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Full Length Research Paper

The Ontological Principle

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ABSTRACT

This paper establishes an ontological principle as a foundation to analytically investigate a previously introduced ontological assumption residing outside any phenomenological reach. The mere analytical result of a logically possible ontological manifestation constitutes a heuristic value for as well as both, statements about ontology itself and statements about the empirically real.

Key words: ontology, epistemology, heuristic, real, reality, hypothetic, axiomatic, deductive, declarative, revelation

1. INTRODUCTION

In [1], a heuristic principle of inability has been formally established.
A major finding of [1] includes that any kind of language, even the most simple and abstract concept such as a single, isolated geometrical point (or it's arithmetic equivalent, e.g., the number 1) and an associated linear continuum of points or numbers, represents a fundamental, epistemological inability. Hence, a consistent concept of *reality* requires an *ontological assumption* which resides outside any phenomenological reach.
[1] formalized this ontological aspect by considering 1, a point, or the very notion "*something exists*" as the *most fundamental inability* of any language in terms of an inability of determination.
Accordingly, an *axiom of reality* was introduced representing a complementary notion where multiple, apparently contradictory properties on highest level of abstraction, i.e., the most undefined expression yields something or:

"*Omnis negatio est determinatio*", which is equivalent to the complementary expression that *total negation yields something* or that the *negation of totality yields something* (in Fig. 1):

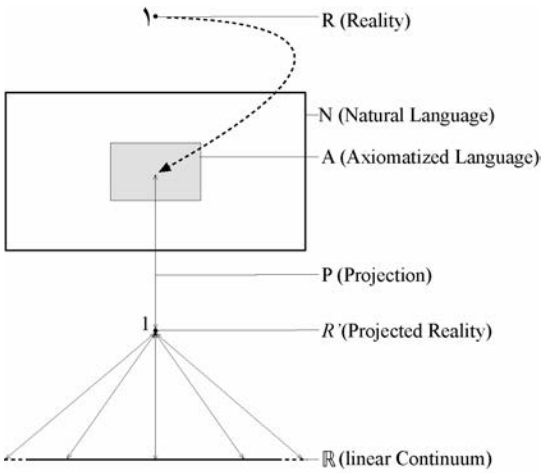

Figure 1 A strict analytical perspective suggests apart natural languages (N) neither the arithmetical language nor the geometrical track with points (in terms of *axiomatized languages* (A)) being able to express the projections of our imagination. At a certain stage of abstraction we rather have to account for the undefined or indefinable constituents of our reality.

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Def. 1 0 = negator
∞ = totality
ا = something

Axiom of reality $0 \cdot \infty = \text{ا}$

This paper will provide with the foundation to analytically investigate such a *reality* and establishes an ontological principle to further analyze the axiomatically introduced notion of an ontological *reality*:

2. EPISTEMOLOGICAL PROCESS

Reviewing the epistemological process how language is projected to the objects of our imagination and perception in Fig. 2 and Fig. 3, we introduced *N* representing natural language with *A* representing axiomatized (or formalized) language (such as mathematics).

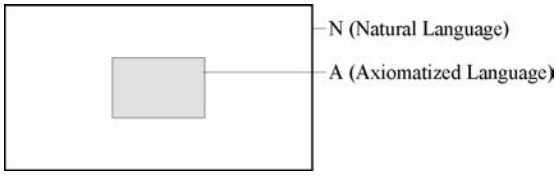

Figure 2 The languages were symbolized in "boxes" to express their actual finiteness in terms of symbols and grammar. The axiomatized language *A* is symbolically a subset of *N* because it is thought to be less expressive than natural language, i.e., natural language generally acts as the meta-language for axiomatized languages.

