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Semi-empirical Approach on the Methanogenic Toxicity of Aromatic Compounds on the Biogas Production

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Authors' contributions

This work was carried out in collaboration among all authors. Authors BMN and NLB designed the study and wrote the protocol. Author DMM performed the statistical analysis. Authors BMN,GNB and PTM wrote the first draft of the manuscript. Authors JCKK, KNN and BMN managed the analyses of the study. Authors GNB, APM and KNN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

In this work, a semi-empirical approach correlating the values of methanogenic toxicity of 22 aromatic compounds was used. The IC_{50} exp, along with the various molecular properties of these compounds were determined using the DFT B3LYP/6-31G (d,p) method. While a conceptual approach of the FDT, was made in order to determine those, which are responsible of this methanogenic activity of the studied aromatic compounds. The Principal Component Analysis method was used in order to describe all the connections and information contained between these

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various variables (IC_{50}exp and molecular properties) of the aromatic compounds. The Hierarchical Cluster Analysis helped to classify the studied aromatic compounds in various classes defining the various types of methanogenic toxicity. The findings show that the electron withdrawing and lipophilic substituents made the aromatic ring more toxic than the electron donating and hydrophilic substituents. The aromatic compounds with -NO_2 and –Cl groups formed the classes of the most toxic with the bactericidal action ofstudied aromatic compounds, with values of IC_{50}exp ranging between 4.19 ± 0.01 and 67.20 ± 1.97 mg/L. The compounds with -OH and -NH_2 groups formed the class of the least toxic of studied aromatic compounds with bacteriostatic action with values of IC_{50}exp ranging between 966.27 ± 7.04 and 3151.49 ± 5.93 mg/L. The benzene (aromatic ring unsubstituted) taken as reference, formed its own class with a value of IC_{50}exp of 208.78 ± 2.80 mg/L. It thus marks the line of demarcation between the classes of studied aromatic compounds with the electron withdrawing and/or lipophilic substituents (-NO_2 and -Cl), more toxic with the bactericide action, and that of the aromatic compounds with electron donating and hydrophilic substituents (-OH and -NH_2), less toxic with the bacteriostatic action.

Keywords: Methanogenic toxicity; semi-empirical approach, methanogenic Archaea; aromatic compounds; Coenzyme M, Biogas production.

1. INTRODUCTION

Methanization is one of the most efficient and less expensive technologies for the production of renewable energy (biomethane) and the treatment of organic waste [1-3]. It currently constitutes one of the elements of response to various problems faced by developing countries in terms of security and diversification of energy supply but also in the fight against environmental pollution. However, several parameters, namely : temperature, pH and potential of the environment, the tightness, cost and permanent monitoring of the digester, the nature of the substrate and the presence of inhibiting substances in the substrate to be biomethanized, limit the development of this technology, by blocking the biological activity of the archaeous plants [3-5]. Of all these parameters, the presence of inhibiting compounds, especially aromatic compounds, is currently one of the main causes of malfunction of this technology, as these compounds are naturally present in most organic waste and industrial effluents [6-9,10].

Our previous studies on methanogenic toxicity (MT) [6-7] have shown that the lipophilic character of the aromatic compound (quantified by the Log Poct value) as well as its electron density (influenced by the electro attracting or electrodonating character of its substituents) are at the basis of this toxicity. Since, the cell membranes of these methanogenic archae are nothing but lipoproteins (lipids), and on the other hand, the modification of the electron density on the aromatic ring considerably affects its reactivity towards the coenzyme M. In addition to their surfactant effects on cell membranes, thus modifying the cell diffusion and surface tension, aromatic compounds are also responsible for the disruption of certain interactions, in particular that between coenzyme M and the acetate substrate, at the expense of the formation of a complex by donor-acceptor charge transfer between coenzyme M and aromatic toxins [6-7]. This interaction probably leads to the blocking of the activity of coenzyme M on the acetate substrate. Nevertheless, the steric effects of the substituents on the aromatic ring may sometimes cause some restriction of the MT of the aromatic ring [7,11]. A good correlation between the structures of these aromatic compounds and their inhibitory effects on the bioactivity of acetoclastic methanogenic archaea. The electro-attractor or electro-donor character, the lipophilic or hydrophilic nature, the number and position of the substituents linked to the aromatic ring had a considerable influence in one way or another on the methanogenic toxicity of the aromatic compounds.

In the current work, a semi-empirical approach was considered, correlating the MT of 22 aromatic compounds experimentally measured (IC_{50} exp.) with their molecular properties, determined using the DFT B3LYP/6-31G (d, p) method [12] and a conceptual approach to Functional Density Theory (FDT) [13,14], which could be at the basis of this toxicity. To understand the main factors underlying this MT of aromatic compounds would lead us to circumvent this problem. To do this, we have used the Principal Component Analysis (PCA) method [15-17], which allows us to explore and describe all the links and information contained between these different variables observed on aromatic compounds (molecular properties and IC_{50} exp.). This in order to highlight the various

influences existing between these different variables. But also we used the method of Hierarchical cluster analysis [18,19], allowing to obtain a simple schematic representation of a partition of different aromatic compounds studied in different defined classes starting from various observations of the aforementioned variables. These different classes define different types of MTof these studied aromatic compounds.

2. MATERIALS AND METHODS

2.1 Biomass

Pig manure from Domaine Agro-Industriel de la N'sele (DAIPN)/ Kinshasa in the Democratic Republic of the Congo (DRC) was digested in the digester at the laboratory for six months. The digested pig manure, not previously acclimated to any aromatic compounds, was utilized as inoculums in anaerobic toxicity tests. The characteristics of inoculums were: total suspended solids (TSS, 94.20 g/L), volatile suspended matter (VSM, 58.18 g/L), and specific acetoslactic methanogenic activity(AMA): 159.74-205.83 mgCOD-CH_4/gs VSS.

2.2 Preparation of Stock Solutions

2.2.1 Substrate stock solution

The stock solution for the substrate consisted of a solution of sodium acetate (pH=7) obtained by reacting acetic acid with NaOH. A concentration of 100 g COD-CH_3COOH/L (chemical oxygen demand per liter) was used in this study.

2.2.2 Stock solution 1

The macronutrient compounds used are the following: NH_4Cl (170 g/L), KH_2PO_4 (37 g/L), $MgSO_4.4H_2O$ (37 g/L) and $CaCl_2.2H_2O$ (10 g/L) [4,20,21].

2.2.3 Stock solution 2

The precursor compounds of trace elements were: $FeCl_3.4H_2O$ (200 mg/L), $CoCl_2.6H_2O$ (2000 mg/L), $MnCl_2.4H_2O$ (50 mg/L), $CuCl_2.2H_2O$ (50 mg/L), $ZnCl_2$ (50 mg/L), H_3BO_3 (50 mg/L), $(NH_4)_3 Mo_7O_2.4H_2O$ (90 mg/L), Na_2SeO_3. $5H_2O$ (100 mg/L), $NiCl_2$. $6H_2O$ (50 mg/L), yeast extract (200 mg/L) and resazurin (500 mg/L) [4,18,19].

