Chapter 4 – Analysis of Particulate Matter Pollutants Biological Mechanism of Action.

The pollutants toxic activity and the verified concentration in the particulate matter, as shown in the previous chapter had to be correlated with the emerging study of their biological mechanisms of action, in order to comprehend and prevent their effects.

To define the analysis protocol Bisphenol A was used as a case of study because of its known toxic effect reported in literature [62, 63, 65-70, 72, 75-77, 79, 80, 108-110]. It is known that BPA interferes with the endocrine system, interacting with some nuclear receptors like the α and β estrogen receptors and androgen receptor [68]. The data of experiments *in vivo* and *in vitro* concerning the BPA related diseases of the cardiovascular system; diabetes; breast and prostate carcinoma, is numerous. This progress in knowledge is directed on the newest effect of the xenobiotic, but the biological mechanism of action remains unknown and the protein involved in the BPA interaction. Our analysis protocol is based on the molecular docking for the identification of the possible target proteins involved; for the study of their interaction properties and bonding affinities.

4.1 Molecular Docking [118-121].

This study uses two main approaches: a Direct and an Inverse Docking Procedure. The Direct Docking explores punctually the conformation of binding of a protein target and a ligand, while Inverse Docking is an automated identification of potential protein targets of a small molecule (such as Xenobiotics, Drugs, Natural Products).

For Inverse Docking the Dock Program was used, for Direct Docking, the Autodock Program was used.

Dock Program is a *matching method while Autodock is a docking simulation method*. In fact, the first creates a model of the active site, and then attempts to dock a given inhibitor structure into this rigid body by matching its geometry: for this reason it is efficient to screen entire chemical ligand and target proteins database rapidly. The second model docks a ligand to a target in greater detail: the ligand begins randomly outside the protein, and explores the conformational active site in order to define its binding mode translations, orientations and conformations. These techniques, for which **AutoDock** is the most successful, are slower but allow flexibility within the ligand to be modelled and can utilize more detailed molecular mechanism to calculate the energy of the ligand in the context of the active site.

AutoDock was created from the Scripps Research Institute to generate a free software that could be installed on any platform that includes a cluster in order to obtain high performances solutions. Many compared results have been reported in literature on the performance of this program even for the enzymes class considered. The accuracy was tested comparing the algorithms coming from different software packaging: in particular it was reported that in 46% of the cases the RMSD are lower than 2 Å [111, 112]. The RMSD of docking of a protein-ligand complex (called DA) was defined as:

$$DA = f_{RMSD\leq 2} + 0{,}5(f_{RMSD\leq 3} - f_{RMSD\leq 2})$$

Where $f_{RMSD\leq a}$ `is the fraction of docking conformations obtained with RMSD≤a Å, Autodock has obtained a mean accuracy of 0.47Å. The DA definition is equivalent to the mean percentage of conformations with an RMSD ≤ 2 Å and 3 Å.

To launch a docking simulation it is necessary to model the protein target and choose the root and the torsions for the ligand. Then it is necessary to create the Grid Box and the maps files with the Autogrid program. With this Autotools program the molecule is positioned in a 3D grid of 0.375Å points (as default) far from each other, ¼ of a C-C bond. The

region limited from the grid defined the molecule portion of ligand interaction. On each grid point a potential ligand atom is located that has a particular interaction energy with the protein. So it possible to build an affinity map for each atom type of the ligand and an electrostatic grid. The configuration and electrostatic energy are obtained with the interpolation of the affinity values of a single cell.

Autodock uses Genetic Algorithms (GA), based on the natural genetics and biological evolution: the ligand's state variables (translation, orientation, conformation of the ligand respect the protein), correspond to a gene; the ligand's state to a genotype; the atomic coordinates to a phenotype.

Another important parameter of docking is the fitness: the total interaction energy of the ligand with the protein, evaluated using the energy function. So the generation selection (GA) is based on the individual's fitness. If the global search uses genetic algorithm GA, the local search, i.e. the method used to perform energy minimization, is adaptive: the torsional space search is adjusted step by step upon recent history of energies. The GA method with the adaptive LS method forms the so called Lamarkian genetic algorithm (LGA). A good description of the method adopted is shown in fig.78 that illustrates genotypic and phenotypic search, and compares Darwinian and Lamarckian search.

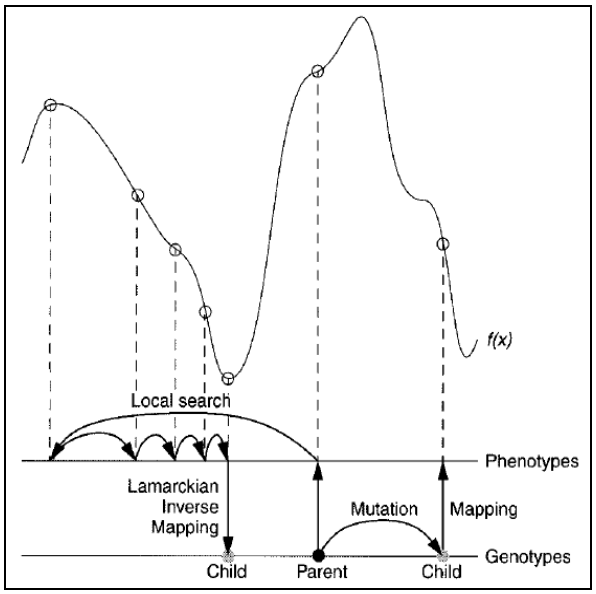

Fig.78 - Genotypic and phenotypic search and compared Darwinian and Lamarckian search. The space of the genotypes is represented by the lower horizontal line and the space of phenotypes by the upper line. The fitness function is f(x). The result of applying the genotype mutation operator to the parent's genotype (on the right-hand side), has the corresponding phenotype shown. Local search (on the left-hand), is performed in phenotypic space and employs information about f(x). The local search is performed by continuously converting from the genotype to the phenotype. The genotype of the parent is replaced by the resulting genotype, in accordance with Lamarckian principles.

The genetic algorithm iterates over generations until one of the termination criteria is met. At the end, the fitness (the docked energy), the state variables, the coordinates of the conformations and the estimated energy of binding, were reported.

AutoDock4.0 has a free-energy scoring function that is based on a linear regression analysis, the AMBER force field, and an even larger set of diverse protein-ligand complexes with known inhibition constants than those used in AutoDock3.0.

The scoring method has two purposes: to detect the correct binding conformation and to estimate the binding affinities of the candidate molecules. The scoring methods used are based on force field calculations, empirical scoring functions, knowledge-based potential of mean force or finally the consensus scoring (evaluation of the best docked conformer with multiple scoring functions).

The best model was cross-validated with a separate set of HIV-1 protease complexes, and it was confirmed that the standard error is around 2.5 kcal/mol. This is enough to discriminate between leads with milli-, micro- and nano-molar inhibition constants. The Amber model (force field model), employs the interaction energy of a molecular system with terms for dispersion/repulsion; hydrogen bonding; electrostatics and deviation from ideal bond lengths and bond angles. This model requires considerable computer time and tends to perform less well in ranking the binding free energies of compounds that differ by more than a few atoms: an empirical relationship is needed between molecular structure and binding free energy that reproduces observed binding constants, adding entropic terms to the molecular mechanics equations:

$$\Delta G = \Delta G_{vdw}+\Delta G_{hbond}+\Delta G_{elec}+\Delta G_{conform}+\Delta G_{tor}+\Delta G_{sol}$$

Where the first four terms are the typical molecular mechanics terms for dispersion/repulsion, hydrogen bonding, electrostatics and deviations from covalent geometry. The latter term includes the restriction of internal rotors and global rotation and translation, the desolvation and the hydrophobic effect (solvent entropy changes at solute – solvent interfaces). It is also the most challenging, because of the grid based method of AutoDock, against methods based on surface area calculations. So for ΔG_{sol} linear regression was used to calibrate the function against a set of different ligand-protein complexes with published binding constants, sufficient to rank inhibitors with millimolar, micromolar and nanomolar binding constants. The equation includes five terms:

$$\Delta G = \Delta G_{vdW}\Sigma(A_{ij}/r^{12}_{ij}-B_{ij}/r^{6}_{ij}) + \Delta G_{hbond}\Sigma E(t)(C_{ij}/r^{12}_{ij}-D_{ij}/r^{10}_{ij}+E_{hbond}) + \Delta G_{elec}\Sigma q_i q_j/\varepsilon(r_{ij})r_{ij} + \Delta G_{tor}N_{tor} + \Delta G_{sol}\Sigma S_i V_j e^{(-r2ij/2\sigma 2)}$$

Empirical regression-based scoring functions estimate the bonding affinity protein-ligand complexes by adding up interaction terms derived from weighted structural parameters of the complexes, assigned by regression methods, as free energy contributions from interactions like hydrogen bonding, ionic interactions, hydrophobic interactions and entropic contributions. A major drawback of any regression scoring function is the dependence on the size, composition and generality of the training set used to derive the weights and the implicit assumption that each occurrence of a particular interaction contributes equivalently, with a consequently overstimation of polar interactions to non polar ones. The first-principle-based approaches approximate the binding free energy of protein-ligand complexes by adding up the individual contributions of different types of interaction: the individual terms are derived from physico-chemical theory and are not determined by fitting experimental affinities. In most cases, gas-phase molecular mechanical energies are combined with solvation free energies and vibrational, rotational and translational entropies.