Eventually, language is projected (*P*) to objects of our imagination and perception, symbolized as reality (*R*):

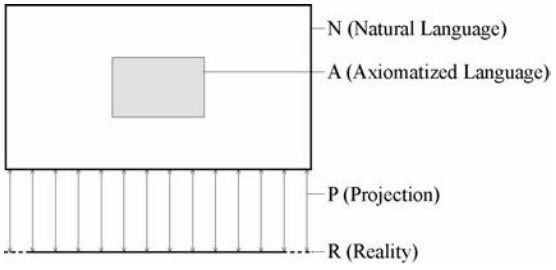

Figure 3

Methodologically, the circularity yielding an infinite regress is obvious:
As visualized in Fig. 4, any object of perception or imagination requires a corresponding term in language and *vice versa*, *reality* can only be perceived or imagined in terms of our language capabilities with the projection (*P*) being reciprocal[1]:

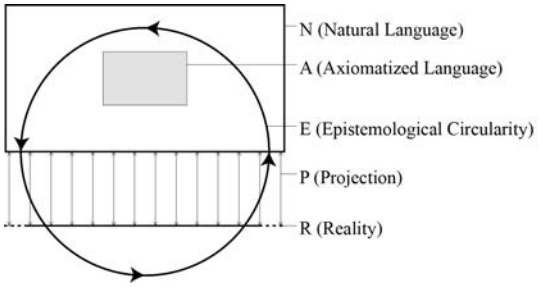

Figure 4 The act of projecting language to reality (and *vice versa*, *reality* to language) necessarily reduces *reality* to the circularity of our language and perceptive capabilities, whether axiomatized, instrument assisted, or not.

In [1], the most critical aspect and fundamental epistemological restriction in perceiving *reality* was identified with the fact of applying language to objects of our imagination and perception at all.

3. THE ONTOLOGICAL PRINCIPLE

While it is impossible for any perceiving subject to perceive *reality* on principle (epistemologically), an inversion of this process yields the logical possibility of an ontological manifestation, i.e., *reality* may manifest itself without being subject to any epistemological restriction.
Methodologically, any projection of perceiving subjects between language and perceivable (empiric) or imaginable phenomena can now be classified as a *hypothetic deduction* (cf. [2]) while any manifestation of ontology can be regarded as *axiomatic declaration* with statements about ontology itself but also about the empirically real (Fig. 5):

[1] An exception may be constituted by *meditation* where any language and affects are kindly released. Consequently, this meditative aspect of perception cannot be communicated in any language.

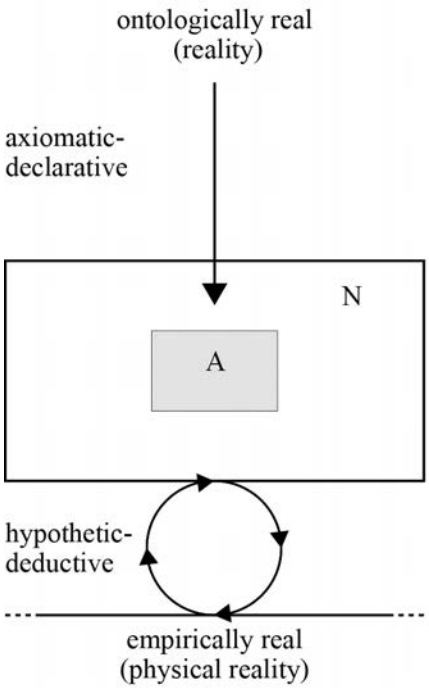

Figure 5 Any hypothetic deduction circularly operates on the empirically real layer (*physical reality*) while the ontologically real (*reality*) must be subject to a declarative process in order to manifest itself.

Since an axiomatic-declarative process and its associated epistemological value may seem very attractive, it is indicated to perform a further attribution:

4. EPISTEMOLOGIC ATTRIBUTION

An immediate and necessary consequence of allowing the logical possibility of an axiomatic declaration is its *perceptibility*.

However, as severally discussed already, *perceptibility* implies the complete spectrum of epistemological inabilities, i.e., an ontological manifestation is totally bound to the limits of language capabilities as illustrated in Fig 5.