2.3 Aromatic Compounds

Twenty-one aromatic compounds with both electron donating and electron withdrawing groups were used. These were Nitrobenzene, o-diNitrobenzene, m-diNitrobenzene, p-diNitrobenzene, Chlorobenzene, o-diChlorobenzene, m-diChlorobenzene, p-diChlorobenzene, Benzoic Acid, Acid 3,5-diNitrobenzoic, Acid 3,4-diNitrobenzoic, Phenol, Aniline, p-Nitrophenol, p-Nitroaniline, p-Aminophenol, Catechol, Resorcinol, Hydroquinone, Phloroglucinol and Pyrogallol. Benzene was used as reference for comparison purposes, making a total of twenty-two compounds studied. All aromatic compounds were pure for analysis products supplied by MERCK VWR (Leuven, Belgium).

2.4 Anaerobic Toxicity Assay [4,20,21]

Specific AMA measurements were performed with one liter of glass serum bottles sealed with rubber septa. These measurements were performed based on the following procedure:

Add to each serum bottle from the digester 1.5 g VSM of digested pig manure, 2 mL of stock solution 1, 1 mL of stock solution 2 and 40 mL of substrate stock solution. Then the serum bottle was filled to about 1000 mL with oxygen free tap water (tap water flushed with nitrogen gas for at least 15 minutes) [22]. After that process, the flask with rubber septum cap was sealed and the mixture was shaken for few minutes at the room temperature. The required quantity of test compounds (toxicants) was added to provide the concentrations to be investigated. No toxicant was added to the controls. The toxicant concentrations were chosen as to induce an inhibition (0-100%) of the acetoclastic methanogenic activity [3,20-23]. The concentrations of inhibitors used in the anaerobic toxicity assay are given in the Table 1. The specific methanogenic activity was calculated from the slope of the cumulative methane production versus time curve and the quantity of VSM. The compound concentration that caused 50% inhibition of the Methanogenic activities of the control and samples containing inhibitory compounds were determined [24].

2.5 Methane Gas Measurement

The volume of methane gas produced was measured by serum bottle liquid displacement systems (Mariotte flask system) as previously described [25]. The liquid used was a solution of NaOH (15 mg/L). As the biogas passes through these high pH solutions, the CO_2 of biogas is converted to carbonate and absorbed into the liquid. Only methane gas passes through the

Table 1. The inhibitory concentration of compounds used in anaerobic toxicity assay

N°	Aromatic compounds	Concentration (mg/L)				
		1	2	3	4	5
1	Benzene	0	150	300	450	600
2	Nitrobenzene	0	2	3	6	9
3	o-diNitrobenzene	0	1	2	6	9
4	m-diNitrobenzene	0	1	2	6	9
5	p-diNitrobenzene	0	1	2	6	9
6	Chlorobenzene	0	10	15	30	50
7	o-diChlorobenzene	0	10	30	50	70
8	m-diChlorobenzene	0	10	30	50	70
9	p-diChlorobenzene	0	10	30	50	70
10	p-Nitrophenol	0	15	20	40	60
11	p-Nitroaniline	0	15	20	40	60
12	Acid 3,5-diNitrobenzoic	0	15	25	50	80
13	Acid 3,4-diNitrobenzoic	0	15	25	50	80
14	Benzoic Acid	0	900	1500	2500	3500
15	Phenol	0	500	700	1500	3000
16	Aniline	0	500	700	1500	3000
17	p-Aminophenol	0	1000	2000	3000	4000
18	Catechol	0	1000	2500	3500	4500
19	Resorcinol	0	1000	2500	3500	4500
20	Hydroquinone	0	1000	2500	3500	4500
21	Pyragollol	0	1500	3500	4500	6000
22	Phloroglucinol	0	1500	3500	4500	6000

solution and an equivalent volume is pushed out of the mariotte flask. The volume of the displaced liquid is then measured in a graduated cylinder [6-7,21].

2.6 Determination of the Molecular Properties of Aromatic Compounds Studied

Complete optimization of all structures has been carried out using FDT. FDT calculations were carried out using the B3LYP functional [12] with 6-31 + G(d,p) basis set. The reactivity of all structures have been assessed by using a conceptual IDFT approach [13,14]. All the investigations were executed with Gaussian 03 package [26].

2.7 Exploration and Description of Different Linkages and Continuous Information between the Molecular Properties and the MT of the Studied Aromatic Compounds

The various links and information between the molecular properties and the MT of the studied aromatic compounds were identified and described using the principal component analysis (PCA) method [15-17]. This chemometric method consists of reducing the number of initial variables to one, two or at most three by transforming interrelated variables, called "correlated", into new variables that are decorrelated from each other, called "principal components". This is allowed in order to highlight a structure, not known a priori, on this set of variables, and it facilitates the perception and interpretation of all the information and links between these variables.

2.8 Ascending Hierarchical Classification of the Aromatic Compounds Studied

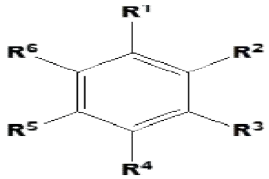
The HCA method [18,19] allowed to divide the aromatic compounds studied into different classes, defining their types of toxicity. The principle of the method is based on the distribution of n individuals in classes defined by the observation of p variables on these individuals, using a certain metric or norm (Euclidean distance, Pearson's dissimilarity) in order to determine the similarity or dissimilarity between the different objects (individuals).

3. RESULTS AND DISCUSSION

3.1 Inhibition of Specific Methanogenic Activity

The inhibitory effect of the test compounds and the reference (Benzene) on the activity of acetoclastic methanogenic archaea was studied

Table 2. IC_{50} exp. values, partition coefficient and electronic parameters of tested compounds



