDF has very low solubility, with negtive influences upon its bioavailability - data reported in the literature indicating that TDF is almost insoluble in water [13].

Only some of the solid forms identified in the literature, ie anhydrous TDF (CSD refcode: IQUMAI) [14], identified as form I [15] and three co-crystals with methylparaben, propylparaben and hydrocinnamic acid were reported in the Cambridge Structural Database (CSD) [16]. In this context, using the screening methodology for this compound we conducted a polymorphism and co-crystallization study with the purpose to obtain new TDF solid forms with improved solubility. Following these experiments, two new solvate forms (TDF-ACE and TDF-MEK) were obtained as single crystals, which were then structurally characterized by an *NMR crystallography* approach [17].

Tabelul 3.1. *Crystallographic data and details of the crystal structures determined.*

	Compus		
	TDF-ACE	**TDF-MEK**	**TDF[a]**
Formula	$C_{25}H_{25}N_3O_5$	$C_{26}H_{27}N_3O_5$	$C_{22}H_{19}N_3O_4$
Molecular mass (g/mol)	447.49	461.52	389.40
Temperature (K)	293	293	293
Crystallographic system	Orthorhombic	Orthorhombic	Monoclinic
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_1$
a (Å)	7.7408(3)	7.7842(4)	9.894(2)
b (Å)	13.7341(5)	13.8562(9)	7.782(1)
c (Å)	21.1301(9)	21.4012(12)	12.395(2)
α (°)	90	90	90
β (°)	90	90	99.75(2)
γ (°)	90	90	90
V (Å^3)	2246.38(15)	2308.3(2)	940.570
Z	4	4	2
F(000)	947	979	408
Calculated density g/cm^3	1.3231	1.3279	1.375
θ-range (°)	3.84-70.84	3.80-70.63	4.53-57.26

	Compus		
	TDF-ACE	TDF-MEK	TDF[a]
Number of collected reflections	4888	5196	1463
Refined parameters	300	309	320
GOF on F^2	1.0316	0.9980	1.019
R1 (F, I>2Σ(I))	0.0617	0.0564	0,0259
wR_2 (F^2, all reflecions)	0.1698	0.1563	0.0712

[a]CSD code: IQUMAI [14]

Following their structural characterization, it was found that the two solvates exhibit a higher symmetry, i.e. orthorhombic, compared to the structures reported in the literature, which are monoclinic (Table 3.1). Although the same TDF molecule chains are observed in all crystalline structures, the inter-molecular interactions between TDF and solvent molecules in the solvates forms induce small conformational modifications of TDF molecules (Figure 3.1).

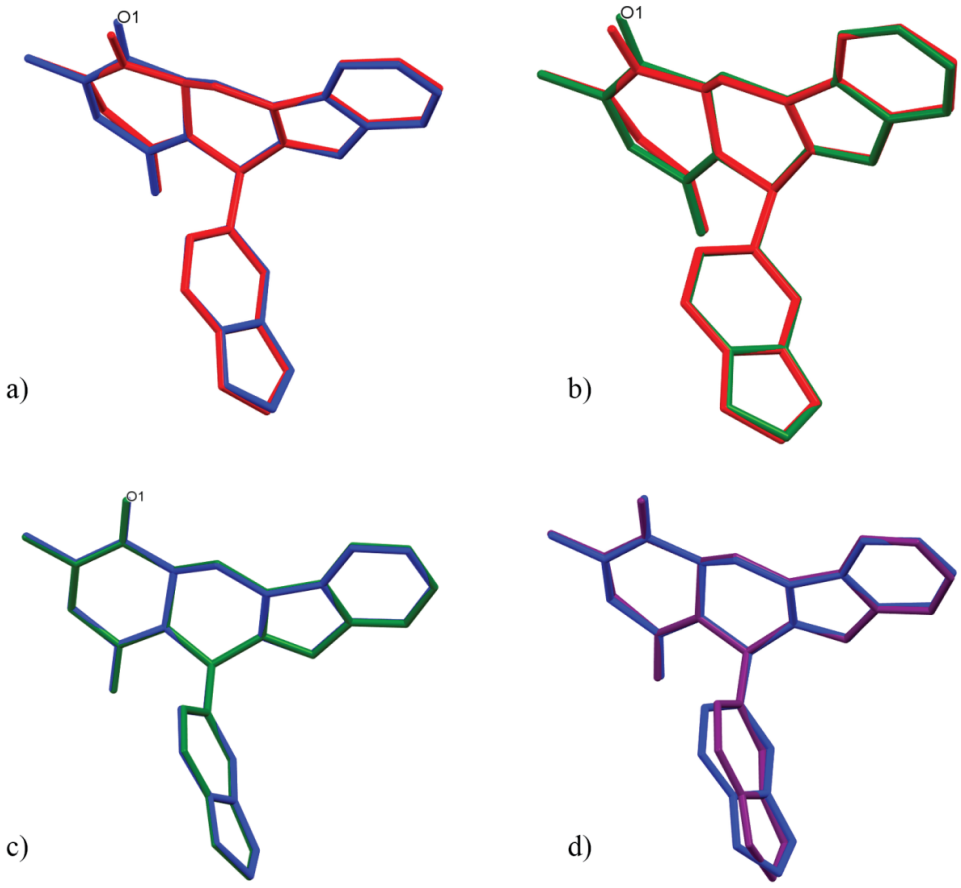

Fig. 3.1. *Overlay of TDF molecules: a) TDF-anhydrous (red) versus TDF-(ACE) (blue), b) TDF-anhydrous (red) versus TDF-(MEK) (green), c) TDF-(ACE) (blue) versus TDF-(MEK) (green), d) TDF-(ACE) (blue) versus TDF propylparaben co-crystal.*

The MEK solvate exhibits higher stability than the solvate with acetone (Figure 3.2), although they have similar molecular conformations and packaging in the unit cell. Considering the low solubility of TDF in a large number of organic solvents, the use of

acetone and methyl ethyl ketone as crystallization solvents may provide the advantage of obtaining higher concentrations in the TDF crystallization process.