For the analysis of Inverse Docking the web-based tool was used: TarFisDock (Target Fishing Dock), ***available online at*** http://www.dddc.ac.cn/tarfisdock/ [107]. This web-based tool uses the DOCK4.0 program to execute both the simulations of docking and scoring function, in order to calculate the energetic interactions. Dock employs the creation of a negative image of a target site; placement of the putative "ligands" into the site; evaluates the quality of fit. To the creation of a negative image of the target or putative site, Dock characterizes the entire surface of the molecule with Connolli method. Each site is filled with overlapping spheres. For the placement of the ligand into the site, the Dock algorithm, (a first principles methods) using van der Walls and Coulumb terms of force field.

Dock is a descriptor matching program, i.e. its analyzes the receptor for regions of similar complementarity: ligand atoms are placed at the "best" positions in the site, generating a ligand-receptor configuration that had to be refined by

optimization. This method is rarely exhaustive, it is sensitive to the quality of the negative image and has a limited conformational exploration but it is fast and it can be used for a particular region of the receptor site.

4.2 Docking Protocol

This study used two main sequential approaches: an Inverse Docking and a Direct Docking procedure. Inverse Docking is useful for the identification of the potential target proteins types involved in the Bisphenol A interaction. Direct Docking explores punctually the conformation of binding of a protein and a ligand.
The molecular structure of BPA in mol2 format has been obtained using Omega (OpenEye Scientific Software), (see Chap. 4.2.1).
In order to research the potential binding proteins of the xenobiotic BPA, the web-based tool TarFisDock was used (Target Fishing Dock) [107].
TarFisDock takes the BPA molecule in mol2 format, dockeing the ligand into the protein targets PDTD (Potential Drug Target Database), and outputs the 10% candidates ranked by the energy score, including their binding conformations and a table of the related target information. The web-based tool uses the DOCK4.0 program to execute both the simulations of docking and scoring function, in order to calculate the energetic interactions.
For Direct Docking simulations each PDB protein crystal structure (downloaded from the Protein Data Bank web site) was refined by PyMOL Viewer program: the chain of interest was selected and ligands and water were removed. The Autodock Tools (ADT) script preplig was used to convert the mol2/pdb and the pdb protein molecules format to the pdbqt format by adding Gasteiger charges, adding all hydrogens and assigning ligand flexibility.
All data is processed with the same Docking parameters:

Number of GA Runs: 100
Population Size: 250
Maximum Number of Evals: 25000000

With the same Grid Box of 60-66-54 points, spaces around the binding site and with 0,375 Å of spacing.
The default parameters were appropriate to reproduce the X-ray ligand pose with an RMSD value lower than 2.0 Å.
All other parameters are settled as default in Autodock Tools.
Docking simulation were run on a Linux Cluster of 32 nodes, each SuperMicro equipped of two processors: the 2.50Ghz INTEL(R) Quad-core and the 16GB Xeon(R), for a 256 total processors and 512 GB of RAM. The cluster has a furniture of 20TB disk and is interconnected with the Infiniband 4X network. For the Audock implementation data, employed with the maximum advising input parameters, a few hours could be required.

4.2.1 Conformations Generations, [117].

To generate the ligand tridimentional structures as imput mol/pdb files for the docking and redocking simulations the Omega program is used. For all analysis obtained with Omega the conformations are compared with the docking of the cocrystallized pdb imput file, in order to validate the method.

The Omega software package, (from OpenEye Scientific Software), generates multi-conformer 3D structures. The multi-conformers structures for a xenobiotic; the inhibitors or a natural ligand are generated in order to move away from the crystallographic ones, maintaining the chirals centres.

The initial conformation of the ligand is more important for Autodock than Dock programs in relation to the Ligand Flexibility Algorithms. Dock 4.0 uses Incremental Construction Algorithm (i.e. a systematic algorithm), based on steric complementarity: there are rigid and flexible regions for the ligand. The flexible parts grow incrementally degree of freedom by degree of freedom and when the molecule is complete, it's reminimized.

Autodock 4.0 uses stochastic methods to find the global energy minimum by LGA to improve convergence for ligands with more than eight rotable bonds as seen. Omega has two main structures: the model binding and the torsion driving. The 2D input molecule is fragmented at exocyclic sigma bonds and carbon to heteroatom acyclic (but not exocyclic) sigma bonds. The conformations for these fragments are retrieved from a pregenerated library "makefraglib" or created using the distance rules followed by a geometric optimization protocol of "makefraglib". At last the molecule is assembled by a simple vector since fragment points are along the sigma bonds. Omega generates additional model enumerating ring conformations (obtained from makefraglib) and invertible nitrogen atoms.

So the "Flipper" utility enumerates defined unspecific stereochemistry (considering that for each atom/bond with a stereochemical state there are 2^N stereoisomers – R/S; Cis/Trans), by graph algorithms to determine which atoms are stereocentres and generates configurations. For our need we had to compare the stereochemistry of the biological active molecule (cocrystallographic inhibitors or natural ligand) and choose the appropriate configuration to improve the conformation molecules. Omega2 in fact starts from the configuration and generates, with the fraglib ausiliary, all the possible conformations, ordering them by Energy level.

In this work the bad energy and the best energy state has been taken conforming to a date inhibitor, as it will be seen later, but all the conformations had to be considered as equal starting point for the automated molecular docking simulations.

4.3 Analysis Results.

It is known that BPA interferes with the endocrine system binding itself to some nuclear receptors like the α and β estrogen receptors and androgen receptor [68]. Also it is cocrystallized with the Estrogen Related Receptor γ (ERR γ). We lack information about the interaction BPA-protein, pertaining to the molecular pathways involved and known in the literature, because the biological mechanisms of action involved, at molecular level, are still unknown..

The molecular docking simulations are a useful analysis to identify the potential protein targets and the interaction of a complex for a given ligand.

From potential binding proteins of BPA, screened from potential drug target database (PDTD), using the reverse molecular docking approach TarFisDock, the human and mammal proteins were selected for this study. The Kegg database interrogation provided the determination of the involved metabolic pathway for the resulting proteins.

Among the found potential targets from reverse docking simulation, there were those referring to coagulation pathway, xenobiotics metabolism by cytochrome P450, MAP kinase and neoplastic disease. The target proteins show an energetic interaction range with BPA from -27.0 kcal/mol to -35,0 kcal/mol. The proteins involved in the coagulation cascade are: the thrombin, present many times with the human PDB entry 1AE8, 1BMM, 1DWC and the bovine 1ETT; the porcine factor IX, (PDB entry 1PFX) and the human factor X, (PBD entry 1XKA). The three proteins target belonging to the

serine proteases family have a catalytic domain structurally similar and bind BPA in the active site pocket with a interaction energies between -27,0 and -29,0 kcal/mol. The obtained results could be linked with the literature data that associated BPA with cardiovascular disturbs (coronary heart disease, heart attack, angina) [68].

The monoxygenase Cytochrome P450 2C9 is an enzyme that recognizes and metabolizes various environmental and polluting compounds, including BPA. CYP2C9 and CYP2C18 exhibited the highest affinity (Km=3.9 μM) for BPA metabolism [108]. TarFisDock characterizes the PDB Entry 1OG5, involved in the metabolism of xenobiotics by cytocrome P450. The complex shows a binding energy of -27,12 Kcal/mol.

Two human enzymes belonging to the hydrolase's class, Raps-related protein, Rap-1A, and the Kinesin-related motor protein, EG5, have been identified with PDB entry 1C1Y and 2FKY. The Raps-related protein, Rap-1, modulates the interaction between the RAS/RAF proteins involved in the MAPK kinase pathway, turned to the expression of genes involved in the cellular division. BPA binds RAP1A protein interfering with the RAS/RAF pathway.

Kinesin-related motor protein EG5 uses ATP hydrolysis to generate force and movement along the microtubules and turns out implied in the cellular division. BPA bound the inhibitor-binding pocket interfering with the cellular division. The result supported the immunofluorescence studies about the spindle aberrations induced by Bisphenol A [109].

The Tarfisdock entries, covering 841 known and potential drug targets with structure from the Protein Data Bank (PDB), could not be comprehensive. The database could be enriched in particular by human proteins to identify the potential binding targets in-silico.

4.3.1 Investigation of the interaction between Bisphenol A and the Coagulation Pathway Proteins.

From the identified target proteins, the punctual BPA investigations were monitored on the Serine Protease alpha Thrombin and the Blood Factor Xa because of the multiple different proteins and same molecules cocrystallized with different inhibitors obtained from TarfisDock outputs. Moreover it is known in literature that Bisphenol A favours the coagulation and other cardiovascular disorders, [68]. TarfisDock selected as site of binding for BPA, the Heavy chain of each protein structure. The modelling of this chain is employed by PyMOL: H chain for the 1dwc and 1ae8 PDB entry, and C chain for the 1xka entry.

In general the Serine proteinases are a group of enzymes that hydrolyze peptide bonds in proteins for a variety of different functions such as food digestion; the cleavage of signal peptides and the control of blood pressure and blood clotting. These molecules have four features in the active site:

- the **catalytic triade** consists of **Asp102**; **His57** and **Ser195** for the formation of the covalent transition state;
- the **Oxyanion Hole** for the stabilization of the transition state **Residues 192, 193, 194**;
- a **Specific Pocket, Residues number 189; 216; 226**, and a **Non Specific Pocket** region, **Residues 215; 227** for the binding of the ligand.