Such a conclusion may render any ontological manifestation trivial since it cannot bypass any of the epistemological restrictions of perceiving subjects.

Nevertheless, not only the mere analytical result of this logical possibility *per se* may proof to be of further heuristic value because it is the *only* logical possibility to perceive ontological manifestation, but also any empirical evidence for such an axiomatic declaration which can be identified as *revelation per definition* (Fig. 6):

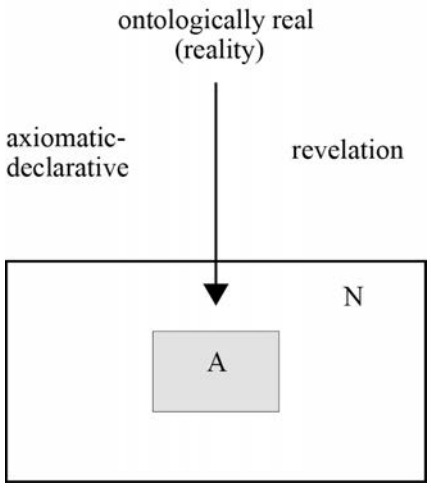

Figure 6

Having identified *revelation* as a possible empirical manifestation of an axiomatic-declarative process, the most critical path of further analysis is constituted by its attributed degree of *authenticity* throughout the whole process of empirical preservation.

Otherwise, any manifestation of axiomatic declaration would be necessarily reduced to the attributes of hypothetic deduction.

The necessary condition of authenticity provided, which itself is subject of empirical analysis, revealed statements of ontology could serve as a heuristic guide for the process of hypothetic deduction as a whole. In this context, revealed statements of ontology about itself would become subject to testability in terms of analytical consistency while statements of ontology about the empirically real would be subject to empirical analysis with regard to refutability.

5. CONCLUSION

Strong evidence has been provided for perceiving subjects not being able to perceive reality on principle (cf. [1]).

This process has been classified *hypothetic-deductive*.
The logically possible inversion of hypothetic deduction yields a process which was classified *axiomatic-declarative*.
This process of axiomatic declaration constitutes the ontological principle.
It has been demonstrated, that any axiomatic declaration which manifests for perceiving subjects, i.e., which becomes perceivable, must necessarily be subject to the complete range of epistemological restrictions, notably represented by the limits of language capabilities.
However, the mere analytical result of a logically possible ontological manifestation constitutes a heuristic value for as well as both, statements about ontology itself and for statements about the empirically real.
With *revelation* having been identified to provide evidence for a possible empirical manifestation of ontology, a necessary condition for further analysis was set to be *authenticity*.
Assuming *authentic revelation*, further analysis will focus on ontological declarations about itself as well as about the empirically real where the critical aspect of *authenticity* will be subject to empirical analysis itself.
The anticipated result of such analysis must provide with consistent statements about ontology itself, i.e., with a *non-determination* in complementary terms, as well as with *non- perceptibility* for any perceiving subject.
On the empirically real layer, any axiomatic declaration is anticipated to provide with restrictions as constituted by the hypothetic deductive methodology on principle.

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Full Length Research Paper

Silymarin Natural Antimicrobiol Agent Extracted from Silybum Marianum

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Accepted December 22 2012

ABSTRACT

The goal of this work is the study and the valorisation of a medicinal plant *Silybum marianum*, widely responded in Mediterranean region, particularly in Algeria. The chloroform and butanolic solvents extracts of *Silybum marianum* were screened for antibiocidal and phytochemical properties. Flavonoids were detected in both extracts. These extracts were active against *Staphylococcus aureus*, *Staphylococcus albus*, *Candida albicans* and *Saccharomyces cerevisiea* with a diameter exceeding (15mm). Flavonoides were separated and identified by a thin layer chromatography (TLC) on silica gel. The TLC results allows to identify 3 different spots S1, S2 and S3.The thermostability essays revealed their resistance at low (-5°C, 4°C) and high temperatures (40°C, 60°C) during 30 min and inactivated at 100°C. These results prove antibiocidal effects of flavonoids extracted from *silybum marianum*, which enlarge the therapeutic properties of this plant.