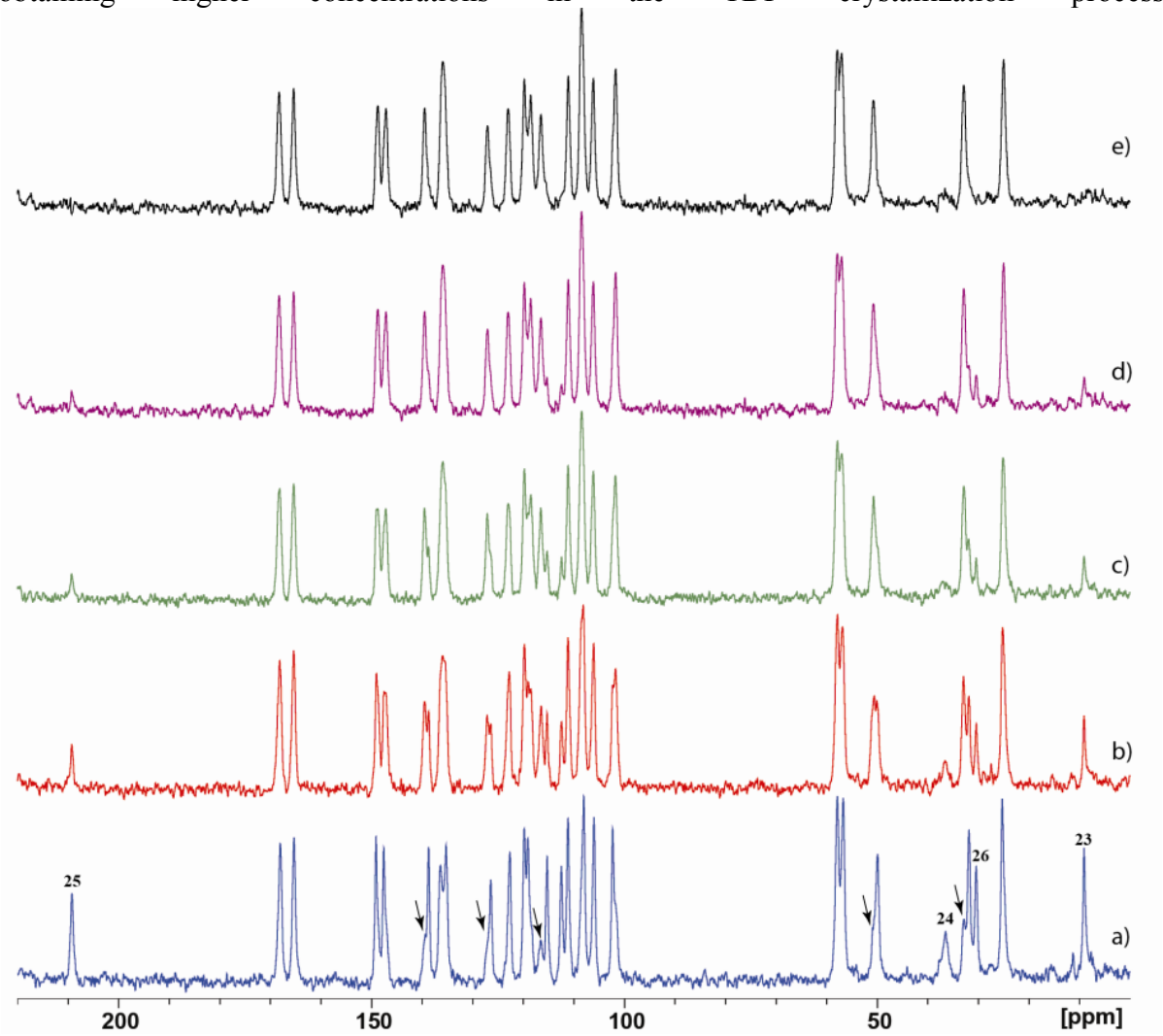

Fig. 3.2. *3C CP-MAS spectra of TDF-(MEK) recorded in the: a) 1st hour; b) 7th hour; c) 14th 49 hour; d) 20th hour, e) 30th hour.*

In the pharmaceutical industry, solvates are commonly used in the purification process [18], as precursors in the production of polymorphs [19], or to control the particle size. Particle size and morphology can be controlled by the desolvation process while at the same time obtaining the desired anhydrous polymorph [20]. For this reason, desolvation of the solvates obtained in our study motivates their use in order to obtain the anhydrous form. This study emphasizes the importance of crystal structure knowledge in understanding the stability of solvates and evaluating the development risks of their use as precursors for obtaining anhydrous forms.

4. ANHYDROUS QUERCETIN SOLVATE FORMS STRUCTURAL CHARACTERIZATION

As the food supplements market is constantly growing all over the world, the second compound studied was Quercetin. For this reason in this thesis I included a systematic study on one of the most widely used natural bioactive substances in the composition of food supplements, Quercetin.

Pharmacokinetic studies show that Quercetin exhibits low bioavailability, with only 20-30% of an oral dose being absorbed [21]. Because of its low bioavailability, this flavonoid has been investigated in recent years from structural point of view, and also new forms with improved bioavailability have been attempted. Although the crystal structures for quercetin monohydrate [22] and dihydrate [23], and also several co-crystals and two metal complexes have been reported at the time of this study, the crystal structure of anhydrous quercetin was not reported in the literature because it could not be obtained in the form of single crystal, and the structure from powders was not obtained most likely due to the ambiguities that appear in the structural patterns. In this context, I proposed as a theoretical objective the determination of the crystal structure, both for anhydrous quercetin and for the obtained new solid forms.

Following the screening experiments, new solid forms with ethanol and methanol were obtained, both showing a reduced crystallinity. By performing the solvent-drop grinding and cooling crystallization experiments the 1,4-dioxane (Quer_diox) solvate form, ethanol (Quer_EtOH) and, respectively, methanol (Quer_MeOH) forms were obtained, with the sufficienly good crystallinity to be structurally characterized.

ANHYDROUS QUERCETIN

In case of the anhydrous form of Quercetin, three distinct structural solutions were investigated. The initial molecular structure used in the first model, *Quer_1*, was sketched, and then its geometry was optimized by classical molecular mechanics methods (MM). The second model considered in the study, *Quer_2*, was obtained from the systematic search over the torsion angles θ_1-θ_5 associated with the hydroxyl groups in the molecular structure considered in *Quer_1* model. The third molecular model, *Quer_3*, was obtained using an isolated molecule extracted from the crystal structure of Quercetin dihydrate [23] determined from single crystal. This model can be used to assess the reliability of the other two models described above.

For the *Quer_1* model an unsatisfactory R_{wp} fitting factor was obtained due to errors induced by the approximation of intra-molecular parameters constrained during XRPD

refining (Table 4.1). The geometric optimization applied across the crystal has corrected most of these errors, except for the conformation of one hydroxyl group. Due to this parameter, a larger lattice energy is obtained for the *Quer_1* model compared to the energy calculated for the *Quer_2* model. This was also confirmed by the comparison of $^{13}C/^{1}H$ experimental chemical shifts with those calculated on the DFT optimized models of *Quer_1/2*.

Tabelul 4.1. *Crystallographic data for anhydrous quercetin and information obtained after refining for the three models*

Chemical formula		$C_{15}H_{10}O_7$		
Crystallographic system		Monoclinic		
Spatial group		$P2_1/a$		
Z		4		
Radiation type		Cu $K\alpha_1$, $\lambda = 1.54056$ Å		
Data collection mode		Reflection		
Scan mode		Continuous		
2θ values (°)		$2\theta_{min}= 3$; $2\theta_{max}= 40$; $2\theta_{step}=0.001$		
Models		*Quer_1*	*Quer_2*	*Quer_3*
Volume (Å^3)		1202.0	1201.3	1198.8
R factors (%)	R_{wp}	10.5	9.1	8.9
	R_p	7.7	6.8	6.6
Lattice parameters	*a* (Å)	19.98(8)	19.99(1)	19.97(1)
	b (Å)	3.71(1)	3.71(1)	3.70(9)
	c (Å)	16.26(2)	16.25(0)	16.24(1)
	β (°)	85.15(5)	85.13(6)	85.15(9)
Density ρ (g/cm^3)		1.670	1.671	1.675

Finally, the formed hydrogen bonding network as well as the 2D ^{1}H DQ spectrum analysis suggest that *Quer_2* model is the most realistic in terms of supra-molecular architecture. The formation of the two distinct molecular superstructures, *Quer_2/Quer_3,* depends on the intermolecular hydrogen bonds which establish between neighbouring cathecol rings, as emphasized by dashed lines in Figure 4.1.