The members of the chymotrypsine superfamily (chymotrypsine; trypsine; elastase) have the same type of specific pocket. The Thrombin and Factor Xa maintain the specific pocket of the trypsine (Gly 226; Asp 189 and Gly 216), [114, 115].

Comparative studies of molecular-structural graphic of the Serine Protease alpha Thrombin 1ae8 and 1dwc showed that there are some different orientations of the pocket residues, in particular for the Oxianion Hole. In fig.79 and 80 the

Connelli Surface was implemented for the two molecules: the cyan molecular surface represented the catalytic triade; the yellow accessible area, the Hoxianion Hole; the green portion, the Specific Binding Pocket and the Orange, the Non Specific Binding Pocket. The overlapping of the Surface Molecule for the two Serine Protease alpha Thrombin, (fig.79-c) and the Connelli surface of the Blood Factor Xa (PDB entry 1xka), underlined the conformation pocket differences, [116].

(a) 1ae8 (b) 1dwc (c) 1ae8-1dwc

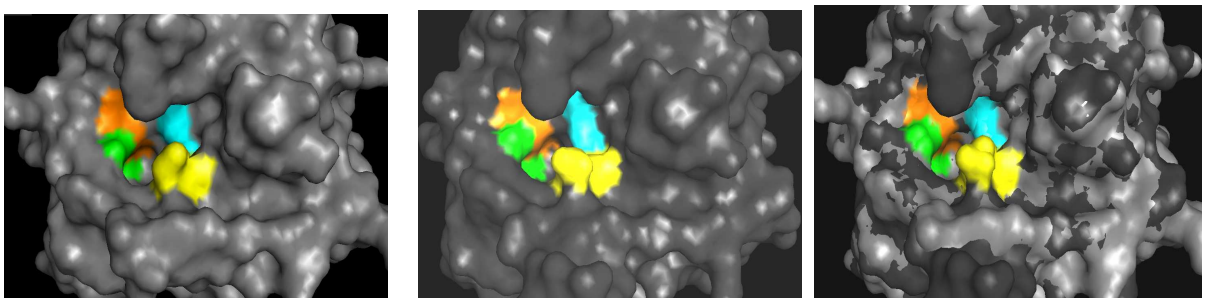

Fig.79 – In order from the left, The Binding Pocket Structure respectively, the 1ae8 and 1dwc Serine Protease alpha Thrombin and their overlap: the cyan portion of surface is referable to the catalytic triade; the yellow surface to the Oxyanion Hole; the green to the Specific Pocket and the orange to the Non Specific Pocket.

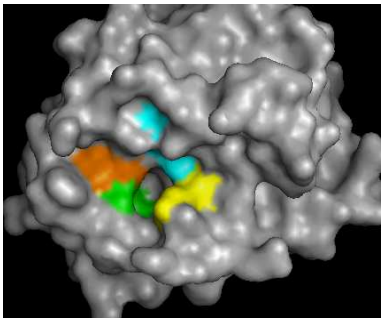

Fig.80 - The Binding Pocket Structure for the 1xka Blood Factor Xa. The cyan portion of surface is referable to the catalytic triade; the yellow surface to the Oxyanion Hole; the green to the Specific Pocket and the orange to the Non Specific Pocket.

Each single situation was analyzed with stick representations shown in fig.81 for the Thrombin. In fig.82, the two crystal structures 1ae8 and 1dwc have some conformational differences located respectively in Catalytic Triade for the residue 195 SER; the Oxyanion Hole 192 GLU; and the Specific Pocket for the 216 GLY.

The differences in the binding pocket are noted for the Thrombin and the Xa factor: only two residues change: the ILE227 residue in the Thrombin Non Specific Pocket becomes the PHE227 in the Xa factor and the GLN192 of the Oxyanion Hole becomes a GLU192, (fig.83).

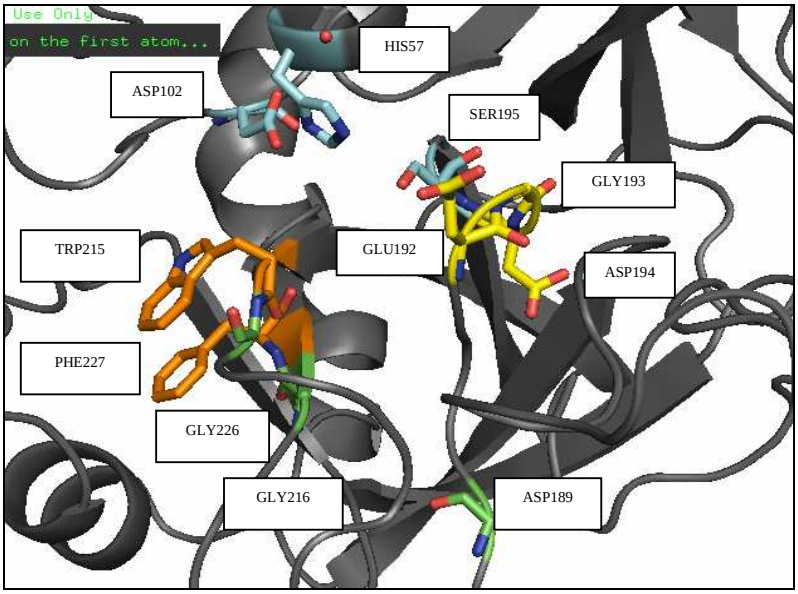

Fig.81 - The Binding Pocket Structure for the **Serine Protease alpha Thrombin**: the catalytic triade coloured in cyan (HIS57; ASP102; SER195); the Oxyanion Hole coloured in yellow (GLN192; GLY193; ASP194); the Nonspecific Substrate Binding Region coloured in orange (TRP215; PHE227); the Substrate Specificity Pocket coloured in green (ASP189; GLY216; GLY226), [116].

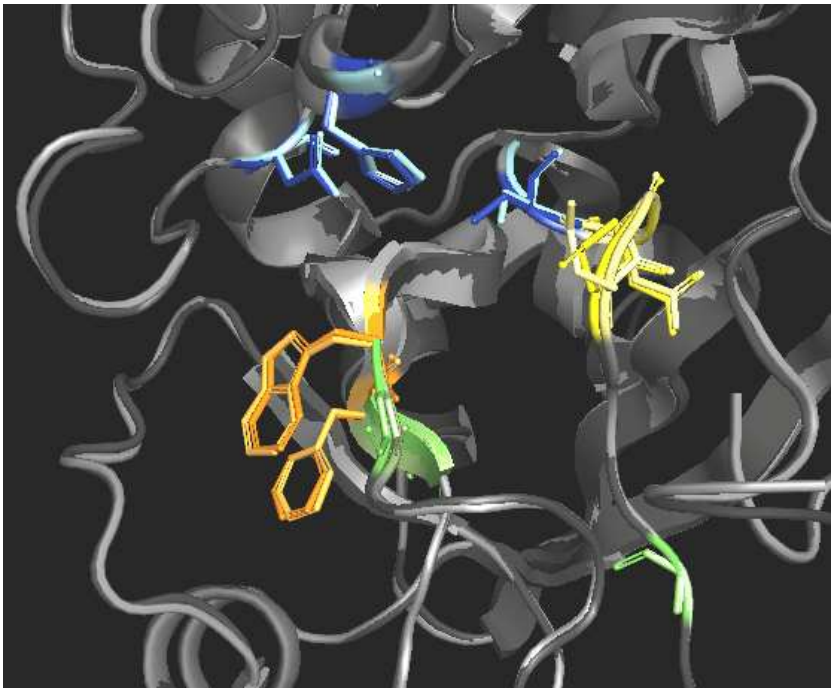

Fig.82 - The Binding Pocket Structure for the **Serine Protease alpha Thrombin**: the Catalytic Triade coloured in cyan for the 1dwc PDB entry and blue for the 1ae8 PDB entry; the Oxyanion Hole coloured in yellow for the 1dwc and pale yellow for the 1ae8; the Nonspecific Substrate Binding Region coloured in orange; the Substrate Specificity Pocket coloured in green for the 1dwc and pale green for the 1ae8, [116].

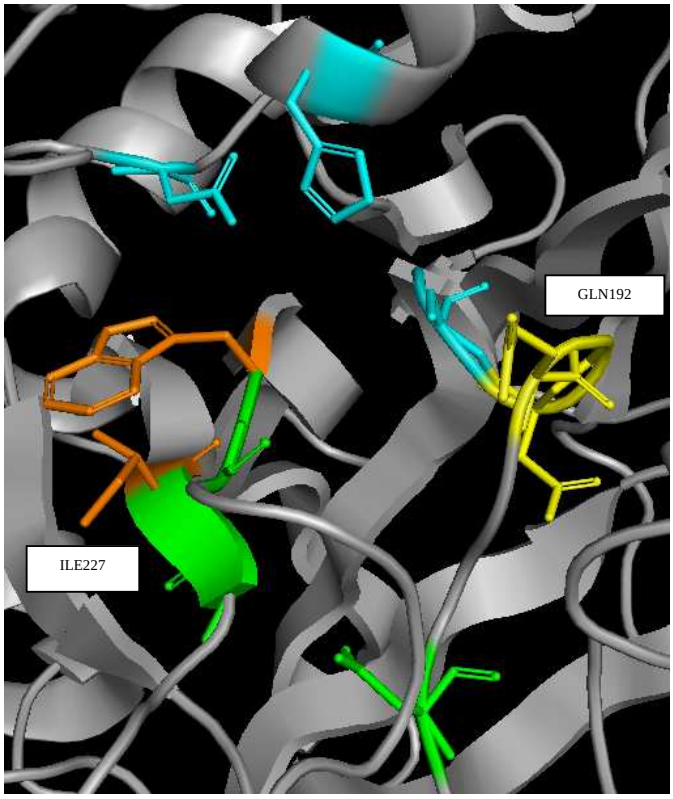

Fig.83 - Conformation pocket of the Blood Factor Xa with the noted structural differences from the Thrombin (the orange residue ILE227 and the yellow GLN192), [116].