N°	R (Substituents)	Compound	IC_{50} exp. (mg/L)	σ	ρ	Log $P_{oct.}$
1	R^1= R^2= R^3= R^4= R^5= R^6= H	Benzene	208,78 ± 2,80	0	0	2.13
2	R^1=NO_2, R^2= R^3= R^4= R^5= R^6= H	Nitrobenzene	4.19± 0.01	0.60	0	1.85
3	R^1= R^3=NO_2, R^2 = R^4= R^5= R^6= H	m-diNitrobenzene	5.44 ± 0.02	0.60	0	1.49
4	R^1= R^4=NO_2, R^2 = R^3= R^5= R^6= H	p-diNitrobenzene	5.60 ± 0.07	0.60	0	1.37
5	R^1= R^2=NO_2, R^3 = R^4= R^5= R^6= H	o-diNitrobenzene	6.28 ± 0.04	0.60	0	1.69
6	R^1=Cl, R^2= R^3= R^4= R^5= R^6= H	Chlorobenzene	32.25 ± 0.23	0.28	0	3.35
7	R^1= R^3=Cl, R^2 = R^4= R^5= R^6= H	m-diChlorobenzene	41.22 ± 1.39	0.28	0	3.53
8	R^1= R^4=Cl, R^2 = R^3= R^5= R^6= H	p-diChlorobenzene	45.05 ± 1.69	0.28	0	3.40
9	R^1= R^2=Cl, R^3 = R^4= R^5= R^6= H	o-diChlorobenzene	47.42 ± 1.03	0.28	0	3.43
10	R^1= NO_2, R^4=OH, R^2 = R^3= R^5= R^6= H	p-Nitrophenol	40.82 ± 0.22	-NO_2=0.60	-OH=1.06	1.91
11	R^1= NO_2, R^4=NH_2, R^2 = R^3= R^5= R^6= H	p-Nitroaniline	44,74 ± 2,54	-NO_2= 0.60	-NH_2= 1.08	1.27
12	R^1= COOH, R^3= R^5=NO_2, R^2 = R^4=R^6= H	3,5-diNitrobenzoic Acid	63.17 ± 1.51	-NO_2= 0.60 -COOH=0.32	-COOH= 0.35	1.75
13	R^1= COOH, R^3= R^4=NO_2, R^2 = R^5=R^6= H	3,4-diNitrobenzoic Acid	67.20 ± 1.97	-NO_2= 0.60 -COOH= 0.32	-COOH= 0.35	1.71
14	R^1=OH, R^2= R^3= R^4= R^5= R^6= H	Phenol	966.27 ± 7.04	0	-OH= 1.06	1.47
15	R^1=NH_2, R^2= R^3= R^4= R^5= R^6= H	Aniline	1038.74 ± 199.38	0	-NH_2= 1.08 -OH= 1.06	0.90
16	R^1= OH, R^4=NH_2, R^2 = R^3= R^5= R^6= H	p-Aminophenol	1721.35 ± 116.60	0	-NH_2= 1.08	0.10
17	R^1= R^2=OH, R^3 = R^4= R^5= R^6= H	Catechol	1436.28 ± 18.02	0	-OH= 1.06	0.88
18	R^1= R^3=OH, R^2 = R^4= R^5= R^6= H	Resorcinol	2014.90 ± 187.91	0	-OH= 1.06	0.80
19	R^1= R^4=OH, R^2 = R^3= R^5= R^6= H	Hydroquinone	2760.01 ±159.66	0	-OH= 1.06	0.59
20	R^1=COOH, R^2= R^3= R^4= R^5= R^6= H	Benzoic Acid	2515.20± 35.21	-COOH= 0.32	-COOH= 0.35	1.87
21	R^1=R^2= R^3= OH, R^4= R^5= R^6= H	Pyrogallol	2826.55 ± 8.41	0	-OH= 1.06	0.97
22	R^1=R^3= R^5= OH, R^2= R^4= R^6= H	Phloroglucinol	3151.49± 5.93	0	-OH= 1.06	0.16

σ and ρ are Hammett parameters representing the inductive strength (withdrawing power) and the sensitivity of donor, respectively

at different concentrations ranging from 1-6000 mg/L. This is exemplified by the experiment with Chlorobenzene as shown in Fig. 2, from which the IC_{50}exp. was calculated as the concentration of Chlorobenzene corresponding to 50% of inhibition.

The method used to calculate the IC_{50} exp.which isillustrated in the Fig. 1 was extended to all test compounds.

Table 2 summarizes the inhibitory concentrations (IC_{50} exp.), the partition coefficient [27] and electronic parameters [28] of tested compounds.

Based on the interpretation of these experimental results, we note that organic compounds behave as inhibitors of the biomethanization process because they all have relatively low IC_{50} exp. values. However, this toxicity depends on the nature of the aromatic compounds, i.e. on the structural effects of the bound substituents on the benzene ring [6-7]. However, the substituents on the benzene ring, depending on their hydrophobic or hydrophilic nature made the latter more lipophilic or hydrophilic [6-7,29]. This hydrophobic/hydrophilic property of the aromatic molecule, quantified by its LogPoct value, was for this purpose, the most decisive molecular property of the MT of aromatic compounds (benzene ring). This is because aromatic compounds behave like surfactants towards the methanogenic archaea and their ability to cross the lipoprotein membranes of the methanogenic archaea depends on this property [6,29,30]. In addition to the above-mentioned phenomena, aromatic compounds were also responsible for blocking the activity of coenzyme M on the acetate substrate [6,7]. This was due to the formation of a charge transfer complex of the donor-acceptor type with coenzyme M.

Compared to benzene, taken as a reference (IC_{50} exp.= 208.78±2.80 mg/L), aromatic compounds with electron-donor substituents make the aromatic ring more toxic to methanogenic archaea and the opposite trend is observed for those with electron-donor substituents. In fact, the substitution of an -H (on the benzene ring) by an electron-withdrawing substituent (-NO_2, -Cl, -COOH, etc...) stabilizes the negative charge developed in the aromatic ring during its interaction with coenzyme M. As a consequence, the complex formed will be stable and will therefore block the activity of coenzyme M on the acetate substrate, which leads to the production of biomethane [7]. Thus, the electro-attractive nature of the substituent remains one of the most important parameters determining the extent of methanogenic toxicity of the aromatic ring. This justifies the greater toxicity of aromatic rings with nitro-NO_2 substituents (IC_{50} exp. in the order of 4 mg/L) than those with chloro-Cl substituents (IC_{50}exp. in the order of 32 mg/L). This is due to the marked electron-withdrawing character of the -NO_2 group (σ = 0.60) compared to the -Cl substituent (σ = 0.28), despite the high lipophilic character of the chloro group ($\boldsymbol{\pi}$ = 0.71 for -Cl and $\boldsymbol{\pi}$ = -0.28 for -NO_2). In addition, the nitro group is highly reactive in nature, so it reacts with the cellular constituents of the methanogenic archaea, which further increases its toxicity.

For aromatic compounds with electron-donating substituents (-OH, -NH_2,...), the presence of the latter increases the electron density on the benzene ring and consequently destabilizes the complex formed between the coenzyme M and the aromatic ring. This is related to the electron-donor strength of each substituent and its hydrophilicity (benzene: IC_{50} exp.= 208.78 ± 2.80 mg/L; phenol: IC_{50} exp.= 966.27 ± 7.04 mg/L, $\boldsymbol{\rho}$ = 1.06 and $\boldsymbol{\pi}$ = -0.67 for the -OH substituent; and aniline: IC_{50}exp.= 1038.74 ± 199.38 mg/L, $\boldsymbol{\rho}$ = 1.08 and $\boldsymbol{\pi}$ = -1.23 for the -NH_2 substituent). However, benzoic acid (IC_{50}exp.= 2515.20 ± 35.21 mg/L, σ = 0.32 and $\boldsymbol{\pi}$ = -0.32 for the -COOH substituent) was found to be less toxic than benzene, despite the presence of the acidic (-COOH) electron-withdrawing group on the benzene ring, thus making an exception. This is justified by the strong tendency of this acid group to dissociate in solution (-COOH ⇋ -COO- + H+), which makes the benzene ring less lipophilic than in the case of benzene (benzoic acid LogPoct = 1.87 and benzene LogPoct = 2.13). And this less lipophilic character of the benzene nucleus increases however its difficulty to cross the cell membrane of methanogenic archaea.