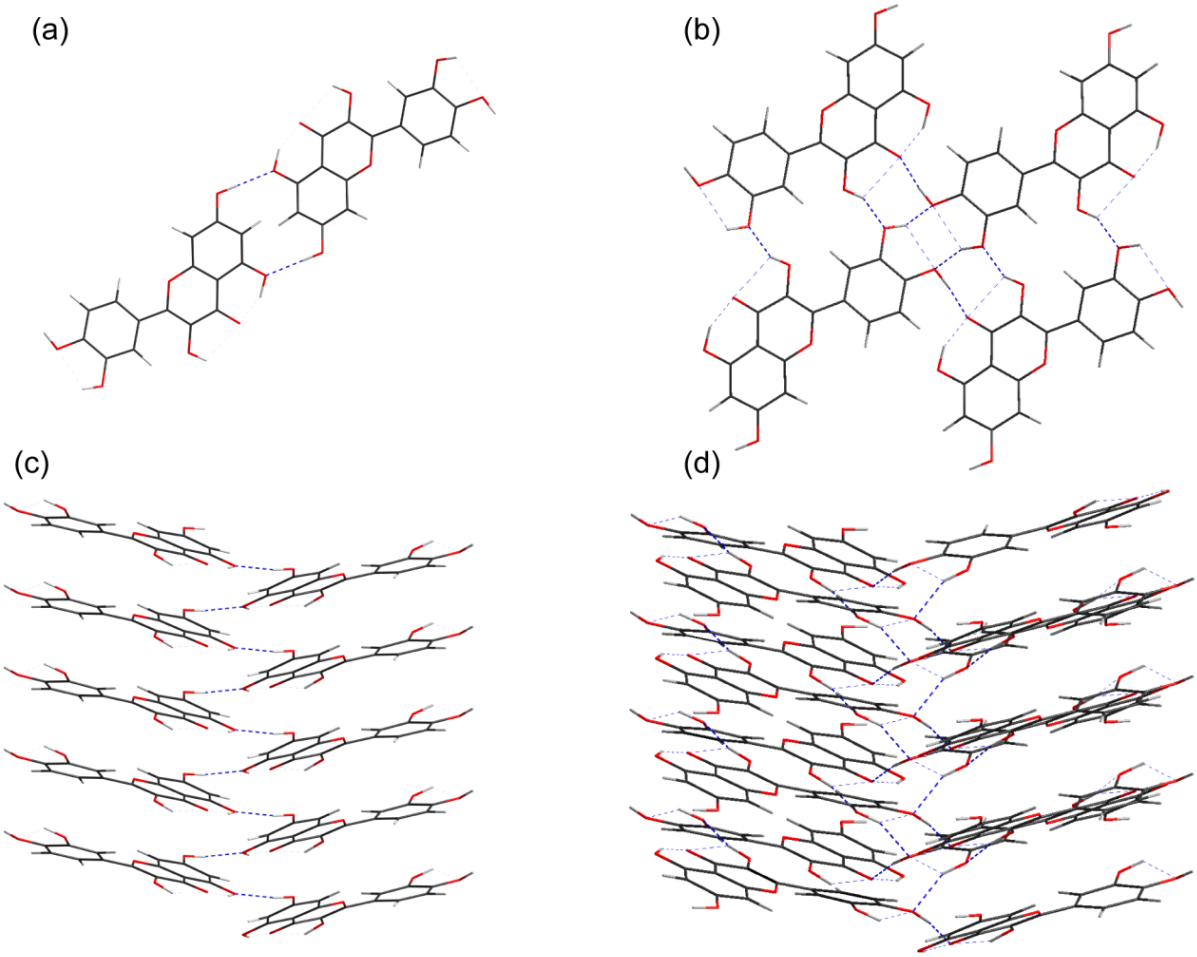

Fig. 4.1. *The identified supra-molecular arrangements in anhydrous quercetin illustrated on the Quer_2 geometrically optimized crystal structure model (similar arrangements are found also in Quer_3). The proposed hydrogen bonding patterns (dotted lines) projected within the (ac) plane are shown in (a) and (b), whereas the corresponding views along the b crystallographic axis are given in (c) and (d).*

For validation, a similar analysis was done in parallel for *Quer_3*. DFT optimized *Quer_2/Quer_3* structural solutions are almost identical, since *Quer_3* starts from a molecular structure determined by X-ray diffraction on the single crystal, and finally confirms the high level of accuracy obtained by using an *NMR crystallography* approach[24].

ETHANOL QUERCETIN AND METHANOL QUERCETIN

We proceeded to determine the crystal structure for the two solvates with alcohols (Quercetin ethanol and Quercetin methanol) obtained from by cooling crystallization experiments. Only for Quercetin methanol solvate (*Quer_MeOH*) the indexing process was successful, while for *Quer_EtOH* we did not obtain satisfactory lattice parameters. This is most likely due to the presence of small diffraction intensities belonging to anhydrous quercetin. Therefore, only the *Quer_MeOH* structure was determined (Table 4.2).

Tabelul 4.2. *Criystallographic data for Quer_MeOH.*

Chemical formula	$C_{15}H_{10}O_7 \cdot CH_3OH$
Crystal system	Triclinic
Space group	P-1
Z	4
Radiation type	Cu $K\alpha_1$, $\lambda = 1.54056$ Å
Data collection mode	Reflection
2θ values (°)	$2\theta_{min} = 3$; $2\theta_{max} = 40$; $2\theta_{step} = 0.001$
Volume (Å^3)	681.614
R_{wp}	8.74%
R_p	5.96%
a (Å)	19.414(2)
b (Å)	9.981(3)
c (Å)	3.552(3)
α	97.445(8)
β	90.806(6)
γ	92.786(9)
Dendity ρ (g/cm^3)	1.62

However, due to the striking resemblance between the XRPD patterns (Figure 4.2) and the ^{13}C NMR spectra of the two solvates (suggesting that they are almost iso-structural), we can consider the crystal structure determined for *Quer_MeOH* as a structurally acceptable model also for the solvate with ethanol.

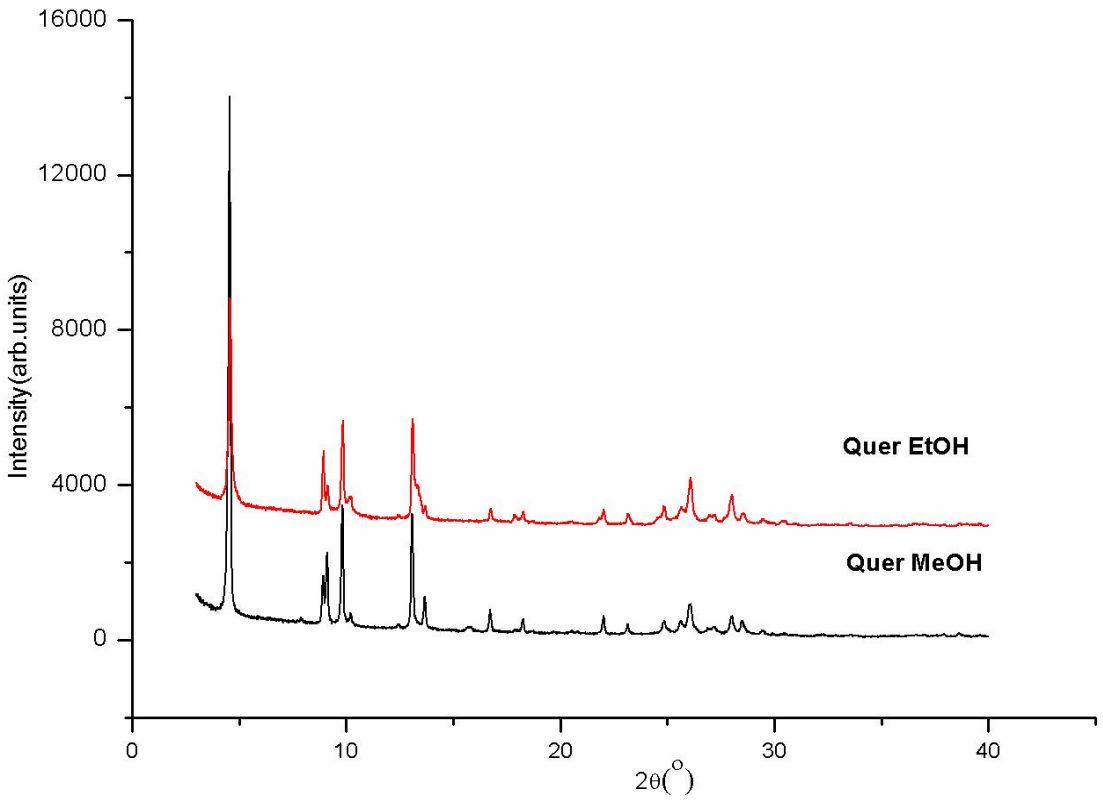

Fig. 4.2. *Comparison of XRPD patterns of Quer_MeOH and Quer_EtOH*

To confirm the XRPD structural solution of *Quer_MeOH*, we compared the experimental chemical shifts ^{13}C NMR, δ^{ex} (^{13}C), with the calculated ones, δ^{calc} (^{13}C). For this, the crystal structure obtained by XRPD has been first geometrically optimized (with the unit cell parameters fixed), and then the chemical shifts parameters were calculated.

In addition to the difficulty of obtaining the two solvates (Quer_MeOH / EtOH) with a high degree of crystallinity and purity, necessary to determine their crystal structure, they also showed a low stability. In order to determine their behavior in time, we have conducted stability studies by solid state NMR spectroscopy over a long period of time, for the methanol solvate almost one year. The results of the study are illustrated in Figure 4.3, considering the solvate with ethanol, because desolvation is much faster than that observed in the solvate with methanol.