Key words: silymarin, flavonoids, antibiocidal activity, TLC, CMB/CMI

1. INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value (Nostro et al., 2000).

Flavonoids are a group of natural compounds known to have various pharmacological actions such us antioxydative, anti-inflammatory and diuretic (Havsteen, 2002).

The development of resistance to the available antibiotics has lead researchers to investigate the antimicrobial activity of medicinal plants. *Silybum marianum* commonly called blessed milk thistle is a small trees belonging to *Asteracea* family with up to 1meter high, widely spread in Mediterranean region notably in Algeria.

Flavonoids are naturally occurring substances that possess various pharmacological actions and therapeutic applications. Some, due to their phenolic structures, have antioxidant effect and inhibit free-radical mediated processes (Montvale; 2000).

The extracts of the flowers and leaves of *Silybum marianum* (St. Mary's thistle, milk thistle) have been used for centuries to treat liver, spleen, and gallbladder disorders (Rainone, 2005). In the 1960s the biologically active principles of the seed and fruit extracts were isolated, and the chemical structures were elucidated. The isolation led first to a mixture that was named *Silymarin*, and it was with this flavonolignan mixture that most of the clinical studies were carried out. The main constituents are *Silibinin*,

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Isosilibinin, *Silicristin*, and *Silidianin* (Sonnenbichler and al., 1999).

One of the important issues about *Silymarin* is that it may be accepted as a safe herbal product, since no health hazards or side effects are known in conjunction with the proper administration of designed therapeutic dosages (Montvale, 2000). In this study, the chloroformic acetate of ethyl and butanolic extracts of *Silybum marianum* were investigated for antimicrobial and antifungal activity. The phytochemical components were also investigated as a scientific assessment for the claim of therapeutic potency.

The study aimed investigating the antimicrobial activity of the plant by preliminary in-vitro bioassay screening using aqueous and petroleum ether as well as chloroform extracts.

2. MATERIALS AND METHODS

2.1. Plant Material

Flowers of *Silybum marianum* were collected and seeds than pulverized into small coarse powder stored until required for use.

2.2. Microorganisms

Microorganisms denoted with ATCC included in this study (*Staphylococcus aureus*, *Staphylococcus albus*, *Pseudomonas* sp, *Escherichia coli*, *Serratia* sp; *Aspergillus* sp, *Penicillum* sp, *Candida albicans* and *Saccharomyces cereviseae*) were provided from the medical institute of microbiology (Constantine; Algeria). The microorganisms were maintained on nutrient agar slants at 4°C, reidentified by biochemical tests and sub-cultured in nutrient broth for 24h prior to testing.

2.3.Extraction and Fraction Procedure

Fractionation of the extracts was fractionated using ethanol-water 80/20 v/v for 24h during three days and different organic solvents (petroleum-ether, chloroform, acetate of ethyl and n-butanol). The powdered extract of the plant 100g was overnight fractionated with ethanol-water (800 ml) at room temperature (Isaac and Chinwe, 2001). The extract was filtered and then partitioned into petroleum-ether than chloroform, acetate of ethyl and butanolic solvents. The different alcoholic extracts were evaporated in Rotavapor at 40-50°C. Finally reconstituted in 6 ml of methanol as a contributory antimicrobial effect of the organic fractions. (Markham, 1982).

2.4. TLC Analysis

Merck silica gel plates Kieselgel F254 (Merck, Germany) and the following mobile phases were used as eluent for TLC:

S1: toluene/butanol/ethanol/petroleum ether 20/10/10/20 v/v.