By comparing the IC_{50}exp. values of aniline (IC_{50}exp.= 1038.74 ± 199.38 mg/L), phenol (IC_{50}exp.= 966.27 ± 7.04 mg/L) and para-aminophenol (IC_{50}exp.= 1721.35 ± 116.60 mg/L) to each other; those of phenol (966.27 ± 7.04 mg/L), catechol (IC_{50}exp.= 1436.28 ± 18.02 mg/L), resorcinol (IC_{50}exp.= 2014.90 ± 187.91 mg/L), hydroquinone (IC_{50}exp.= 2760.01 ± 159.66 mg/L), pyrogallol (IC_{50}exp.= 2826.55 ± 8.41 mg/L) and that of phloroglycinol (IC_{50}exp.= 3151.49 ± 5.93 mg/L) also with each other, and finally that of benzoic acid (IC_{50}exp.= 2515.20 ± 35.21 mg/L) with that of 3,5-dinitrobenzoic acid (IC_{50}exp.= 63.17 ± 9.51 mg/L) and 3,4-

dinitrobenzoic acid (IC_{50}exp.= 67.20 ± 1.97 mg/L). We highlight the additivity of the effects of the substituents on the aromatic ring. This, on the one hand, as a result of the increase in the IC_{50}exp. values of the aromatic compounds corresponding to the increase in the number of electron-withdrawing substituents and, on the other hand, as a result of the decrease in the IC_{50}exp. values of the aromatic compounds corresponding to this increase in the number of electron-withdrawing substituents. For aniline (IC_{50}exp.= 1038.74 ± 199.38 mg/L) and phenol (IC_{50}exp.= 966.27 ± 7.04 mg/L) compared to para-nitroaniline (IC_{50}exp.= 44.74 ± 2.54 mg/L) and para-nitrophenol (IC_{50}exp.= 40.82 ± 0.22 mg/L) We note that the electro-attracting effect of the nitro group (-NO_2) decreases the hydrogen bond basicity (HBB) of the -OH and (-NH_2) substituents by decreasing the electron density on these two heteroatoms. As a result, their ability to form intermolecular hydrogen bonds isreduced, which also reduces their hydrophilicity. For this purpose, the Nitro group acts as an inducer and the -OH and (-NH_2) groups as responders (donors) [28]. Since their effects (inducer and responder) are in opposition, the hydrophilic and electro-donor characters of the substituents (-NH_2) and -OH are thus diminished when we switch from phenol or aniline to para-nitrophenol or para-nitroaniline, respectively, which explains the increase in toxicity of the latter two compounds.

Referring to the different IC_{50}exp. values of the studied aromatic molecules (Table 2), we see that these IC_{50}exp. values vary according to the number and position of the substituents according to the following sequences:

1) For phenolic compounds: phenol > catechol (1,2-dihydroxybenzene) > resorcinol (1,3-dihydroxybenzene) > hydroquinone (1,4-dihydroxybenzene) > pyrogallol (1,2,3-trihydroxybenzene) > phloroglucinol (1,3,5-trihydroxybenzene). In this case, we show the decrease in the MT of the aromatic ring following the increase in the number of hydroxyl groups. For having the electron-donating and hydrophilic characters [26], an additional hydroxyl substituent on the aromatic ring will increase its electron density and decrease its lipophilic character. As a consequence, it weakens its interaction with coenzyme M, and in addition, it increases its difficulty to cross the lipidic cell membranes of methanogenic archae. However, by comparing the toxicity of catechol with that of resorcinol and hydroquinone, we see that due to its ability to form the intramolecular hydrogen bond in the case of catechol (ortho-dihydroxybenzene, IC50exp.= 1436.28 ± 18.02 mg/L and LogPoct = 0.88), the aromatic nucleus is more lipophilic and less bulky than in the case of resorcinol (meta-dihydroxybenzene, IC_{50}exp.= 2014.90 ± 187.91 mg/L and LogPoct = 0.80) and hydroquinone (para-dihydroxybenzene, IC_{50}exp.= 2760.01 ± 159.66 mg/L and LogPoct = 0.59) and therefore more toxic than these two isomers.

(2) For compounds with nitro substituents: nitrobenzene (IC_{50}exp.= 4.19 ± 0.01 mg/L) > 1,3-dinitrobenzene (IC_{50}exp.= 5.44 ± 0.02 mg/L) > 1,4-dinitrobenzene (IC_{50}exp.= 5.60 ± 0.07 mg/L) > 1,2-dinitrobenzene (IC_{50}exp.= 6.28 ± 0.04 mg/L). We note that m-, o- and p-dinitrobenzene were found to be less toxic than nitrobenzene, despite the presence of an additional second electron-withdrawing nitro group on the aromatic ring. This is justified by the very pronounced steric constraints on the aromatic ring due to the presence of this second bulky nitro group. The latter increases the difficulty of the aromatic ring through the cell membranes of methanogenic archaeas. However, m-dinitrobenzene has been shown to be more toxic than p-dinitrobenzene, which in turn is more toxic than o-dinitrobenzene, due to the strong steric constraints in the latter isomer, which has two large nitro groups in the ortho position. The same trend is also observed for chloro-substituted compounds, where methanogenic toxicity varies according to the following sequence: chlorobenzene (IC_{50}exp.= 32.25 ± 0.23 mg/L) > 1,3-dichlorobenzene (IC_{50}exp.= 41.22 ± 1.39 mg/L) > 1,4-dichlorobenzene (IC_{50}exp.= 45.05 ± 1.69 mg/L) > 1,2-dichlorobenzene (IC_{50}exp.= 47.42 ± 1.03 mg/L).

(3) For benzoic acid (IC_{50}exp.= 2515.20 ± 35.21 mg/L), 3,5-dinitrobenzoic acid (IC_{50}exp. = 63.17 ± 1.51 mg/L) and 3,4-dinitrobenzoic acid (IC_{50}exp. = 63.17 ± 1.51 mg/L).= 67.20 ± 1.97 mg/L), we show a considerable increase in the MT of the aromatic ring following the substitution of the two -H with two highly electro-attracting and reactive -NO_2 groups. However, 3,5-dinitrobenzoic acid was found to be more toxic than 3,4-dinitrobenzoic acid due to the very marked steric constraints in the latter compound.