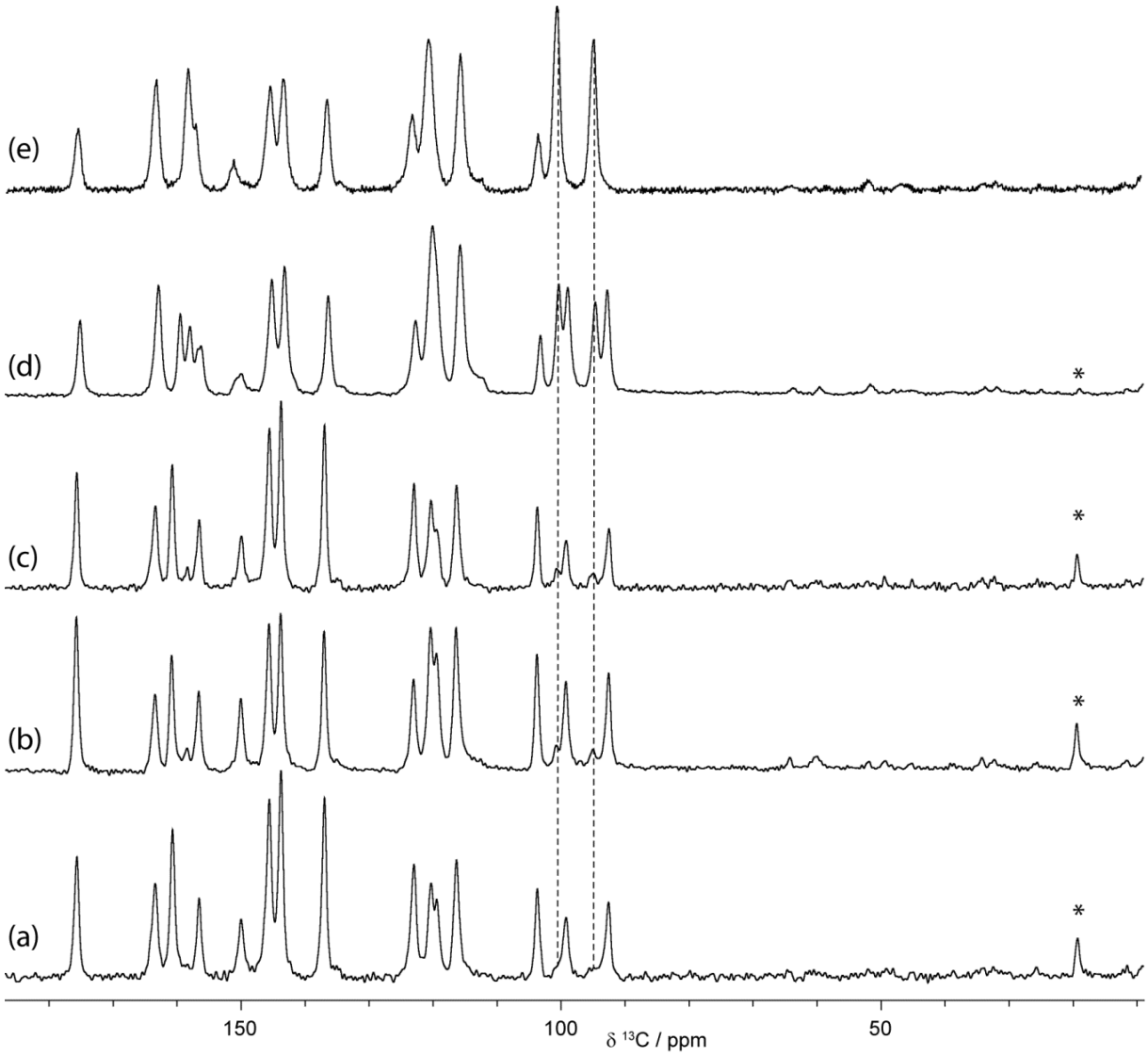

Fig. 4.3. *Comparison of ^{13}C CP-MAS spectra of Quer_EtOH recorded immediately after preparation (a), after one day (b), three days (c), two months (d), anhydrous Quercetin (e).*

Specifically, we monitored the evolution over time of the ^{13}C CP-MAS spectra for *Quer_EtOH* over two months (Figure 4.3). The spectra recorded immediately after preparation shows that the samples contained a small amount of anhydrous Quercetin, this form was identified by the presence of characteristic ^{13}C NMR peaks of anhydrous Quercetin (marked by dotted lines in Figure 4.3).

In time, these peaks become more and more defined, indicating a continuous transformation from the solvate to anhydrous form. This is most likely due to the fact that each ethanol molecule has weak hydrogen bonds in the lattice and only binds to one quercetin neighbor molecule, in other words, the solvent molecules are not involved in an extended network of hydrogen bonds, they can easily "escape" from the crystalline lattice, finaly leading to the formation of anhydrous Quercetin.

DIOXANE QUERCETIN

A new solvate of quercetin, 1,4-dioxane-quercetin, was obtained and its crystal structure was determined by *NMR crystallography* protocol adapted to systems with multiple hydroxyl groups [25]. For this purpose, a multi-step approach was considered, where finer and finer structural parameters could be constrained by adding at each stage. Following these steps, we came to the conclusion that the structural model *Quer_D_DFT* is the closest to the actual crystal structure of the solvate.

In this case, the structure appears to be stabilized by the hydrogen bonding network: a Quercetin molecule binds with two molecules of dioxane through O-H catechol and benzopyran groups and is also involved in an inter-molecular hydrogen bond with a neighboring quercetin molecule (O-H catechol and O with ketone benzopyran OH). Conversely, a molecule of 1,4 dioxane forms hydrogen bonds with two quercetin molecules with each O cyclic atom which interacts with the O-H groups belonging to the catechol ring, and, respectively, benzopyran. After a closer inspection of the crystal structure of Quer-dioxane, formation of structural units hierarchically interconnected by a high level of complexity is observed (Figure 4.4).

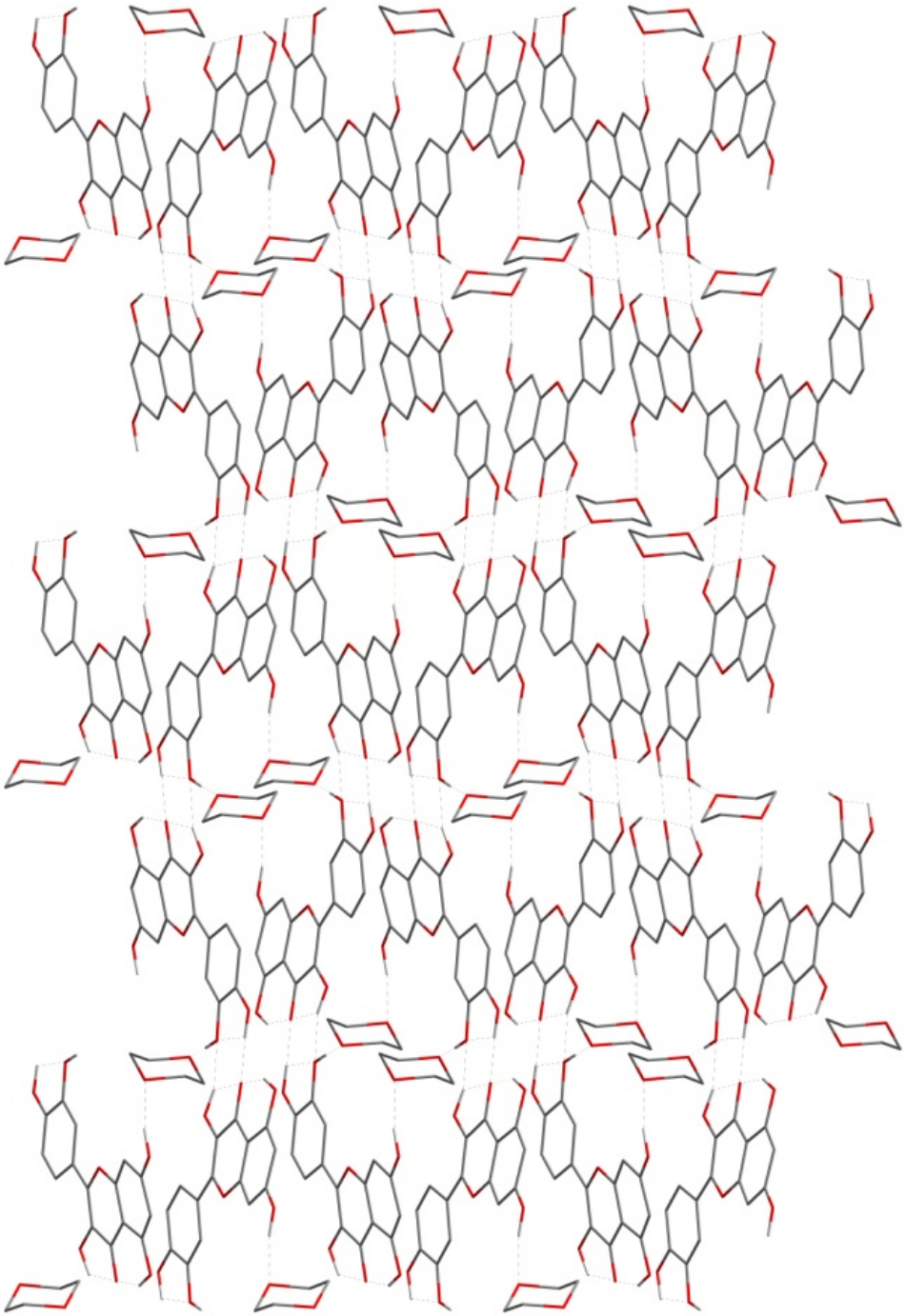

Fig. 4.4. *Extended 2D hydrogen bonding network in the (bc) plane.*

The stability of the compound was investigated by solid state NMR in ambient contitions and compared to the stability of alcohol Quercetin. The 1,4-dioxane solvate proved to be stable for a long period of time due to the way the solvent molecules are involved in the hydrogen bonding.