S2: chloroform/acetone/formic acid 75/16,5/8,5 v/v.

S3: ethyl acetate /methanol/water 50/20/10 v/v.

The chromatogram was evaluated under light after spraying the plate with godin reagent (1% ethanolic solution of vanillin, following by 3% of perchloric acid solution).

2.5. Antimicrobial Assays

Pure culture of the organisms were inoculated onto Muller-Hinton nutrient broth (Oxoid, England), incubated for 24h at 37°C. Diluted with sterile nutrient broth to a density of 9×10^8 cfu/ml equivalent to *McFarland* test. The suspension was used to streak for confluent growth on the surface of *Muller-Hinton* agar on *Petri* dishes with sterile swab. Using a sterile 6mm disk contained methanol as positive control. The *Petri* dishes were placed in the incubator overnight at 37°C. The

antimicrobial activity was recorded if the zone of inhibition was greater than 9mm (Hassan et al; 2006).

Antimicrobial activity was investigated by the disk diffusion method and the broth two fold macro dilution methods. Results of the diffusion method were expressed as the diameter of the inhibition zone around the hole filled with investigated solution.

Dilution method results were recorded as the minimum inhibitory concentration (MIC) and minimum microbiocidal concentration (MMC). Details of both methods are described else-where.

The determination of the minimum inhibitory concentration MIC for butanol and chloroform extracts showed significant activity (d > 9mm) and were chosen for MIC assay. MIC was determined by the standard method (Kamagate and al; 2002) in there nutrient broth was prepared and sterilized. 5 ml of the prepared broth was dispensed in to the test tubes.

Serial dilutions of plant extract (chloroform and butanol) were undertaken to test the growth capacity of the different microorganisms. 200 µl of each extract dilution were transferred into each tube with exception of control tubes and incubated 24-48 h at 37°C.

2.6. Effect of Temperature

Chloroform and butanolic extracts were treated at: 4°C, 40°C, 60°C and 100°C during 30min. 100 µl of the extracts were added to suspension of nutrient broth and *Yeast* extract glucose medium (YG) inoculated by *Staphylococcus albus* and *Candida albicans*. The suspension was used to streak for confluent growth on the surface of *Muller-Hinton* agar on *Petri* dishes with sterile swab. Using a sterile disk of 6mm diameter, tow disk contained methanol were used as reference or positive control. The Petri dishes were placed in the incubator at 37°C overnight.

2.7. Effect of Variation

400µl of extract (chloroform, ethyl acetate) were distributed in curved *Eppendorf* tubes then dried by evaporation and treated (NaOH/HCl) 1N/1N, fitted by means of a PH-meter to the following PH values: 1.16; 3.01; 6.5; 8.6; 9.57; 11.9; 12.8; 13.15 and finally left reacting for 30min.

3. RESULTS AND DISCUSSION

3.1. TLC

TLC was employed to determine the composition of flavonoids in each fraction. Flavonoids of *S. marianum* were separated in three main fractions, which were evaporated and redisoleved in 98% methanol. The results lead to different spots S1, S2, S3 with an RF (Rf1 = 0.36cm; Rf2 = 0.40cm; Rf3 = 0.53cm) subsequently corresponding to *Silydianine*, *Silychristine*, and *Silybine*, the active constituent of *Silymarine* (Fig. 1).

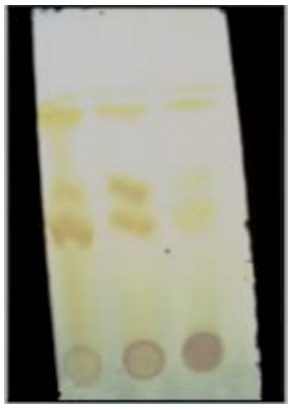

Figure 1 TLC results of the different extracts (1: chloroform; 2: ethyl acetate; 3: butanol).