The treated graphic studies could be important for the analysis of the BPA binding mode obtained from molecular docking.

The first PDB entry considered is 1dwc, a thrombin cocrystallized with the MIT inhibitor, Argatroban [Md-805; mitsubishi inhibitor] with chemical formula: $C_{23}H_{36}N_6O_5S$. The second PDB entry for the Thrombin 1ae8, cocrystallized with the AZL inhibitor, the oligopeptide ASP-PHE-GLU-GLU-ILE-PRO-GLU-GLU-TYS-LEU - O-sulfo-l-tyrosine with the chemical formula: $C_9H_{11}NO_6S$. At last the 1xka has been tested, the PDB entry for the Blood Coagulation Factor Xa. Its synthetic inhibitor is the 4PP (2s)-(3'-amidino-3-biphenyl)-5-(4-pyridylamino) pentanoic acid with chemical formula: $C_{23}H_{24}N_4O_2$, [116].

4.3.2 Redocking.

A docking analysis protocol could not be tested in accuracy and reproducibility by a simple evaluation of the RMSD between the cocrystallized ligand conformations. In fact the results of docking simulation depend heavily on the input of the ligand 3D structure. Feher and Williams, 2009 demonstrated how it could be used a conformational research or molecular dynamics simulation on ligand preceding the docking, [113].

For the evaluation of the choice conformations for redocking the AZL was used because of its nature like oligopeptide, the more complex structure to be docked. First the configurations were generated with Flipper: AZL has two chiral centres, so the software generates four molecules. From the comparison of the first chiral centre two possible molecules

have been selected (first chirality center of the cocrystallized ligand); from the second only the structure was selected corresponding to the second cocrystallized stereocenters, [116]:

CCOC(=O)N[C@H](Cc1ccccc1)C(=O)N2CCC[C@H] 2C(=O)NNCCCCN	Cocrystallized AZL
CCOC(=O)N[C@@H](Cc1ccccc1)C(=O)N2CCC[C@@H]2C(=O)NNCCCCN	flipper_1
CCOC(=O)N[C@H](Cc1ccccc1)C(=O)N2CCC[C@@H]2C(=O)NNCCCCN	flipper_2
CCOC(=O)N[C@@H](Cc1ccccc1)C(=O)N2CCC[C@H]2C(=O)NNCCCCN	flipper_3
CCOC(=O)N[C@H](Cc1ccccc1)C(=O)N2CCC[C@H]2C(=O)NNCCCCN	flipper_4

First selection

CCOC(=O)N[**C@H**](Cc1ccccc1)C(=O)N2CCC[C@@H]2C(=O)NNCCCCN	flipper_2
CCOC(=O)N[**C@H**](Cc1ccccc1)C(=O)N2CCC[C@H]2C(=O)NNCCCCN	flipper_4

Second Selection

CCOC(=O)N[C@H](Cc1ccccc1)C(=O)N2CCC[**C@H**]2C(=O)NNCCCCN	flipper_4

The final selected configuration (flipper_4) was given as an ism file to the Omega program that has generated 177 conformations ordering them by internal free energy: for the redocking demonstration the first energetically favoured configuration (Flipper_4; Omega_1) and the last (Flipper_4; Omega_177) were considered and processed. The autodock program ranked the best energy conformation obtained by RMSD scores: in a same cluster the molecules that differ in structure from each other less than 2 Å could be found. The most populated clusters are those that contain many conformations. For Flipper_4; Omega_1 and Flipper_4; Omega_177 the docking simulations are shown in tab.36 For each Autodock dlg file the most populated cluster with the lowest energy binding mode was chosen.

The free binding energy of omega-conformations-thrombin complexes is in both cases, even for 1 Kcal/mol, better that the cocrystallized-thrombin complex.

In fig.84 the three conformations of interest are shown: the AZL for the 4-1 conformation; the AZL in the 4-177 conformation and the crystallized conformation: as it could be seen even the synthetic inhibitor is an oligopeptide, i.e. a big structure, in general difficult to dock with a good accuracy, the RMSD are respectively 1,4834 Å; 1.5740 Å if compared with the crystal and 0.5704 Å for the two omega-generated conformations. The RMSD reported are under the 2 Å of accepted error from Autodock4.0 standard parameter.

PDB Entry	1ae8	1ae8 Omega Conformations	
Ligand	**AZL Redocking**	**4-1**	**4-177**
Run	96	39	3
Number of Conformations in cluster	35	15	18
Estimated Free Energy of Binding (kcal/mol)-(1+2+3+4)	-8.01	-9.28	-9.00
Estimated Inhibition Constant, ki (298,15K) μM	1.35	0.16	0.25
1-Intermolecular Energy (a+b)	-10.78	-11.01	-10.52
a-vdW+Hbond+desolv Energy	-9.65	-10.72	-10.32
b-Electrostatic Energy	-1.13	-0.29	-0.20
2-Internal Energy	-1.47	-2.11	-2.31
3-Torsional Free Energy	+3.29	+3.57	+3.57
4-Unbound System's Energy	-0.95	-0.27	-0.26

Tab.36 - The docking results for the redocking of AZL with the crystallographic structure and with the Omega conformation, compared with the best cluster for the Bisphenol A.

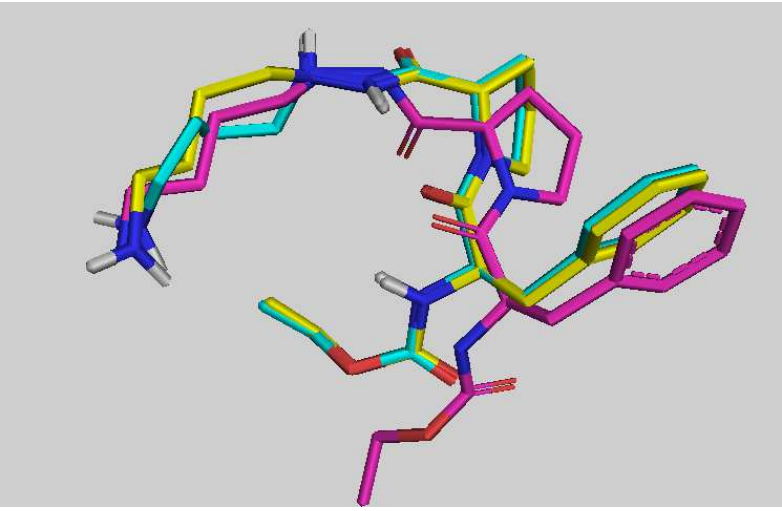

Fig.84 - Redocking results: the AZL crystal is the pink molecule; the best Autodock4.0 binding modes for 4-1 AZL conformation is the cyan inhibitor and the 4-177 AZL Autodock4.0 conformation is the yellow ones, [116].

4.3.3 Binding Mode between BPA and the Serine Protease Alpha Thrombin and the Blood Factor Xa.

The conformations for Bisphenol A generated with Omega are two, called BPA-1 and BPA-2. Each xenobiotic Conformer is docked with the proteintarget and compared with the best score of the synthetic inhibitor crystal.
The Free Energy of Binding are higher in any case for the Bisphenol A, but only compared with MIT the xenobiotic

has an energy higher than 2 Kcal/mol, the fixed accepted calculation error, fig.37.
Even the estimated Inhibition Constant suggests that BPA has a lower affinity for the proteins tested, but if we consider that a synthetic inhibitor has an affinity higher than the natural ligand, it could be concluded that BPA has a good affinity with these proteins as the TarfisDock scores suggest.

PDB Entry	**1dwc**			**1ae8**			**1xka**	
Ligand	**MIT**	**BPA-1**	**BPA-2**	**AZL**	**BPA-1**	**BPA-2**	**4PP**	**BPA-1**
Run	71	11	36	96	8	75	17	60
Number of Conformations in cluster	6	93	80	35	75	85	80	100
Estimated Free Energy of Binding (kcal/mol) (1+2+3+4)	-9.80	-6.71	-6.70	-8.01	-6.07	-6.07	-8.95	-6.82
Estimated Inhibition Constant, ki (298,15K) µM	0.07	12.14	12.24	1.35	35.59	35.53	0.27	9.99
1-Intermolecular Energy (a+b)	-10.78	-7.97	-7.94	-10.78	-7.24	-7.26	-11.23	-7.96
a-vdW+Hbond+desolv Energy	-9.47	-7.72	-7.69	-9.65	-6.83	-6.85	-10.81	-7.77
b-Electrostatic Energy	-1.32	-0.25	-0.25	-1.13	-0.41	-0.41	-0.41	-0.18
2-Internal Energy	-2.70	-0.28	-0.31	-1.47	-0.37	-0.36	-0.43	-0.41
3-Torsional Free Energy	+2.47	+1.10	+1.10	+3.29	+1.10	+1.10	+2.47	+1.10
4-Unbound System's Energy	-1.21	-0.45	-0.45	-0.95	-0.45	-0.45	-0.24	-0.45

Tab.37 - Analysis results for the Serine Protease alpha Thrombin and Blood Factor Xa with their inhibitor and the BPA.