DISCRIMINATION OF THE COMPONENTS IN QUERCETIN DIATARY SUPPLIMENTS

Despite their documented health benefits, a significant percentage of the population is not consuming sufficient quantities of dietary polyphenols as a result of inadequate fruit and vegetable intakes caused by the modern lifestyle. Consequently, an alternative source of flavonoids that has become extremely popular in the recent past is dietary supplements. The biggest problem with the increase consumption of nutraceuticals is that they are not regulated to the degrees that are medicines. This allows manufacturers to market supplements without fully testing them for efficacy or potential side effects [26], this is the reason why I considered important to conduct this study. The most common solid forms in quercetin-based food supplements are quercetin monohydrate [22] and dihydrate [23], but a recent study [27] shows that the final product also contains a small amount of anhydrous Quercetin, a residue resulting from the manufacturing process involving the extraction of quercetin with solvents from different natural sources. The solvents most commonly used in the extraction process are alcohols (ethanol and methanol), but also acetonitrile and ethyl acetate.

Solid formulations (tablets, capsules) of food supplements give rise to additional concern for consumers. On the one hand, the precise knowledge of the solid form in which the active compound is present is important because it directly influences the dissolution rate and the bioavailability of the final product, analogously to what happens with the synthetic pharmaceutical compounds. On the other hand, such information is very rarely presented by the producers. The present study aims to make an exact contribution in this direction and is a direct practical application for both *NMR crystallography* investigation methods, as well as for the previously discussed results regarding the obtaining and characterization of new solid forms of Quercetin.

Using an approach derived from *NMR crystallography*, we have developed a reliable and rapid procedure for characterizing quercetin-based food supplements in terms of the purity of the solid form of the quercetin extract incorporated into the tablets/capsules. The food supplements analyzed are: Quercetin (Natrol Inc., USA) denoted as *Quer_N* and Quercetin (Organika, Canada) denoted as *Quer_O*. The challenge was to distinguish the solid form present in the tablet/capsule, since the tablet/capsule is a complex mixture which also contains a number of excipients along with the active ingredient(s).

The results obtained for the investigated quercetin commercial products demonstrate the importance of complementarity between the two techniques (NMR and XRPD) for a complete approach of this problem. In particular, XRPD is very useful for a rapid

identification of the main crystalline components (active substances and excipients) mentioned by the manufacturers in the prospectus of the two products (Figure 4.5). The detection limit was quite small, but this seems to be inevitable when the XRPD technique is applied to complex mixtures of powdered components.

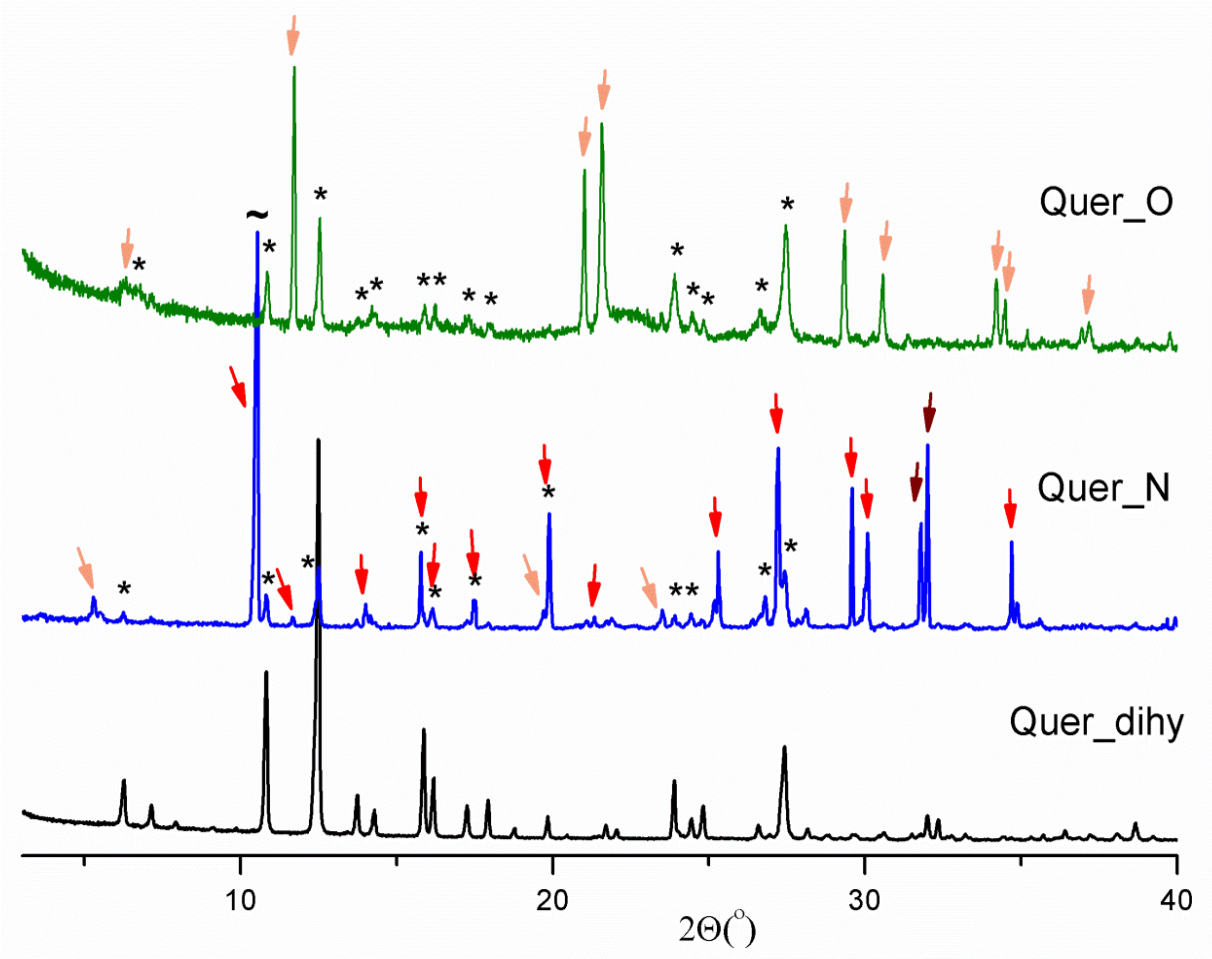

Fig. 4.5. *PXRD patterns of Quer_dihy (*), Quer_N, and Quer_O. The excipients are indicated by arrows: orange - magnesium stearate / stearic acid, red – ascorbic acid, brown – silica.*

By contrast, solid state NMR spectra showed a better sensitivity in distinguishing the solid forms of quercetin present in the commercial formulation even in the presence of other active ingredients and excipients. In addition to the majority of Quercetin dihydrate, small amounts belonging to another solid form were identified in both supplements. Most ^{13}C-NMR lines indicate the presence of anhydrous Quercetin, but small discrepancies observed in the other two signals indicate the presence of a solid form of Quercetin not reported. In the case of CP-MAS spectra (Figure 4.6), most lines inside the gray rectangle, covering the spectral region 90-190 ppm, belong to Quercetin. The ascorbic acid RMN lines (marked with red arrows in Figure 4.6) appear differently from XRPD, the intensities being very small, despite

the fact that vitamin C is the major component of the capsule. This is a consequence of the fact that the CP-MAS spectrum is not quantitative.