From these experimental results on the MT of aromatic compounds, we draw the following conclusion: The nature, number and position of the substituents on the aromatic ring have a considerable influence on its MT. Because the electroattractor or electrodonor and lipophilic or

hydrophilic characters of the substituents on the aromatic ring have been shown to be parameters influencing this toxicity. However, steric constraints impose restrictions on this methanogenic toxicity of aromatic compounds to some extent.

3.2 Molecular Properties of the Aromatic Compounds Studied

Fig. 2 and Table 3 report respectively the optimized structures and molecular property values of the investigated aromatic compounds, evaluated using the FDT B3LYP/6-31 G (d,p) method.

3.3 Influences of Molecular Properties of Aromatic Compounds on their Methanogenic Toxicity

The PCA method allowed us to explore and describe with the help of a graph (component diagram) all the bonds and especially all the information contained between different variables (molecular properties and MT) observed on the studied aromatic compounds, with the least possible destruction. To achieve this, we first found the different correlations (Pearson correlation) [31,32] existing between these variables, and this allowed us to reduce them into different groups representative of each variable.

Table 4 reports the different values of correlations (Pearson correlation) between these different variables observed on the studied aromatic compounds.

From this Table 4, it can be seen that the variables v1, v2, v4 and v6 of the aromatic compounds, respectively their HOMO energy, LUMO energy, electronegativity and overall electrophilicity index are highly correlated with each other (with correlation coefficients of the order of 0.902 to 0.995). And these variables also showed a good correlation with the IC_{50}exp: a correlation coefficient of 0.627 with the variable v1; of 0.565 with the variable v2; of 0.607 with the variable v4; and finally, a correlation coefficient of 0.568 with the variable v6. In the light of this Pearson correlation between these different variables (v1, v2, v4, v6 and IC_{50}exp.), we are already highlighting the possible influences that these molecular (electronic) properties may have on the MT of aromatic compounds, as demonstrated by our previous experiments on the inhibition of the methanogenic activity of archaea by aromatic compounds [6,7]. Following the example of the above molecular properties, the variable v8 (water/n-octanol partition constant) showed only a good correlation, negative R= -0.651, with the IC_{50}exp. Furthermore, we notice from this table that there is a very strong negative correlation (R= -0.991) between the variables v3 (hardness) and v5 (softness). And in turn, these two variables are correlated with the variable v7 (dipole moment) with correlation coefficient values of 0.688 and - 0.703 with v3 and v5 respectively.

These found correlation values highlight the different influences that can exist between these different molecular properties, between them, and especially with the MT of these aromatic compounds. However, this may or may not be significant, i.e. in reality, there may or may not be a causal relationship between these different molecular properties of aromatic compounds and their MT [10]. Therefore, we have used the PCA method to explore and describe by means of a graph all the links and especially all the information contained between these different variables.

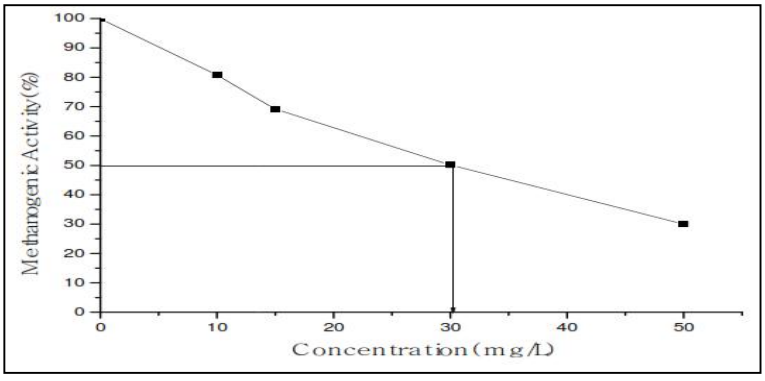


Fig. 1. Methanogenic activity of digested pig manure exposed to Chlorobenzene as a function of Chlorobenzene concentration