For each result the protein residues interaction is located for both the Pollutants and the synthetic inhibitors ligands.
In fig.85 the overlap of BPA on the inhibitor binding mode in the Serine Protease alpha Thrombin is shown, 1dwc PDB entry. The xenobiotic bonds with the residues of the Specific Binding Site like MIT.

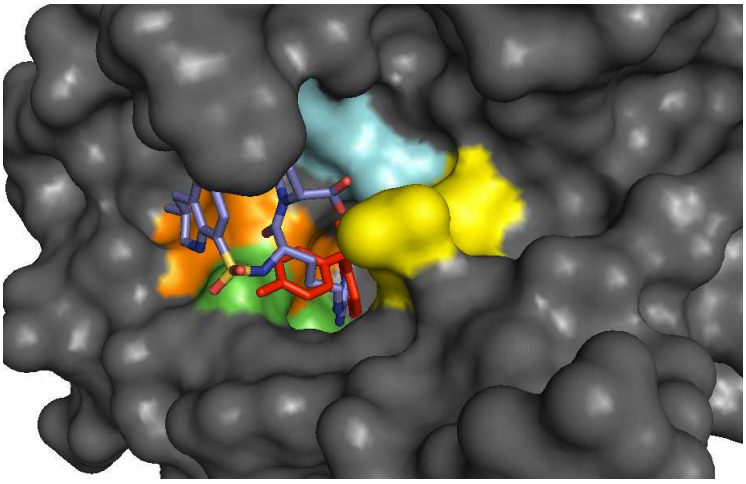

Fig.85 - Interactions between BPA and co-crystallized MIT with the Binding Pocket of the Serine Protease alpha Thrombin 1dwc.

The same types of interactions are visualized in fig.86 where the hydrogen bonds between the ligand and the protein target are measured. The Bisphenol A forms a hydrogen bond with the backbone of the 216GLY and 226GLY of the Specific Binding Pocket. In fig.86 the ligand molecules present two colours because the two conformations of BPA, (1 and 2) are completely overlapped.

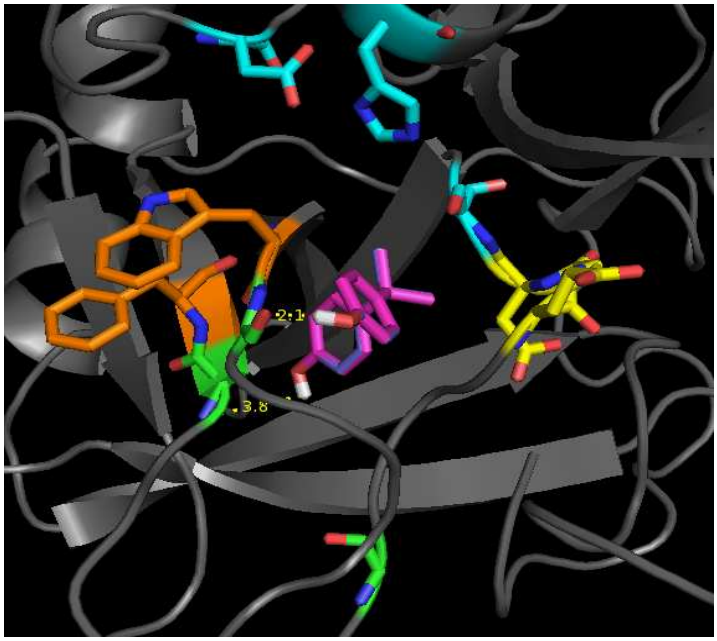

Fig.86 - The BPA interactions with the Serine Protease alpha Thombin Glycine of the Specific Binding Pocket.

Even for the second Thrombin considered PDB entry 1ae8 the Bisphenol A bonds in the deep pocket binding, as it could be seen in fig.87, and the binding omega-conformations overlap the co crystallized AZL inhibitors. The two pollutants conformations BPA-1 and BPA-2 overlap as in the previous case.

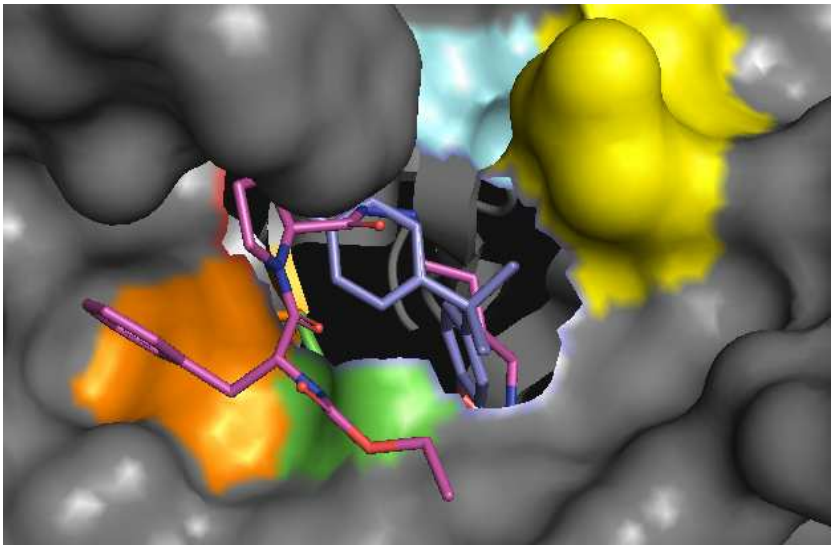

Fig.87 - The Binding Mode of Bisphenol A and AZL with the Serine Protease alpha Thrombin 1ae8 PDB entry.

Also in these cases a hydrogen bond is involved with 189 Glycine of the Specific Binding Pocket, see fig.88, and the second hydroxyl of BPA forms hydrogen bonds with the 57 Histidine ring of the Catalytic Triade.

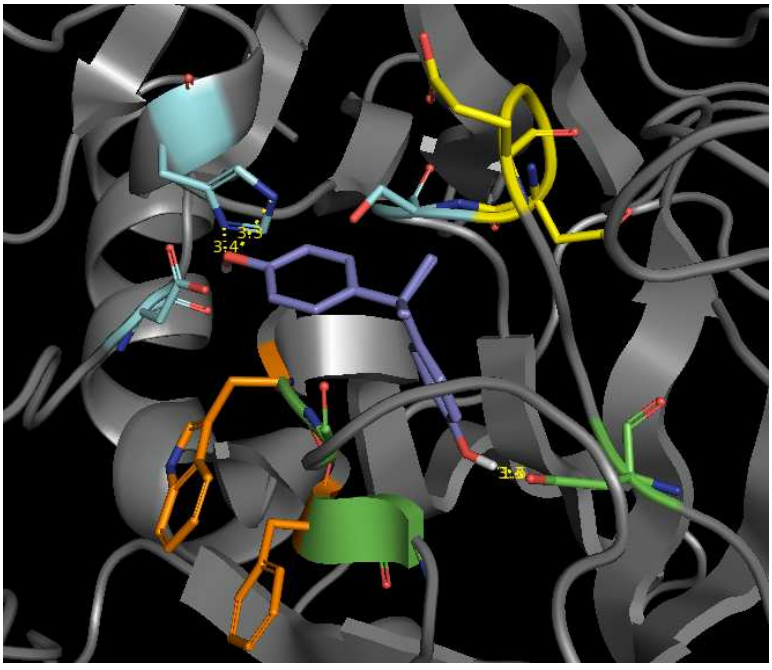

Fig.88 - Interactions between Bisphenol A and the Bisphenol A and AZL active site residues with the Serine Protease alpha Thrombin 1ae8 PDB entry .

In Factor Xa Binding Pocket, the omega-conformations of the xenobiotic interact with the Glycine 216 of the Specific Binding Pocket and external residues to the four binding features pockets. The Autodock results are explained by the overlapping of the BPA ligand with the 4PP inhibitor, fig.89.

The biphenylamidine group of the 4PP interacts with the Specific binding Pocket, while the pyridine ring is totally overlapped with the Bisphenol A, in a near active site, fig.90.