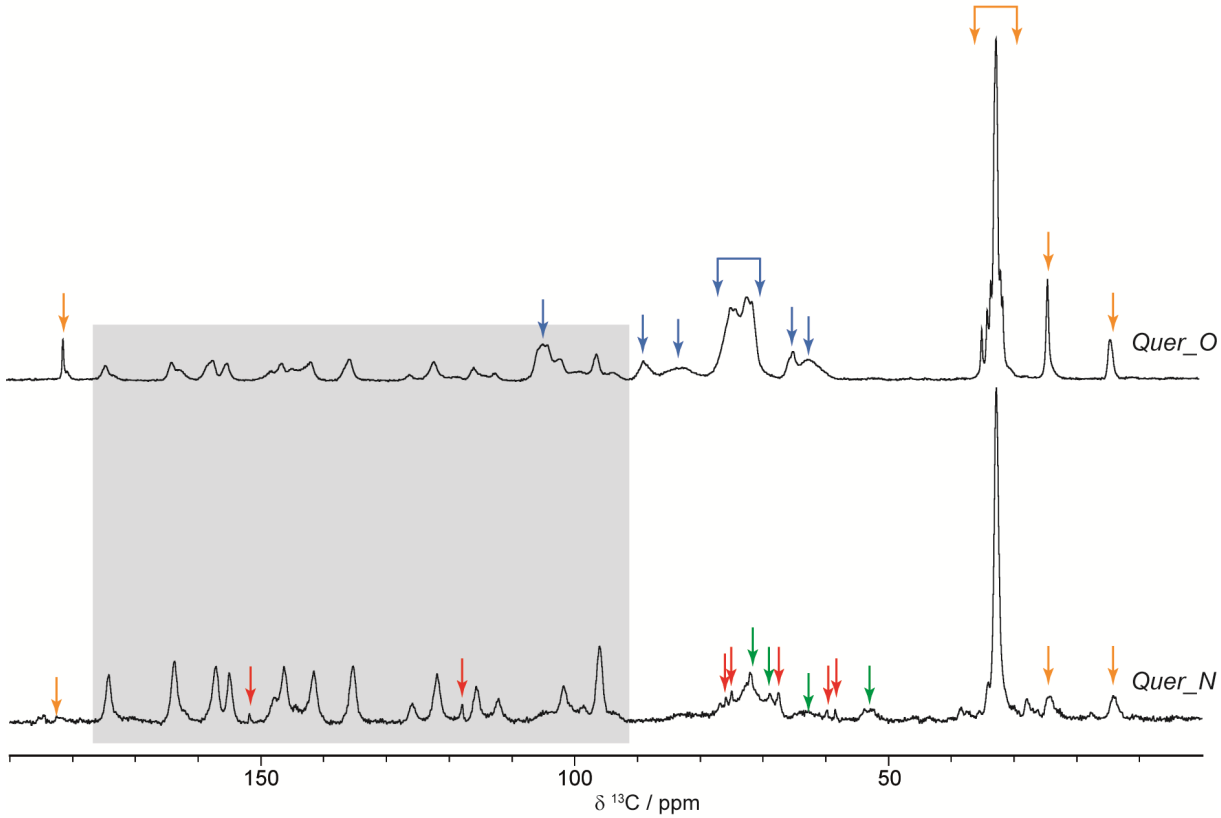

Fig. 4.6. *^{13}C CP-MAS spectra of the two investigated dietary supplements. The spectral window where the quercetin NMR lines are expected is highlighted by the gray rectangle. Characteristic lines of other components in the samples are marked by arrows: orange - magnesium stearate / stearic acid, red – ascorbic acid, green – citrus bioflavonoid complex, blue – cellulose / croscarmellose sodium.*

After extensive studies, this new solid form was identified as solvate with ethanol or methanol (both exhibited XRPD patterns and ^{13}C-NMR spectra, almost identical) of Quercetin. Although this approach has only been tested on two dietary supplements of Quercetin, it can be generalized easily with other commercial products containing Quercetin/flavonoids.

5. LISINOPRIL DIHYDRATE STRUCTURAL CHARACTERIZATION

The last of the bioactive compound studied in this thesis is Lisinopril dihydrate, a cardiovascular drug that acts as an angiotensin converting enzyme (ACE) inhibitor. The single crystal structure was obtained only in the case of Lisinopril - ACE [28] complex, and for the hydrate and the anhydrous forms the structures reported are determined using experimental data collected at synchrotron [29]. In this context, the main objective of the investigation was to determine if the use of a conventional laboratory X-ray source in an *NMR crystallography* approach can lead to a crystal structure of Lisinopril dihydrate with a confidence level and a high degree of precision. Achieving this goal has both a practical and a theoretical reasoning. The practical importance is related to the industry's current trends in using rapid and effective analytical methods to characterize active substances, from the new molecule to the final formulation, and to shorten as much as possible the entire process of developing new drugs. Basically, lisinopril dihydrate is a challenge in the field of structural characterization from powders, primarily because of its hight number of degrees of freedom that need to be explored to achieve a structural solution, but also by the difficulty of characterizing a hydrogen bonding network that we assume to be very complex, given the large number of moieties capable of participating to its formation - two carboxyl groups, two amino groups, a carbonyl group, as well as the water molecules.

To obtain the structural model from the X-ray pattern, two types of searches were performed:

- a complete search, which uses the maximum number of steps/cycle suggested by the fitting program according to the number of the refined degrees of freedom
- a restrictive search through which we explored the possibility of reducing the time required to obtain a structural model, by employing 50 times less steps/cycle.

Following these searches, three structural models denoted with *Lisi_0*, *Lisi_1* and *Lisi_2* were obtained. Figure 5.1 illustrates the long range order and the packaging of the molecules in the crystal lattice. In this figure the following characteristic features (which are valid for all three models) can be identified: the formation of two channels along the crystallographic axis *b* in which the water molecules (A) and (B) are found, and the fact that the bonding between Lisinopril molecules are made along the *a* axis through hydrogen bonds (C).

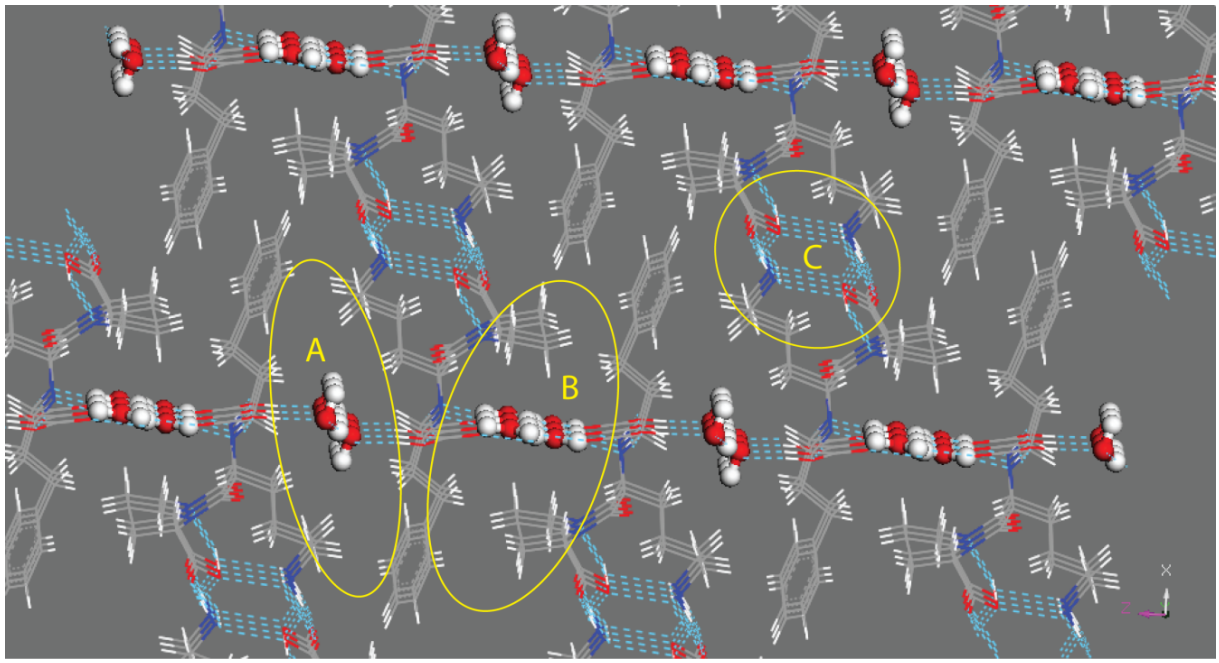

Fig. 5.1 *Long range ordering and the major crystal packing patterns identified in the Lisi_1 crystal structure model. Similar patterns have been found in the Lisi_0 and Lisi_2 models.*

After an analysis of Lisinopril molecules in the three models, we noticed significant differences in conformation of carboxyl groups, as seen in Figure 5.2. These differences also lead to large differences between the associated hydrogen bonding networks and prevent us from defining a unique structural model. In order to remove this ambiguity, we have turned to *NMR crystallography*.