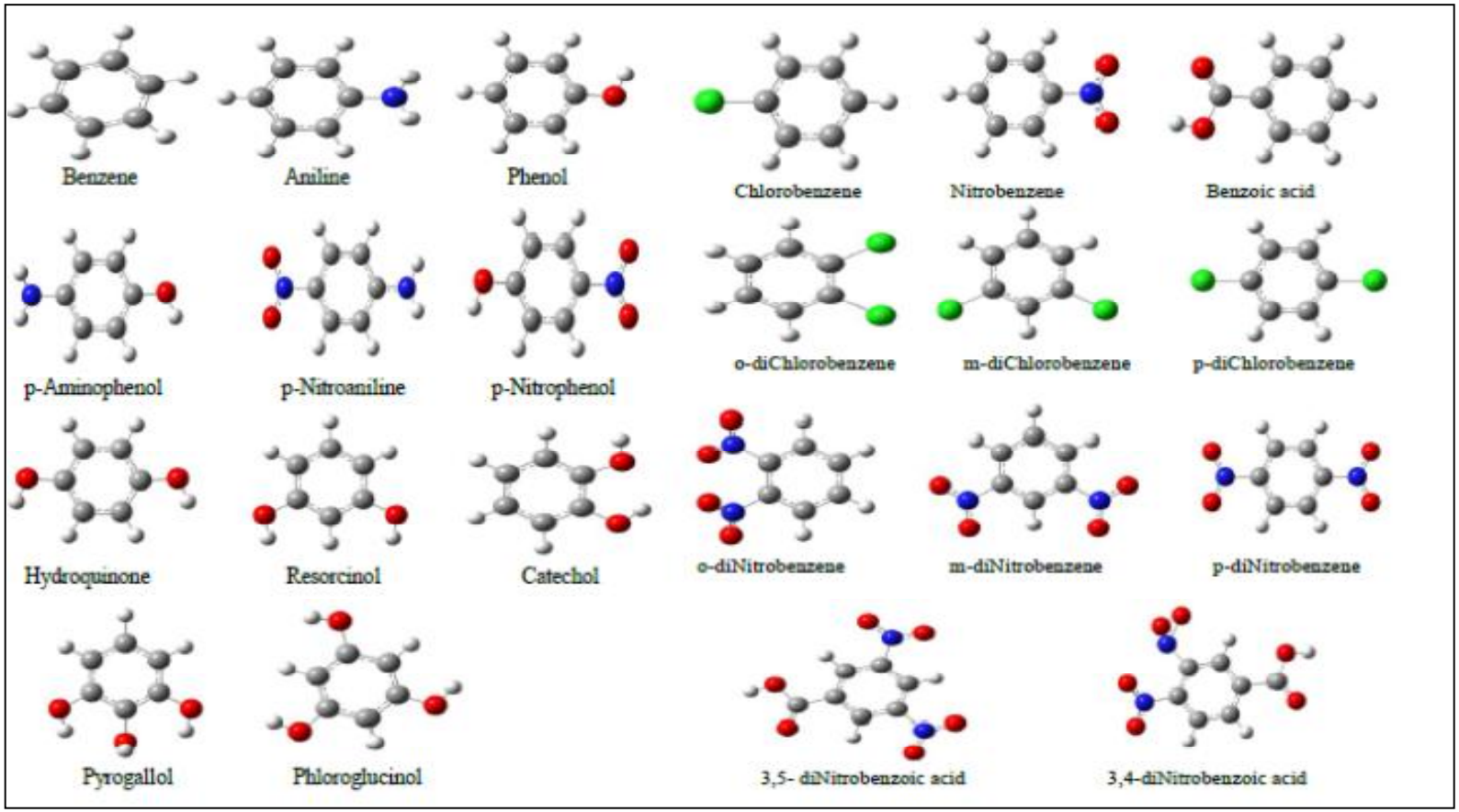


Fig. 2. Optimized structures of the studied molecules

Table 3. Molecular property values and concentrations of different compounds aromatic compounds studied

Molecules	H (kcal/mol)	L (kcal/mol)	N	X	S	W	U (Debye)	K
Nitrobenzene	-182,1095	-67,3756833	-57,3669085	-124,742592	-0,01743165	-135,624479	4,9637	1,85
m-diNitrobenzene	-201,443065	-83,057143	-59,1929609	-142,250104	-0,0168939	-170,924817	4,5657	1,49
p-diNitrobenzene	-199,842916	-91,277516	-54,2826999	-145,560216	-0,01842208	-195,16141	0,0004	1,37
o-diNitrobenzene	-191,597442	-79,9321462	-55,8326481	-135,764794	-0,01791067	-165,065423	7,1492	1,69
Chlorobenzene	-160,3663	-18,511527	-70,9273865	-89,4389135	-0,01409893	-56,3909066	1,8821	3,35
p-Nitrophenol	-168,241543	-63,3031474	-52,4691977	-115,772345	-0,0190588	-127,724803	5,5978	1,91
m-diChlorobenzene	-164,539237	-26,0792903	-69,2299736	-95,3092639	-0,01444461	-65,6063791	1,7704	3,53
p-Nitroaniline	-151,788247	-56,864901	-47,4616728	-104,326574	-0,02106963	-114,661298	7,6206	1,27
p-diChlorobenzene	-160,309824	-26,7005246	-66,8046498	-93,5051744	-0,01496902	-65,4386907	0,0026	3,4
o-diChlorobenzene	-162,913988	-24,8619221	-69,026033	-93,8879551	-0,01448729	-63,8523447	2,695	3,43
3,5-diNitrobenzoic acid	-205,590902	-87,0104522	-59,2902248	-146,300677	-0,01686619	-180,500986	3,7932	1,75
3,4-diNitrobenzoic acid	-190,10397	-83,4838494	-53,3100603	-136,79391	-0,01875819	-175,506965	4,4143	1,71
Benzene	-161,658969	-9,72012047	-75,9694244	-85,6895449	-0,01316319	-48,3266667	0,0001	2,13
Phenol	-145,921034	-11,7344256	-67,0933041	-78,8277297	-0,01490462	-46,3072363	1,3949	1,47
Aniline	-131,783247	-6,95907915	-62,412084	-69,3711632	-0,01602254	-38,5530972	1,6331	0,9
Catechol	-139,413761	-9,01730995	-65,1982257	-74,2155357	-0,01533784	-42,2399971	2,5476	0,88
p-Aminophenol	-123,362071	-10,1719272	-56,595072	-66,7669992	-0,01766938	-39,383572	2,1517	0,104
Resorcinol	-141,164513	-9,33733974	-65,9135864	-75,2509262	-0,01517138	-42,9554982	2,2994	0,80
Benzoic acid	-170,877082	-41,1206904	-64,8781959	-105,998886	-0,0154135	-86,5912171	2,1749	1,87
Hydroquinone	-134,330935	-14,2946639	-60,0181357	-74,3127996	-0,01666163	-46,006029	2,7611	0,59
Pyrogallol	-143,442372	-19,6033934	-61,9194892	-81,5228825	-0,01615	-53,666305	1,9682	0,97
Phloroglucinol	-142,990565	-9,26203861	-66,8642632	-76,1263018	-0,01495567	-43,335659	2,7676	0,16

With H : HOMO Energy ; L : LUMO Energy ; N : Hardness; X : Electronegativity; S : Softness; W : Electrophile global index; U : Dipolar Moment ; K : Partition constant Water /n-Octano

Table 4. Correlation of Pearson (correlation 2 by 2) between different molecular proprieties and values of IC_{50}exp. of studied aromatic compounds

		V1	v2	v3	v4	v5	v6	v7	v8	v9	v10	v11	IC_{50}exp.
v1	Correlation of Pearson	1	,902**	-,283	,969**	,277	,912**	-,289	-,352	-912**	-,872**	-,908**	,627**
	Sig. (bilateral)		,000	,203	,000	,212	,000	,192	,108	,000	,000	,000	,002
	N	22	22	22	22	22	22	22	22	22	22	22	22
v2	Correlation of Pearson	,902**	1	-668**	,981**	,660**	,995**	-,533*	-,120	-995**	-,983**	-,995**	,565**
	Sig. (bilateral)	,000		,001	,000	,001	,000	,011	,594	,000	,000	,000	,006
	N	22	22	22	22	22	22	22	22	22	22	22	22
v3	Correlation of Pearson	-,283	-,668**	1	-,512*	-,991**	-,642**	,688**	-,340	,642**	,682**	,647**	-,177
	Sig. (bilateral)	,203	,001		,015	,000	,001	,000	,121	,001	,000	,001	,431
	N	22	22	22	22	22	22	22	22	22	22	22	22
v4	Correlation of Pearson	,969**	,981**	-,512*	1	,505*	,983**	-,437*	-,227	-983**	-,958**	-,981**	,607**
	Sig. (bilateral)	,000	,000	,015		,017	,000	,042	,310	,000	,000	,000	,003
	N	22	22	22	22	22	22	22	22	22	22	22	22
v5	Correlation of Pearson	,277	,660**	-991**	,505*	1	,633**	-703**	,302	-,633**	-,672**	-,638**	,227
	Sig. (bilateral)	,212	,001	,000	,017		,002	,000	,172	,002	,001	,001	,309
	N	22	22	22	22	22	22	22	22	22	22	22	22
v6	Correlation of Pearson	,912**	,995**	-642**	,983**	,633**	1	-,490*	-,094	-1,000	-,994**	-1,000**	,568**
	Sig. (bilateral)	,000	,000	,001	,000	,002		,021	,677	,000	,000	,000	,006
	N	22	22	22	22	22	22	22	22	22	22	22	22
v7	Correlation of Pearson	-,289	-,533*	,688**	-,437*	-,703**	-,490*	1	-,149	,490*	,486*	,490*	-,236
	Sig. (bilateral)	,192	,011	,000	,042	,000	,021		,508	,021	,022	,021	,290
	N	22	22	22	22	22	22	22	22	22	22	22	22
v8	Correlation of Pearson	-,352	-,120	-,340	-,227	,302	-,094	-,149	1	,094	,012	,085	-,651**
	Sig. (bilateral)	,108	,594	,121	,310	,172	,677	,508		,677	,957	,707	,001