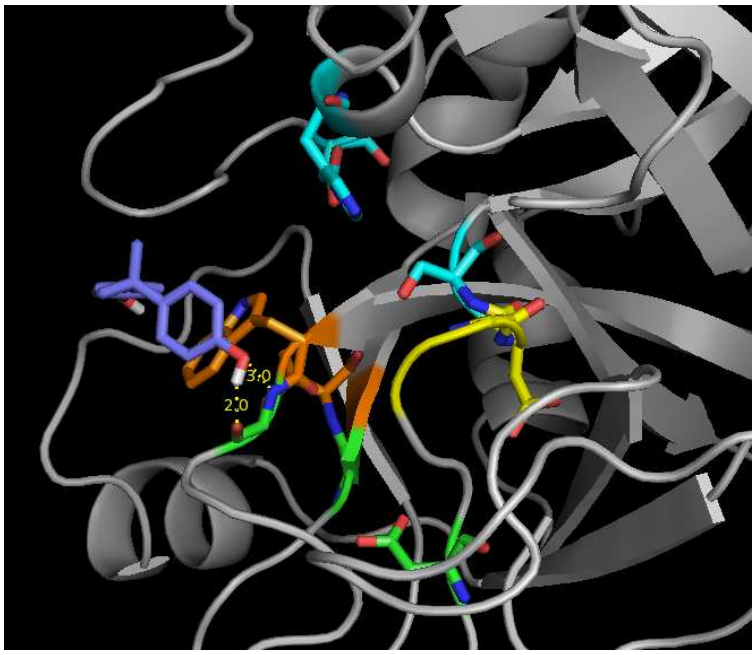

Fig.89 - Interaction between the Bisphenol A and the Specific Binding Pocket of the Blood Factor Xa.

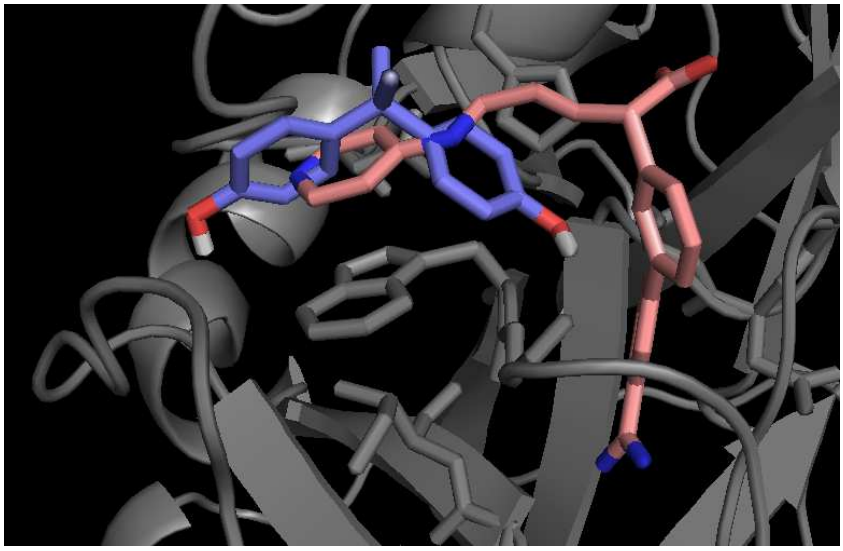

Fig.90 - Overlap of the 4PP inhibitor crystal pose and the docking best score result for the BPA.

At last the xenobiotic Bisphenol A has a good binding affinity with the Blood Serine Proteases involved in the coagulation cascade pathway. The free energy of binding and the inhibition constant are comparable with the co crystallized synthetic inhibitors.

The procedure of docking and of preparation of ligand result accurate and the data is reproducible.

In general, the results of this study suggest that it is possible to explore in silica the Bisphenol A targets proteins and localize its binding interactions by means of the docking procedure,.

In order to explain the Bisphenol A biological function, the docking simulations allowed supplying predicted results about the involved mechanisms of action, correlated to the xenobiotic toxicity, widely documented in literature [68, 75, 97, 107-110].

Chapter 5 – Conclusions.

Three main researches have been employed for the implementation of a protocol analysis for the characterization and quantification of the lignin fraction in the particulate matter at the concentration matrix level; the implementation of different methods of analysis of the toxic interesting pollutants, Oxy-PAHs; Nitro-PAHs and the Bisphenol A, that together with the large set of performed analysis, allowed the characterization of some PM fractions in relation with Indoor and Outdoor concentrations, human exposure and Urban – Rural – Remote sites composition. At last an in silica method was developed for the research of the proteins involved in the interaction with the pollutants of interest, optimized on Bisphenol A because of its history and recent interaction study with the Nuclear Receptors. From the involved pathway the Blood Serine Proteases are used to test the accuracy and reproducibility of obtained Autodock4.0 and Dock4.0 data. The method results useful for research on the biological mechanism of action in relation with both matrix concentrations and *in vivo* and *in vitro* studies. The data predicted will be confirmed by NMR analysis. The newest docking program gives more and more reproducible data, accurate and empirically shaped on the domain problem, at last the experimental data had to confirm or not confirm the predictions.

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to his future learns,
could they be clear, many and interesting.

Bibliography

[1] Dockery, D.W., Pope, CAIII., Acute respiratory effects of particulate air pollution, Ann. Rev. Pub. Hlth., 1994, 15:107-113.

[2] Schwartz, J., Dockery, D.W., Neas, L.M., Is daily mortality associated specially with fine particles?, Journal of the Air and Waste Management Association, 1996, 46, 927–939.

[3] Ackermann-Liebrich U., Environmental Epidemiology Exposure and Desease, Occup. Environ. Med. 1997, 54: 287.

[4] Liu L.J.S., Phuleria, H.C., Arx, M.E.H., Ducret Stich, R., Ineichen, A., Ragettli, M., Braun Fahrländer, C., Schindler, C., Respiratory health impact of diesel exhaust from truck traffic among adults and asthmatic children in trans-alpine highway valleys in Switzerland, Epidemiology, 2008, V19, N6, NOV, SS, p S228.

[5] Pope, C.A., Cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution, J. Amer. Med. Assoc., *2002,* 287, 1132–1141.

[6] Seinfeld, J.H., Pandis, S.N., Atmospheric chemistry and phisycs: from air pollution to climate change, J. Wiley, New York, 1998.

[7] Pope, C.A., Burnett, R.T., Thun, M.J., Calle, E.E., Krewski, D., Ito, K., Thurston, G.D., Lung cancer, cardiopulmonary mortality and long-term exposure to fine particulate air pollution. Journal of the American Medical Association, 2002, 287 (9), 1132–1141.

[8] Intergovernmental Panel on Climate Change IPCC, Geneva, Switzerland Climate Change 2007: Synthesis Report Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change Core Writing Team, Pachauri, R.K. and Reisinger, A. (Eds.).

[9] Kalberer, M., Paulsen, D., Sax, M., Steinbacher, M., Dommen, J., Prevot, A.S.H., Fisseha, R., Weingartner, E., Frankevich, V., Zenobi, R., Baltensperger, U., Identification of Polymers as Major Components of Atmospheric Organic Aerosols, Science, 2004, Vol.303, 1659.

[10] D.R. Oros, M. Radzi bin Abas, N.Y.M.J. Omar, N.A. Rahman, B.R.T. Simoneit, Identification and emission factors of molecular tracers in organic aerosols from biomass burning: Part 3, Grasses Applied Geochemistry, 2006, Vol.21, 919.

[11] Vecchi, R., Marcazzan, G., Valli, G., Cerini, M., Antoniazzi, C., The role of atmospheric dispersion in the seasonal variation of PM_1 and $PM_{2.5}$ concentration and composition in the urban area of Milan (Italy). Atmospheric Environment, 2004, Vol.38, 4437–4446.

[12] Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe.

[13] Freudenberg, K., Biosynthesis and Constitution of Lignin, Nature, 1959, Vol.183, 1152-1155.

[14] Freudenberg, K.; Neish, A. Constitution and biosynthesis of lignin, 1968, Springler Verlag.

[15] Dianin (1891). Zhurnal russkogo fziko-khimicheskogo obshesrva Vol.23, 492.

[16] Dodds EC, Lawson W., 1936. Synthetic oestrogenic agents without the phenanthrene nucleus. Nature 137: 996.

[17] Villavecchia V., Elgenmann G., Ubaldini I., "Nuovo Dizionario di merceologia e chimica applicata". Hoepli Editore, 1976.

[18] Goldblum, K.B., Goldbrum's personal account of working with Dr. Fox. Accessed online: 31 July 2007.

[19] Environmental Protection Agency – EPA; www.epa.gov (2008)

[20] W.C. Hinds, Wiley Interscience Pubblication – Aerosol Technology, Properties, Behaviour and Measurement of Airborne Particles (1982).

[21] P.C. Reist, MacMillan New York – Introduction to Aerosol Science.

[22] M. Sillampää, Finnish Meteorological Institute, Helsinki (2006).

[23] F. Raes, E. Vignati, J. Wilson, L, Seinfeld, P, Adams, Atmospheric Environment, 33: 2715-2724 (2000).

[24] J. Heintzenberg, Tellus, 41B:149-160 (1989).

[25] Purves, W.K.; Sadava, D.; Orians, G.H.; Heller, H.C. Biologia- la biologia delle piante 2001 Zanichelli.

[26] Campbell, N.A. Biologia 1995 Zanichelli.

[27] Taiz, L.; Zeiger, E. Fisiologia vegetale 1998 Piccin.

[28] Lewis, N.G.; Davin, L.B.; Sarkanen, S. Lignin and Lignan Biosynthesis Symposium Series 697 1998, ACS,1-28, American Chemical Society.

[29] Davin, L.B.; Wang, H.B.; Crowell, A.L.; Bedgar, D.L.; Martin, D.M.; Sarkanen, S.; Lewis, L.G. Science 1997, 275, 362-366.

[30] Lewis, N.G.; Davin, L.B. Part.1 Poliphènols Actualitès 2000, 20, 18-25.