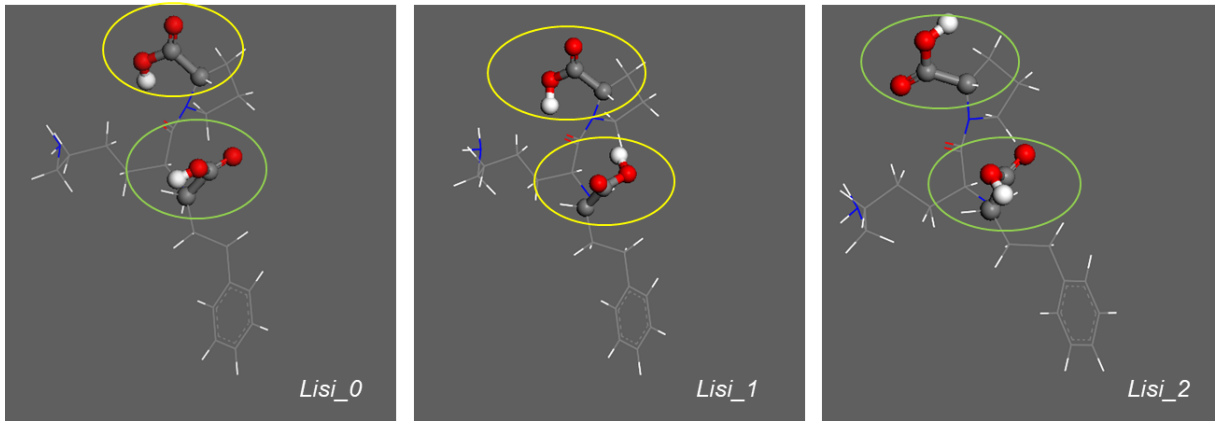

Fig. 5.2. *The conformatin of carboxil grups C10–OOH and C21–OOH in the models selected for the structural analysis for Lisi_0, Lisi_1 and Lisi_2: syn (yellow) și anty (green).*

We conducted a correlated analysis of the information obtained by molecular DFT modeling and NMR spectroscopy in order to finally select the most realistic crystal structure model of the three structural solutions resulting from the XRPD data. The DFT geometry optimization of structural solutions has allowed us not only to order the XRPD structural models, but also the exclusion of the *Lisi_2* model from the analysis: the much smaller lattice

energies and the hydrogen bonds of $N1H_3^+$ with the C21-OO groups from the three neighboring molecules in *Lisi_0*/*Lisi_1*, much more realistic than in *Lisi_2*, shows that the latter model is too far from the actual crystal structure of Lisinopril dihydrate.

Using NMR spectroscopy on solids, ^{15}N, ^{1}H and ^{13}C spectra were recorded (Figure 5.3).

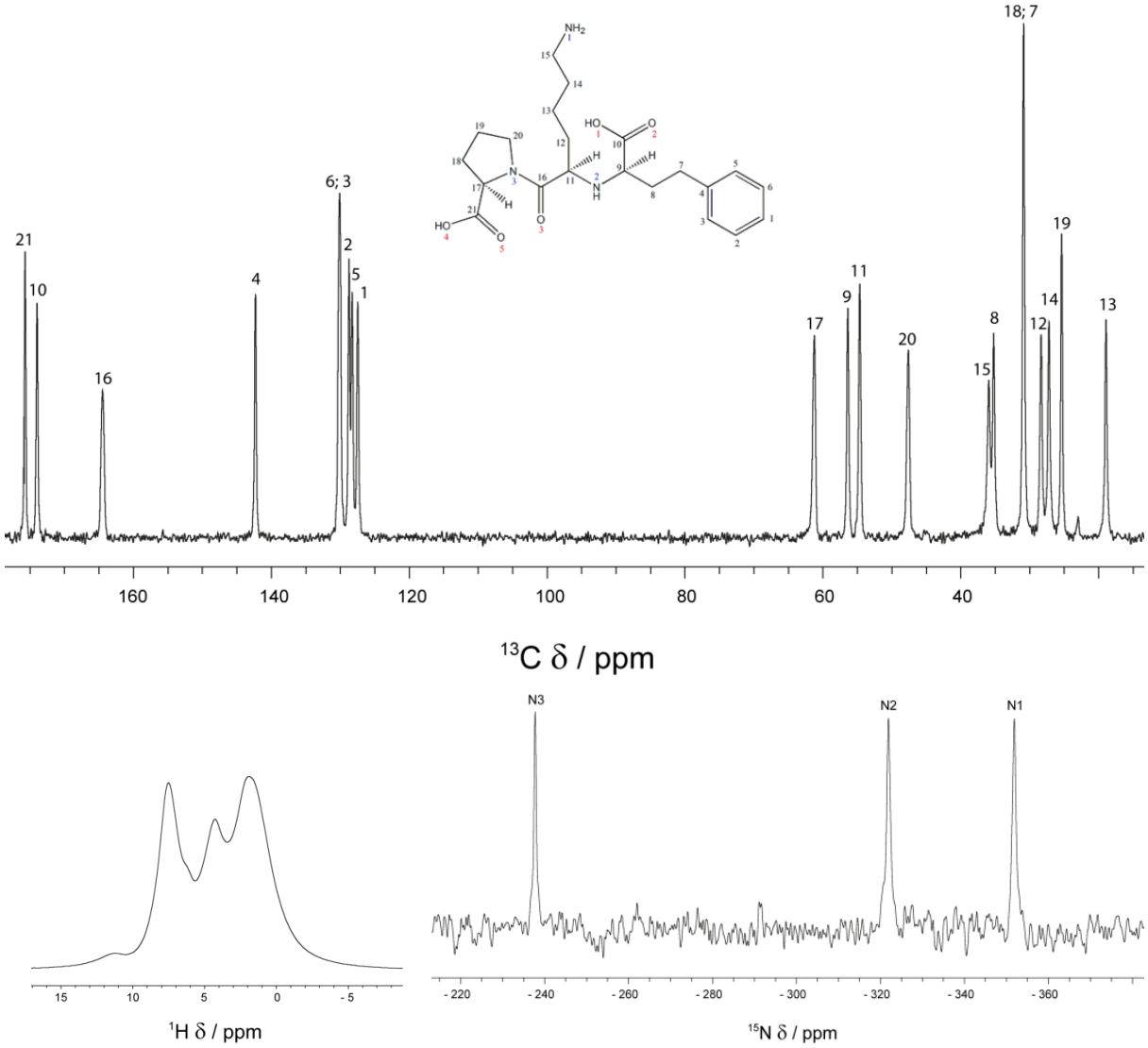

Fig. 5.3. *^{13}C / ^{15}N CP–MAS and ^{1}H la ultra-fast MAS (65 kHz) spectras of Lisinoprilului dihydrate.*

In conclusion, in the present study we have demonstrated in the case of Lisinopril dihydrate, a molecular system with 24 degrees of freedom, the possibility to solve the crystal structure from powder using conventional laboratory X-ray sources with a level of precision close to the X-ray diffraction on single crystal. Although we had a high-quality experimental pattern, we found that the XRPD method provides results with a high degree of confidence in the remote ordering and global conformation of the Lisinopril molecules in the lattice, but not

for a range of local parameters such as the ionization state of the Lisinopril molecule, the geometry of the hydrogen bonding network, and the characteristic parameters for arranging the water molecules in the two channels that are formed. All these characteristics depend on the local structural features around the carboxyl and amine groups centered on the two of the nitrogen atoms: due to the fundamental limitations associated with the XRPD method, multiple conformations of these groups are obtained, and the structural ambiguities generated could be solved only using complementary information obtained by DFT calculations and solid-state NMR spectroscopy, combined in an NMR crystallography approach [**Error! Reference source not found.**]. The applied methodology has proven to be useful both to ultimately achieve a high quality crystalline structure model, but also to characterize the rapid molecular dynamics with atomic resolution.