		V1	v2	v3	v4	v5	v6	v7	v8	v9	v10	v11	IC_{50}exp.
	N	22	22	22	22	22	22	22	22	22	22	22	22
v9	Correlation of Pearson	-912**	-,995**	,642**	-983**	-,633**	-1,000**	,490*	,094	1	,994**	1,000**	-,568**
	Sig. (bilateral)	,000	,000	,001	,000	,002	,000	,021	,677		,000	,000	,006
	N	22	22	22	22	22	22	22	22	22	22	22	22
v10	Correlation of Pearson	-872**	-,983**	,682**	-958**	-,672**	-,994**	,486*	,012	,994**	1	,995**	-,540**
	Sig. (bilateral)	,000	,000	,000	,000	,001	,000	,022	,957	,000		,000	,009
	N	22	22	22	22	22	22	22	22	22	22	22	22
v11	Correlation of Pearson	-908**	-,995**	,647**	-981**	-,638**	-1,000**	,490*	,085	1,000**	,995**	1	-,565**
	Sig. (bilateral)	,000	,000	,001	,000	,001	,000	,021	,707	,000	,000		,006
	N	22	22	22	22	22	22	22	22	22	22	22	22
IC_{50}	Correlation of Pearson	,627**	,565**	-,177	,607**	,227	,568**	-,236	-651**	-568**	-,540**	-,565**	1
	Sig. (bilateral)	,002	,006	,431	,003	,309	,006	,290	,001	,006	,009	,006	
	N	22	22	22	22	22	22	22	22	22	22	22	22

***. The correlation is significant at p= 0.01 (bilateral). * The correlation is significant at p= 0.05 (bilateral). With : v1= H ; v2= L ; v3= N ; v4= X ; v5= S ; v6= W ; v7= U ; v8= K ; v9= Vd= [(3I+(A²)]/[16(I-A)]; v10=Va= [(I+3A)²/[16(I-A)] et v11=Vnet= Va + Vd= V10 +V9*

For the first two factorial axes explaining the 80.79% of information carried by these different variables, we obtained the following results:

From Fig. 3, we can see that the IC_{50}exp. is best explained by the axis of the first main component because the angle formed between it and the IC_{50}exp. is small (compared to the angle it forms with the axis of the second main component and the projection of IC_{50}exp. is almost the entire length of the segment). Hence its correlation coefficient (square cosine of the angle formed) with this new variable will be close to 1 (in absolute value).

Therefore, we notice that the group of variables v1, v2, v4 and v6, respectively the HOMO energy, LUMO energy, electronegativity and the global electrophilic index of aromatic compounds, represented in this diagram by the variable v1, are also close to this axis of the first main component; hence they are also better explained by it. As a result, these variables have a clear (positive) influence on the IC_{50}exp. Therefore, the information contained or carried by the variables v1, v2, v4, v6 and IC_{50}exp. is better explained by this axis of the first main component. Similarly, the information carried by the variable v8 (water/n-octanol partition constant) is also well explained by this axis of the first main component and therefore also has a good (negative) influence on the IC_{50}exp.

On the other hand, the information contained or carried by the variables v3 (hardness), v5 (softness) and v7 (dipole moment) is very well explained by the axis of the second main component. The latter being perpendicular to the axis of the first principal component (which best explains the information carried by the IC_{50}exp.), this implies that there is no significant influence between the IC_{50}exp. and these molecular properties. This is because the empirical correlation coefficients of these variables with the axis of the first principal component are far less than 1 (in absolute value).

From all above, we can see that the electronic properties and the lipophilic character of the aromatic compounds are really determining factors of their MT. Indeed, during the formation of the complex by charge transfer between the coenzyme M and the aromatic nucleus : The capacity to accept the electrons of the aromatic nucleus, quantified by its LUMO energy value (v2), its capacity or tendency not to let its acquired electrons escape, quantified by its electronegativity value (v4) and its capacity to bind strongly to a nucleophilic partner by charge transfer, quantified by its overall electrophilicity index (v6), are therefore molecular properties that determine the stability of the complex that it forms with the coenzyme M. However, the more stable the complex formed, the more the biological activity of the methanogenic archaea is inhibited because the coenzyme M, blocked in this complex with the aromatic ring, will no longer be able to carry out the methylation reaction on the acetate substrate. However, to achieve this, the aromatic compounds must cross the lipoprotein (phospholipid) cell membrane of the methanogenic archaea, hence their lipophilic character, quantified by their LogPoct (v8) value, has also proved to be a determining factor in their MT.

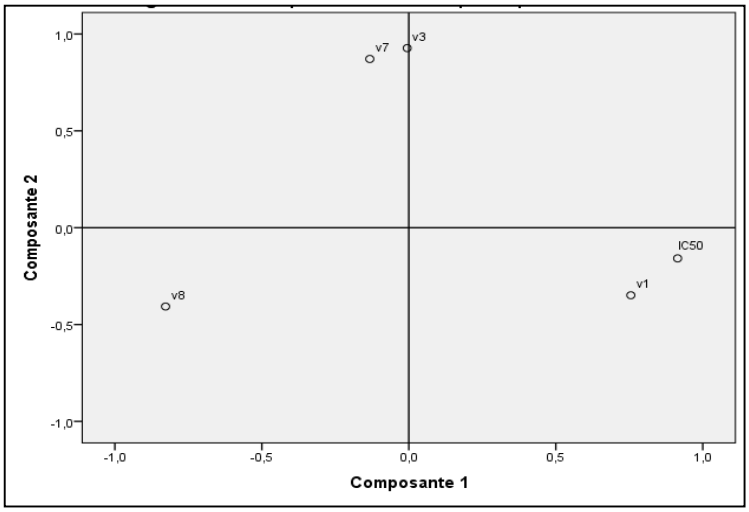


Fig. 3. Spatial component diagram after varimax rotation

Table 5. Total variance explained by the first two main components

Constituent	Initial values			Extraction Sum of squares of selected factors		
	Total	% of variance	% cumulés	Total	% of variance	% cumulés
1	2,232	44,636	44,636	2,232	44,636	44,636
2	1,808	36,154	80,790	1,808	36,154	80,790
3	,481	9,612	90,402			
4	,305	6,099	96,501			
5	,175	3,499	100,000			