3.1. Antimicrobial Results

98% methanol showed no inhibition zones. Both flavonoid fractions inhibited the growth of most of the microbial strains tested. The only exceptions were gram negatives and *Mycelium fungi*. The

majority of yeasts were sensitive to both flavonoids fractions. *Candida albicans* and *Saccharomyces cereviseae* were the only yeast inhibited by both extracts. Among *Mycelium fungi* were resistant.
The size of all inhibition zones was between 9 and 16mm for fungi. Average size of inhibition zones was around 11mm for chloroform and ethyl acetate fractions.
Bacteria most susceptible to both fractions were gram-positive bacteria: *Staphylococcus aureus*, and *Staphylococcus albus* with a diameter of 17 and 18mm.

Dilution phase	C0	C1/2	C1/4	C1/8	C1/16	C1/32
chloroforme	---	---	---	+++	+++	+++
Butanol	---	---	---	+++	+++	+++

(+) resutlts; (-): results

Table 1 Liquid dilution method results against *S. albus* using chloroformic exacts.

Phase Dilution	C0	C 1/2	C 1/4	C1/8	C1/16	C1/32
Chloroforme	---	---	---	+++	+++	+++
Butanol	---	---	---	+++	+++	+++

(+) results; (-): results

Table 2 Liquid dilution method results against *C. albicans* using chloroformic exacts.

3.2. MIC and CBM Results

98% methanol showed no inhibition zones. Both flavonoid fractions inhibited the growth of most of the microbial strains tested. The only exceptions were gram negatives and *Mycelium fungi*. The majority of yeasts were sensitive to both flavonoids fractions. *Candida albicans* and *Saccharomyces cereviseae* were the only yeast inhibited by both extracts, *however mycelium fungi* were resistant.

	CMI (mg/ml)	CMB (mg/ ml)	CMI (mg/ml)	CMB (mg/ml)	Chloroforme Extract	Butanolic Extract
	chloroformic Extract		butanolic Extract		CMB / CMI ratio	
S. albus	10 ,25	41	7	28	4	4
C. albicans	20,5	41	14	28	4	2

Table 3 Results of CMI and CMB; CMI/CMB of actives extracts against *Staphylococcus albus* and *Candida albicans*.

The MIC of chloroform and butanolic extracts ranged from 10, 25 - 20, 5 mg/ml and 7- 14 mg/ml respectively (Table 3). The chloroform extract has the lowest MIC compared to butanolic extract.
The CMB of the extracts ranged from 41 and 28 mg/ ml respectively. The CMB / CMI ratio ranged from 4 and 2 mg/ml subsequently indicated a bacteriostatic action of flavonoids (Archambaud, 2001).
The antimicrobial properties of this plant probably explain its traditional use for treating bacterial diseases. In 1996 Freiburghans indicate that different solvent extracts of some plant may exhibit pharmacological properties.
The mechanism of action of constituents of *S. marianum* may be difficult to speculate; however, many antibacterial agents may exhibit their action through inhibition of nucleic acid, protein and membrane phospholipids biosynthesis (Palanichamy et al., 1990). It is probable that the antimicrobial agents in the extracts act via some of the above cited mechanisms. Further studies for in-*vitro* activity, isolation and structural elucidation of the active components of the plant extracts are recommended.

3.3. Effect of Temperature

Chloroform and butanolic extracts were treated at: 4°C; 40°C; 60°C; 100°C during 30min.
100µl of the extracts were added to suspension of nutrient broth and YG inoculated by *Staphylococcus albus* and *Candida albicans*.

The suspension was used to streak for confluent growth on the surface of *Muller-Hinton* agar on *Petri* dishes with sterile swab. Using a sterile disk of 6mm diameter, tow disk contained methanol were used as reference or positive control. The Petri dishes were placed in the incubator overnight at 37°C. The antimicrobial activity recorded if the zone of inhibition was greater than 9mm (Hassan et al; 2oo6).