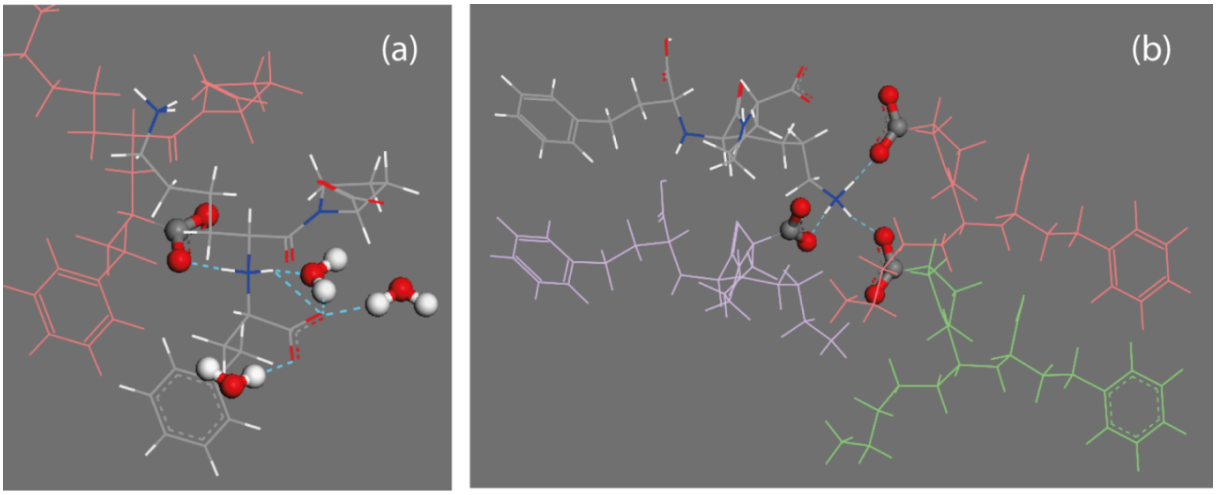

Fig. 5.4. *Hydrogen bonding patterns of the Lisinopril molecules: (a) in the median plane, and (b) around the N1 amine in the Lisi_1 crystal structure model, which was found to best fit the combined XRPD and ss-NMR constraints.*

In conclusion, in the present study we have demonstrated in the case of Lisinopril dihydrate, a molecular system with 24 degrees of freedom, the possibility to solve the crystal structure from powder using conventional laboratory X-ray sources with a level of precision close to the X-ray diffraction on single crystal. Although we had a high-quality experimental pattern, we found that the XRPD method provides results with a high degree of confidence in the remote ordering and global conformation of the Lisinopril molecules in the lattice, but not for a range of local parameters such as the ionization state of the Lisinopril molecule, the geometry of the hydrogen bonding network, and the characteristic parameters for arranging the water molecules in the two channels that are formed. All these characteristics depend on the local structural features around the carboxyl and amine groups centered on the two of the nitrogen atoms: due to the fundamental limitations associated with the XRPD method,

multiple conformations of these groups are obtained, and the structural ambiguities generated could be solved only using complementary information obtained by DFT calculations and solid-state NMR spectroscopy, combined in an NMR crystallography approach [30]. The applied methodology has proven to be useful both to ultimately achieve a high quality crystalline structure model, but also to characterize the rapid molecular dynamics with atomic resolution.

FINAL CONCLUSIONS

In this Ph.D. thesis we obtained new solid forms of bioactive compounds using various screening and crystallization methods and performed their full structural characterization. We also conducted a broad structural investigation for two bioactive compounds incorporated into existing products on the market. The structural characterization of all the compounds analyzed in the present thesis was carried out by a modern approach, which combines complementary X-ray diffraction techniques with solid state NMR and molecular modeling, a methodology known as *NMR crystallography*.

Several pharmaceutical active compounds and natural compounds were studied, out of which three were exhaustively treated in the present thesis.

The first compound studied is Tadalafil, the active substance in Cialis®, a compound with a low solubility. For Tadalafil, we conducted a polymorphism and co-crystallization study to obtain new solid forms with improved solubility. Following these experiments we obtained single crystals for two new solvates forms (Tadalafil acetone and Tadalafil methyl-ethyl-ketone). These two new solid forms were structurally characterized by single crystal X-ray diffraction, nuclear magnetic resonance on solids and molecular modeling. This study underscores the importance of knowing, in a first step, the crystal structure to understand the stability of the solvates and to assess the risks when they are used as precursors in obtaining the anhydrous form.

The second compound studied was Quercetin. For this compound, polymorphism and co-crystallization experiments, we obtain three solvates of Quercetin (with 1,4 dioxane, ethanol and methanol), with high degree of crystallinity that allowed their characterization from the point of view of structure crystal.

A practical application of the structural studies of anhydrous Quercetin and of new solvates is the analysis of solid forms existing in commercial food supplements. Using an approach derived from *NMR crystallography,* we have developed a reliable and rapid procedure for the characterization of food supplements of Quercetin, in terms of the purity of the solid form of the active ingredient, Quercetin, incorporated into tablets or capsules. XRPD was useful for quick identification of the major crystalline components (active ingredients and excipients) specified by the manufacturer on the products, and NMR spectra showed a much higher sensitivity in distinguish the solid forms of Quercetin in commercial formulations, even in the presence of other active ingredients and excipients. Although this approach has

only been tested on two food supplements, it can be easily generalized to other commercial products containing flavonoids.

The last of the active substances studied in this thesis is Lisinopril dihydrate, an active substance that is used for the treatment of hypertension and other cardiac conditions. We have shown that in the case of Lisinopril dihydrate - a molecular system with 24 degrees of freedom - the ability to solve the crystal structure from powder using laboratory X-ray sources (not only X-ray obtained from synchrotron), with a level of accuracy close to that of single crystal X-ray diffraction. Lisinopril dihydrate has been a challenge in the field of structural characterization from powders, primarily because of the large number of degrees of freedom that had to be explored to arrive a structural solution, but also by the difficulty of characterizing a complex hydrogen bonds network. Due to the fundamental limitations associated with the XRPD method, multiple conformations of these groups are obtained, and the structural ambiguities generated could only be solved using complementary information obtained by DFT calculations and solid state NMR spectroscopy - *NMR crystallography*. The applied methodology has proven to be useful to achieve a high-quality crystal structure model, but also to characterize the rapid molecular dynamics with atomic resolution.

LIST OF PUBLICATIONS

Related to the topic of Ph.D. thesis

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Scientific conferences

Oral presentation

Miclaus M, Grosu IG, Filip X, Tripon C, Filip C: *Advanced NMR Crystallography Techniques for structural Characterization of Organic Solids from Powders*, 9th Processes in Isotopes and Molecules International Conference, Cluj-Napoca, Romania, 2013.

Posters presented at international conferences

1. **Miclaus M**, David L, Martin F, Kacso I: *Structural caracterization for Cyclobenzaprine Hydrochloride by X-Ray diffraction and FTIR spectroscopy*, Advanced Spectroscopies on Biomedical and Nanostructured Systems, Cluj-Napoca, Romania, 2014.
2. **Miclaus M**, Grosu I G, Borodi G, David L, M. Pop M: *Polymorfism of fenpiverinium bromide*, 9th Processes in Isotopes and Molecules International Conference, Cluj-Napoca, Romania, 2013.
3. **Miclaus M**, Grosu IG, Filip X, Filip C, Muresan-Pop M, David L: *Solvatomorphism of Quercetin*, 9th Processes in Isotopes and Molecules International Conference, Cluj-Napoca, 2013.
4. **Miclaus M**, Grosu IG, Filip X, Martin F, Filip C: *NMR Crystallography techniques for structural characterization of pharmaceutical compounds from powders*, 15th European Powder Diffraction Conference, Bari, Italy, 2016.
5. **Miclaus M**, Filip X, Grosu IG, Filip C: *NMR Crystallography techniques for structural characterization of pharmaceutical compounds*, 17th International Balkan Workshop on Applied Physics and Materials Science, Constanta, Romania, 2017.
6. Tripon C, **Miclaus M**, Filip X, Filip C: *Molecular architecture characterization of a new Acyclovir polymorph*, European Biophysics Congress EBSA, Lisbon, Portugal, 2013.
7. Tripon C, **Miclaus M**, Kacso I, Filip X, Filip C: *Solid-state NMR: a tool for investigating molecular architecture of new pharmaceutical solid forms*, European Conference on Magnetic Resonance, Dublin, Ireland, 2012.
8. Tripon C, **Miclaus M**, Kacso I, Filip X, Filip C: *NMR crystallography for structural investigation of new pharmaceutical solid forms*, Central and East European Bruker User Meeting, Varna, Bulgaria, 2012.

Posters presented at national conferences

1. **Miclaus M**, David L, Filip C, Filip X, Pop M: *Crystal structure determination of quercetin anhydrate by a multi-technique approach*, 32th National Chemistry Conference, Valcea, Romania, 2012.
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