[31] Ralph, J.; Peng, J.P.; Lu, F.C.; Hatfield, R.D.; Helm, R.F. J. Agric. Food Chem. 1999, 47, 2991-2996.

[32] Meyermans, H. et al. J. Biol. Chem. 2000, 275, 36899-36909.

[33] Blanchette, A.R.; Crueler, W.E.; Height, D.E.; Akhtar, M.; Akin, D.E. J. Biotechnol. 1997, 53, 203-213.

[34] Eglinton, T.I.; Goni, M.A.; Boon, J.J.; Van der Hage, E.R.E.; Terashima, N.; Xie, Y. Holzforschung 2000, 54, 39-54.

[35] Doree, C.; Cunningham, M. J. Chem.Soc. 1913, 103, 677-686.

[36] Matsumoto, Y.; Ishuzu, A.; Nakano, J. Holzforschung 1986, 40, 81-85.

[37] W.F. Rogge, L.M. Hildemann, M.A. Mazurek, G.R. Cass, B.R.T. Simoneit, Environmental Science and Technology, 27: 2700-2711 (1993).

[38] G.P. Moss, IUPAC Nomenclature for fused-ring systems (2008).

[39] G. Portella, J. Poater, M. Solà, J, of Physical organic chemistry, 18: 785-791 (2005).

[40] H. Hosoya, K Hosoi, I. Gutman, Theor. Chim. Acta, 38:33-47 (1975).

[41] E. Clar – Academic Press: London (1964).

[42] E. Clar, Wiley: New York (1972).

[43] M. Randic, Chem. Rev., 103: 3449-3605 (2003).

[44] Y.J. Ruiz Moralez, Phys. Chem. A, 108:10873-10896 (2004).

[45] J.J. Aihara, Chem. Soc., Perkin Trans. 2: 2185-2195 (1996).

[46] M.M.C. Ferreira, Chemosphere, 44: 125-146 (2001).

[47] A.M. Sehili, G. Lammel. Atmospheric Environment 41, 8301 (2007).

[48] R. M. Kamens, H. Karam, Z. Guo, J. Perry, L. Stockburger, Environ. Sci. Technol., 23: 801-806 (1989).

[49] R.M. Kamens, Z. Guo, J.N. Fulcher, D.A. Bell, Environ. Sci. Technol., 22: 103-108 (1988).

[50] T. Spitzer, T. Takeuchi, J. of Chromatography A, 710: 109-116 (1995).

[51] J.Oda, I.Maeda, T. Mori, Y. Yashuara, Y, Saito, Environ. Technology, 19: 961-976 (1998).

[52] P.M. Fine, G.R. Cass, B.R. Simoneit, Environ. Sci Technol, 35: 2665-2675 (2001).

[53] NO. A. Kwamena, J.A. Thornton, J.P.D. Abbat, J. Phys Chem. A, 108: 11626-11634 (2004).

[54] P.S. Bailey, Academic Press: Toronto, 1982.

[55] E.J. Moriconi, B.Rakoczy, W.F. O'Connor, The J. Of Organic Chemistry, 83: 4618-4623 (1961).

[56] K.A. Van Cauwenberghe, Dekker, New York, 1985, pp 351-384.

[57] National Cancer Institute, Division of Cancer Biology, http://dcb.nci.nih.gov (2008).

[58] J.T. Barbas, M. E. Sigman, R. Dabestani, Environ. Sci. Technol. 30: 1776-1780 (1996).

[59]F. Marino, A. Cecinato, P,A, Siskos, Chemosphere, 40 (2000),

[60] T. Ramdahl, T. Nielsen, B. Seitz, Atmos. Environ. ISSN 004-6981 CODEN ATENBP.

[61] J. Sasaki, S.M. Ascmann, E.S.C. Kwok, R. Atkinson, J. Arey. Environ. Sci. Technol., 31: 3173-3179 (1997).

[62] J-H Kang, F, Kondo, Y, Katayama. Human exposure to bisphenol A. Toxicology, 2006, 226: 79-89.

[63] V. Wade, S. C. Nagel, F. S. vom Saal. Large Effects from Small Exposures. III. Endocrine Mechanisms Mediating Effects of Bisphenol A at Levels of Human Exposure. Endocrinology 2006, 147 (6): 56-69.

[64] Liu, X., Matsushima, A., Okada, H., Tokunaga, T., Isozaki, K., and Shimohigashi, Y., Receptor binding characteristics of endocrine disruptor bisphenol A: Chief and corroborative hydrogen bonds of bisphenol A phenol-hydroxyl group with Arg316 and Glu275 residues in the human nuclear receptor of estrogen-related receptor γ (ERRγ),*FEBS Journal* ,274, 6340-6351,2007.09.

[65] H. H. Le, E. M. Calson, J.P. Chua, S.M. Belcher. Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons. Toxicol Lett. 2008 30;176(2):149-56.

[66] A.M. Calafat, Z. Kuklenyik, J.A. Reidy, S.P. Caudill, J. Ekong, L.L. Needham. Urinary Concentrations of Bisphenol A and 4-Nonylphenol in a Human Reference Population. Environ Health Perspect. 2005; 113(4): 391–395.

[67] Environ International Corporation. 2003. Tier 1 Assessment of the Potential Health Risk to Children Associated with Exposure to Commercial Pentabromodiphenyl Ether Product. Prepareted for Great Lakes Chemical Corporation as sponsor to EPA's Voluntary Children's Chemical Exposure Program.

[68] Lang, I., A., Galloway, T., S., Scarlett, A., Henley, W., E., Depledge, M., Wallace, R., B., Melzer, D., Association of Urinary Bisphenol A Concentration With Medical Disorders and Laboratory Abnormalities in Adults, JAMA, Vol. 300, no. 11, 2008, pp. 1303-1310.

[69] Takeuchi T., Tsutsumi O., Ikezuki Y., Takai Y., Taketani Y., Positive relationship between androgen and the endocrine disruptor, bisphenol a, in normal women and women with ovarian dysfunction Endocrine journal 2004, vol. 51, n°2, pp. 165-169.

[70] European Food Safety Autority (EFSA) Scientific Documents Opinion of the Scientific Panel on food additives flavourings, processing aids and materials in contact with food (AFC) related to 2,2-BIS(4-HYDROXYPHENYL)PROPANE. 29 January 2007.

[71] Integrated Risk Information System. 12/03/2002 I.A.6 Screening-Level Literature Review Findings message has been added. http://www.epa.gov/iris/subst/0356.htm.

[72] NTP Finalizes Report on Bisphenol A. U.S. Department of Health and Human Services - National Institute of Environmental Health Sciences (NIEHS). September 03, 2008.

[73] Health Canada Media Relations (613) 957-2983. Environment Canada Media Relations (819) 934-8008 1-888-908-8008 News Release 2008-167 http://www.chemicalsubstanceschimiques.gc.ca/challenge-defi/batch-lot-2/bisphenol-a/index-eng.php.

[74] 111th U.S. Congress 1st session, "To ban the use of bisphenol A in food containers, and for other purposes." March 13, 2009.

[75] Hugo, E., R., Brandebourg, T., D., Woo, J., G., Loftus, J., Alexander, J., W., Ben-Jonathan, N., Bisphenol A at environmentally relevant doses inhibits adipopectin release from human adipose tissue explants and adipocytes, Environ. Health Perspect., Vol. 116, no 12, 2008, pp. 1642-1647.

[76] Prins, G., S., Birch, L., Tang, W. Y., Ho, S., M., Developmental estrogen exposure predispose to prostate carcinogenesis with aging, Reprod. Toxicol., Vol. 23, no. 3, 2007, pp. 374-382.

[77] Dekant Wolfgang; Völkel Wolfgang. Human exposure to bisphenol A by biomonitoring: methods, results and assessment of environmental exposures. Toxicology and applied pharmacology 2008;228(1):114-34.

[78] Rudel RA, Camann DE, Spengler JD, Korn LR, Brody JG. Phatalates, alkylphenols, pesticides, polybrominated diphenyl ethers and other endocrine-disrupting compounds in indoor air and dust. Environ. Sci. Technol. 2003, 38: 2531-2537.

[79] Rudel RA, Brody JG, Spengler JD, Vallarino J, Geno PW, Sun G. Identification of Selected Hormonally active agents and animal mammary carcirogenesis in commercial and residential air and dust samples. J. Air Waste Manage Assoc. 2001 51(10):1386-90.

[80] Matsumoto, H., Adachi, S., Suzuki, Y., Bisphenol A in ambient air particulates responsible for the proliferation of MCF-7 human breast cancer cells and its concentration changes over 6 months, Arch. Environ. Contam. Toxicol., Vol. 48, no. 4, 2005, pp. 459-466.

[81] M. Radzi Bin Abas, B.R.T. Simoneit, Atmospheric Environment 30, 2779 (1996).

[82] M.L. Magnuson, C.A. Kelty, C.M. Sharpless, K.G. Linden, W. Fromme, D.H. Metz, R. Kashinkunti, Environ. Sci. Technol. 36, 5252 (2002).

[83] A. Cappiello, E. De Simoni, C. Fiorucci, F. Mangani, P. Palma, H. Trufelli, S. Decesari, M.C. Faccini, S. Fuzzi, Environ. Sci. Technol. 37, 1229 (2003).