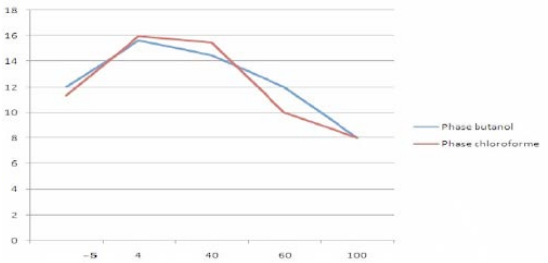

Figure 2 Inhibition of microbial growth via butanolic and chloroformic extracts treated at different temperatures.

These results indicate that flavonoids of *Silybum marianum* conserves their activity at moderate temperature [-5; 4; 40; 60°C]; and inactivated at 100°C, this results are relatively different comparative at those obtained in 2006 by Daughari. The optimum activity of biological molecules was located at 40°C and 60°C.

3.4. Effect of Ph Variation

According to the face (Fig. 3) three intervals of Ph appear: from 1, 16 to 3, 01.

The value of the diameter of inhibition increases slightly; it is situated between 10mm and 11mm for the extract of chloroform, and of 15mm in 17mm for ethyl acetate extract. From 3,01 to 9,75: the diameter of inhibition increases and remains relatively constant in value, it is situated between 15mm and 16mm for the chloroform extract and 9mm and 12mm for the ethyl acetate extract with a decrease of diameter of inhibition. Slightly until reach15 mm for the extract of ethyl acetate (Laleh et al., 2006).

From9, 75 to 13, 16: the value of the diameter of inhibition decreases until it reaches the 10mm value for the extract of chloroform and then increases. The PH influences the activity of flavonoids by substitution of the groupings (OH) which surmount the structure three-dimensional of these compounds what is confirmed by the statistical study: Analysis of the variance (ANOVA): Fobs > F 1, 14, 5%; (6,68 >2, 4):

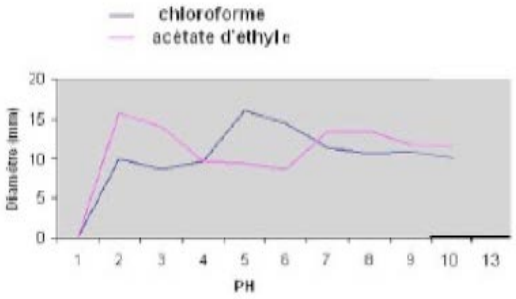

Figure 3 Influence of PH variation.

4. CONCLUSION

Both flavonoid fractions inhibited the growth of most of the microbial strains tested: Gram-positive bacteria and Yeast. The CMB/CMI ratio ranged from 2 and 4 mg/ml subsequently indicated a bacteriostatic action of flavonoids. TLC results led to obtaining different spots S1, S2, S3 with an RF of Rf1=0.36cm; Rf2=0.40cm; Rf3=0.53cm subsequently corresponding to *Silydianine*, *Silychristine* and *Silybine* the active constituent of *Silymarine*.

The optimum activity of biological molecules was located at 40°C and 60°C and at moderate Ph [6,5 - 8,5].

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Full Length Research Paper

Elaboration of Surface Plasmon Resonance Sensor Based on Calix[4]Arene Self-Autoassembeld Monolayer of Cysteamine for Heavy Metals Detection in Water

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Accepted December 30 2012

ABSTRACT

In this work, a new chromogenic calix[4]arene was functionalized onto self-autoassembled monolayer of cysteamine for heavy metals detection and tested by using SPR measurement. To functionalize SPR sensor, two procedures were proposed. The first is based on the oxidation of calixarene-SAM alcohol group to quinone. In the second procedure, pyridine was made to protect oxygen donor group and catalyse the reaction between SAM and calixarene alcohol group. Heavy metals were used Cu^{+2}, Co^{+2}, Cd^{+2}, Mg^{+2}. Detection limit reached are lower than $10^{-5}\mu M$ for the most heavy metals. PH of detection was optimised for each ion and detection was made in basic solution. Several detection zones were observed and linear sensitivities were showed for different heavy metals detection at different zones. The sensitivities obtained are 151.46, 210, 103.99 and 43.75μM^{-1} for M, Co^{+2}, Cu^{+2} and Cd^{+2}, respectively.