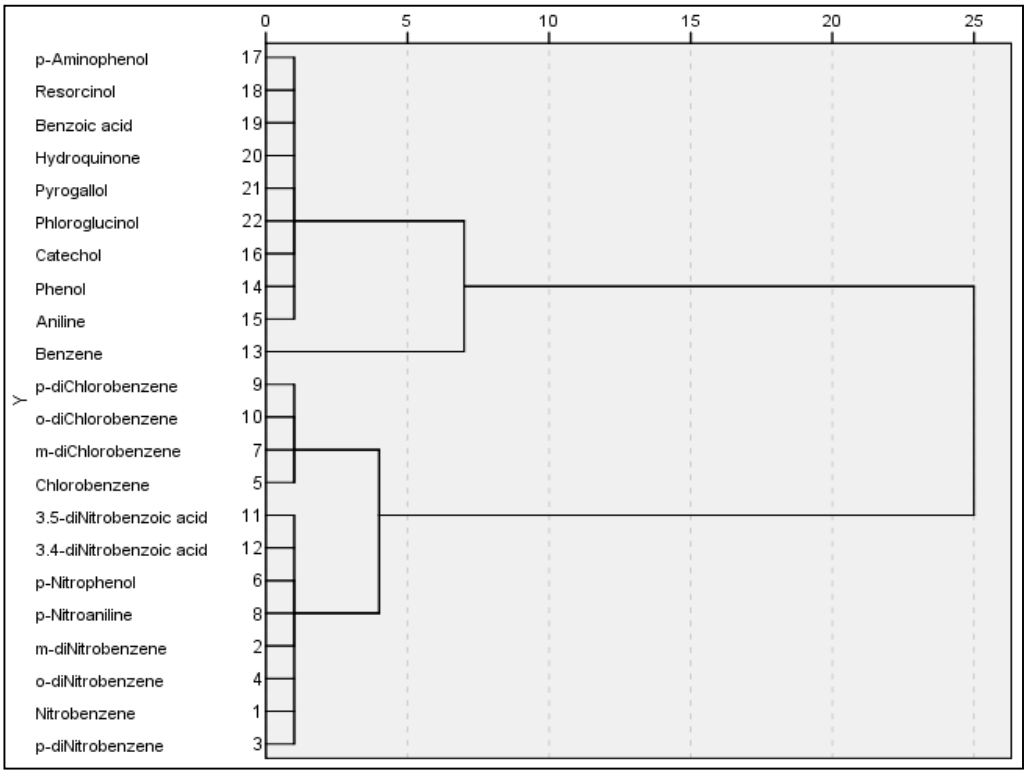


Fig. 4. Dendrogram of the Ascending Hierarchical Classification of Aromatic Compounds Studied

3.4 Ascending Hierarchical Classification of the Aromatic Compounds Studied

Fig. 4 illustrates the dendrogram of the Ascending Hierarchical Classification of the aromatic compounds studied, starting from the various observations on their variables: Molecular properties and MT (IC_{50}exp.). The Pearson distance is taken as a parameter for measuring the dissimilarity between objects (aromatic compounds).These results obtained from the theoretical approaches using the PCA method ultimately show that an aromatic compound with electron-withdrawing (L low, χ high and ω high) and hydrophobic (LogPoct high) substituents will therefore be more toxic than one with electron-donating (L high, χ low and ω low) and hydrophilic (LogPoct low) substituents.

The analysis of this dendrogram highlights the existence of four classes that define the magnitude and type of MT of these different aromatic compounds studied, considering the first interaction.

The first class, formed by compounds with nitro (-NO_2) substituents: very attractive (σ = 0.60) and of a very reactive nature. As a result, it was observed a very pronounced toxicity of all aromatic compounds studied and form for this purpose, the class of aromatic compounds

studied with a very bactericidal action against methanogenic archaea because they cause the death of all strains of methanogenic archaea. However, the opposition of the electronic attracting and giving effects of the substituents on the aromatic ring (case of Nitrophenol and Nitroaniline), the tendency of the acid group to dissociate in the medium (case of 3,5 di-Nitrobenzoic acid and 3,4 di-Nitrobenzoic acid) and steric constraints, mean that in practice some aromatic compounds with nitro substituents are less toxic than other aromatic compounds with cloro substituents that are less electro-attractive (σ = 0.28).

The second class consists of aromatic compounds with cloro (-Cl), lipophilic (π = 0.71) and electro-attracting (σ = 0.28) substituents, with IC_{50}exp. values between 32.25 ± 0.23 and 47.42 ± 1.03 mg/L. It thus defines the class of studied aromatic compounds also with bactericidal action, the most toxic after that of compounds with nitro substituents.

Benzene taken as reference (unsubstituted benzene ring), forms its own class (third class) with an IC_{50}exp. value of 208.78 ± 2.08 mg/L. Thus, it marks the demarcation line between the classes of studiedaromatic compounds with the electron-withdrawing and/or lipophilic (-NO_2 and -Cl) substituents, more toxic to bactericidal action (IC_{50}exp. of the order of 4.19 ± 0.01 to 67.20 ± 1.97 mg/L), and that of aromatic compounds with -OH and -NH_2 electron-withdrawing and hydrophilic substituents (IC_{50}exp. of the order of 966.27 ± 7.04 to 3151.49 ± 5.93 mg/L) less toxic. The latter compounds form the fourth class, consisting of the least toxic studiedaromatic compounds and with bacteriostatic action, since the strains of methanogenic archaea recovered resumed their biological activity after an acclimatization period. Benzoic acid is the exception by being in this fourth class, despite the electro-attracting nature of the acid substituent (-COOH). This is due to its strong tendency to dissociate in the medium, which considerably reduces the capacity of the benzene nucleus to cross the lipophilic cell membrane of the methanogenic archaea.

4. CONCLUSION

A semi-empirical approach was used by correlating the MT of 22 aromatic compounds experimentally measured (IC_{50} exp.) with their molecular properties using the DFT method. The MT of aromatic compounds highlight the different molecular properties of these compounds, which are the basis of their toxicity. The findings show that electro-attracting and lipophilic substituents make the aromatic ring more toxic than electro-donor and hydrophilic substituents. However, the LUMO energy (L), electronegativity (χ), global electrophilicity index (ω) and water/n-octanol partition constant (LogPoct.) of aromatic compounds were found to be the molecular properties influencing their MT.

From these experimental findings on the MT of aromatic compounds, we conclude that: The nature, number and position of the substituents on the aromatic ring have a considerable influence on its MT. For the electro-attractor or electro-donor and lipophilic or hydrophilic characters of the substituents on the aromatic ring have been shown to be parameters influencing this toxicity. However, steric constraints impose restrictions on this MT of aromatic compounds to some extent.

From all above, we notice that all these different classes defined by the HCA method of the studied aromatic compounds are in perfect agreement with our theoretical results found by the PCA method and with those found experimentally on the MTof aromatic compounds.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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