[84] C.D. Simpson, M. Paulsen, R.L. Dills, L.J.S. Liu, D.A. Kalman, Environ. Sci. Technol., 39, 631 (2005).

[85] C. Crestini, M. Orlandi, E-L Tolppa, L. Zoia, R. Saladino. Characterisation of lignin by different spectroscopic and aromatic techniques. Sample preparations from the COST E41 Joint Analysis Effort on wood and fiber characterisation. Vienna 31-agosto-1 settembre 2006.

[86] M. P. Colombini, M. Orlandi, F. Modugno, E-L. Tolppa, M. Sardelli, L. Zoia,C. Crestini. Archaeological Wood Characterisation By Py/Gc/Ms, Gc/Ms, Nmr And Gpc Techniques. *Microch. J.* 2007, *85*, 164-173.

[87] L. Zoia, G. Sestetti, B. Rindone. Tesi di Dottorato presso l'Università di Milano Bicocca, «Processi di bioconversione di fenoli, polifenoli e fibre lignocellulosiche mediati da laccasi e perossidasi", Novembre 2007.

[88] L. Zoia, M. Orlandi, A. Salanti. Tesi di Dottorato presso l'Università di Milano Bicocca, «Caratterizzazione e consolidamento di legni archeologici", Anno Accademico 2005-2006.

[89] L. Zoia, Ma. Orlandi, E.-L. Tolppa, D.I. Donato, P. Agozzino, P. Guerra. "Polimerizzazione in situ per il consolidamento di materiali lignei attraverso tecniche ambientalmente compatibili" in La diagnostica e la conservazione dei manufatti lignei. Nardini Editore Firenze 2006 ISBN 88-44-4151-4.

[90] M. Girardin, M. Metche. Microdosage rapide des groupements alkoxyles par chromatographie en phase gazeuse. Application à la lignine. *Journal of Chromatography*, 1983, *264*, 155-158.

[91] Di Tullio, N. Spreti, F. De Angelis. Mass Spectrometry in the Biosynthetic and Structural Investigation of Lignins. *Mass Spectrometry Reviews*, 2004, 23, 87-126.

[92] D. V. Evtuguin, F. M. Amado. Application of Electrospray Ionization Mass Spectrometry to the Elucidation of the Primary Structure of Lignin. *Macromol. Biosci*. 2003, *3*, 339-343.

[93] Cappiello A.; Famiglini G.; Berloni A.; Palma P.; Mangani F., New liquid chromatography/electron ionization mass spectrometry methods in water analysis. Annali di chimica 2002;92(7-8):623-36.

[94] De Angelis F., Nicoletti R., Spreti N., Veri' F. A New in Vitro Model of Lignin Biosynthesis. Angewandete Chemie-International Edition. 1999; vol. 38 pp. 1283-1285.

[95] F. De Angelis, P. Fregonese, F. Verì, "Structural Investigation of Synthetic Lignins by Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry", Rapid Commun. Mass Spectrom., 10, 1304 (1996).

[96] Evtuguin D.V., Domigues P., Amado F.L., Pascoal Neto C., Ferrer Correia A.J. Electrospray Ionization Mass Spectrometry as a Tool for Lignins Molecular Weight and Structural Characterisation. Holzforshung. Vol. 53, Issue 5, pp. 525-528, 1999.

[97] Reistad, T., Maiussen, E., Fonnum, F., The effect of a Brominated Flame Retarant, Tetrabromobisphenol-A, on Free Radical Formation in Human Neutrophil Granulocytes: The Involvement of the MAP Kinase Pathway and Protein Kinase C, Toxicological Sciences, Vol.83, no. 1, 2005, pp. 89-100.

[98] Gagnaire, D.; Robert, D. Proceedings of the Proc. Eur. Conf. NMR Macromol. 1978, 517-519 Rome, Italy.

[99] M.G. Perrone. Semivolatile Organic Compounds in the Atmospheric Particle Matter of urban Area transport and deposition at high altitude alpine sites. November 2004.

[100] UNICHIM 179/0 (1999) Manual: “Linee guida per la validazione di metodi analitici nei laboratori chimici – criteri generali”,

[101] National Institute of Standards & Technology – Certificate of Analysis – Standard Reference Material 1649a: “Urban Dust”.

[102] Gambaro A., Zangrando R., Gabrielli P., Barbante C., Cescon P., “Direct Determination of Levoglucosan at the picogram per milliliter level in Antartic Ice by High-Performance Liquid Chromatography/Electrospray Ionization Triple Quadrupole Mass Spectrometry”. Anal. Chem. 2008 80: 1649-1655.

[103] Inoue K., Yoshida S., Nakayama S., Ito R., Okanouchi N., Nakazawa H., “Development of Stable Isotope Diluition Quantification Liquid Chromatography – Mass Spectrometry Method for Estimation of Exposure Levels of Bisphenol A, 4-tert-Octylphenol, 4-Nonylphenol, Tetrabromobisphenol A, and Pentachlorophenol in Indoor Air”. Arch. Environmental Contamination and Toxicology, 2006 51: 503-508.

[104] Kamuura, T.; Tajima, Y.; Nakahara, T. “Determination of bisphenol A in air by GC-MS”. J. Environ. Chem. Jpn. 1997, 7, 275-280.

[105] Petrovic, M.; Eljarrat, E.; Lopez de Alda, M. J.; Barcelo, D. “Recent advances in mass spectrometric analysis related to endocrine disrupting compounds in aquatic environment samples”. J. Chromatogr. A 2002, 974, 23-51.

[106] Decreto Legislativo 21 maggio 2004, n. 183 "Attuazione della direttiva 2002/3/CE relativa all'ozono nell'aria" pubblicato nella *Gazzetta Ufficiale* n. 171 del 23 luglio 2004 - Supplemento Ordinario n. 127.

[107] Li, H., Gao, Z., Kang, L., Zhang, H., Yang, K., Yu, K., Luo, X., Zhu, W., Chen, K., Shen, J., Wang, X., Jiang, H., TarFisDock: a web server for identifying drug targets with docking approach, Nucleic Acids Research, Vol. 34, 2006, pp. W219-224.

[108] Niwa, T., Fu Jimoto, M., Kishimoto, K., Yabusaki, Y., Ishibashi, F., Katagiri, M., Metabolism and Interaction of Bisphenol A in Human Hepatic Cytochrome P450 and Steroidogenic CYP17, Biological & Pharmaceutical Bulletin, Vol. 24, no 9, 2001, pp. 1064-1067.

[109] Johnson, G., E., Parry, E., M., Mechanistic investigations of low dose exposures to the genotoxic compounds bisphenol-A and rotenone, Mutation research, Vol. 651, no 1-2, 2008, pp. 56-63.

[110] Hanioka, N., Jinno, H., Tanaka-Kagawa, T., Nishimura, T., Masanori, A., Interaction of bisphenol A with rat hepatic cytochrome P450 enzymes, Chemosphere, Vol. 41, 2000, pp. 973-978.

[111] Bursulaya BD, Totrov M, Abagyan R, Brooks CL. 3rd (2003) Comparative study of several algorithms for flexible ligand docking. J Comput Aided Mol Des 17: 755-763.

[112] Giannini, G., Carloni, P., Lupieri, P., Approcci di "Docking" Molecolare alle Interazioni Biologiche Rilevanti per la Terapia di Cattura di Neutroni su Boro "BNCT" – Tesi di Laurea in Fisica – 2005-2006 Università degli Studi di Trieste.

[113] Feher, M., Williams, C.I., Effect of Imput Differences on the Results of Docking Calculations, J.Chem. Inf. Model., 2009, 49, 1704-1714.

[114] Banner, D.W., Hadvary, P. Crystallographic Analysis at 3.0 Å Resolution of the Binding to Human Thrombin of Four Active Site-directed Inhibitors, The Journal of Biological Chemistry, 1991, Vol.266, No 30, 20085-20093.

[115] Kamata, K., Kawamoto, H., Honma, T., Iwama, T., Kim, S-H., Structural basis for chemical inhibition of human blood coagualtion factor Xa, Proc. Natl. Acad. Sci., 1998, Vol.95, pp. 6630-6635.

[116] RCSB PDB Protein Data Bank http://www.ebi.ac.uk/pdbsum/ and http://www.pdb.org/pdb/home/home.do

[117] OpenEye Scientific Software http://www.eyesopen.com/science/index.html

[118] Morris, G.M., Huey, R., Olson, A.J., Using AutoDock for Ligand-Receptor Docking, Current Protocols in Bioinformatics, John Wiley & Sons, Inc., 2008, Unitt Number: UNIT 8.14.

[119] Morris, G.M., Goodsell, D.S., Halliday, R.S., Huey, R., Hart, W.E., Belew, R.K., Olson, A.J., Automated Docking Using a Lamarkinan Genetic Algorithm and Empirical Binding Free Energy Function, Journal of Computational Chemistry, 1998, Vol. 19, No. 14, 1639-1662.

[120] Brooijmans, N., Kuntz, I.D., Molecular Recognition and Docking Algorithms, Annu. Rev. Biophys. Struct., 2003, Vol.32, 335-373.

[121] Ewing, T.J.A., Kuntz I.D., Critical Evaluation of Search Algorithms for Automated Molecular Docking and Database Screening, J. Comput. Chem., 1997, Vol.18, No.9, pp. 1175-1189.