Key words: calxarene, SAM, SPR, ions, heavy metals, sensor, sensitivity

1. INTRODUCTION

Calixarenes are currently the subjects of study as chemical sensors and selective receptors due to their important functionalisation and complexations possibilities. Among this sensors, different calixarene derivative may be found, thanks of their importance in the fields the medicine and the environment, in ions selective electrodes and in chromogenic sensors (David, 1989; Antesberger et al., 2005; McMahon et al., 2003; F.F. Nachtigall et al., 2002; Atwood et al., 2002; Thallapally et al., 2005; Purse et al., 2005; Kumar, et al., 2006).

Many work was carried out based on calixarene molecule using polymer support and different measurement techniques to detect traces of ions were reported (Jain et al., 2005; Lu et al., 2004; Duncan and Cockayne, 2001; Pérez-Jiménez et al.,1998; Chen et al., 2000; Bouazizi et al., 1996; Lu et al., 2002). A Poly(vinyl chloride) (PVC) based membrane calixarene was largely used to ions and heavy metals detection (Jain et al., 2005; Lu, et al., 2004; Duncan and Cockayne, 2001; Pérez-Jiménez et al., 1998; Chen et al., 2000). For example, it is used as CrO2-4 selective sensor (Jain et al., 2005), silver ion selective electrode (Duncan and Cockayne, 2001, Chen et al., 2000) and caesium-selective sensor (Pérez-Jiménez et al., 1998).

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The use of polymer as support for calixarene, decrease the sensitivity of the sensor (Levit et al., 2002; Park et al., 1999). It is shown that surface adsorption and bulk adsorption of polymers affect the sensitivity and the response time of sensor and exhibiting low vapour permeability. Therefore, we are directed to another effective functionalization technique largely used for self-autoassembled monolyer (SAM). The advantages of SAMs include simplicity of preparation, versatility, stability, reproducibility and the possibility of introducing different chemical functionalities with high-level of order on a molecular dimension (Ulman, 1991; Gao and Siow, 1996; Aizenberg et al., 1998; Sat et al., 1996). One of the most widely used systems in the molecular self-assembled method is the chemisorption of sulfur derivatives (i.e. thiols, disul- fides) on gold surfaces (Steinberg and Rubinstein, 1992; Mandler and Turyan, 1996; Flink et al., 1998).

There are several reports on the use of SAMs to improve selectivity and/or sensitivity of gold electrodes in a broad range of electro-analysis. A feasible method of fabricating phthalocyanine sensors was developed by a covalent attachment of cobalt tetracarboxylic acidchloride phthalocyanine (CoTCACIPc) onto a preformed 2-mercaptoethanol (2-ME) self-assembled monolayer (SAM) modified gold electrode (designated as CoTCACIPc-2-ME-SAM) (Mashazi et al., 2007). Concentration range 0.28^{-20} µM with a detection limit of 5×10^{-7} M. Copper sensors based on SAM were developed (Freire and Kubota, 2004; Arrigan, Bihan, 1999). In reference (Freire and Kubota), a gold-electrode modified with a self-assembled monolayer (SAM) of 3-mercaptopropionic acid (MPA) was evaluated a highly sensitive voltammetric sensor for copper ions. The detection limit reached 1.8 10^{-14}M for copper. In (Arrigan and Bihan, 1999), reported a cysteine monolayer-coated gold-electrode and its Cu^{2+} complexation, which allowed a detection of Cu^{2+} down to 10^{-7}M. A selective molecular interaction at an interface formed by self-assembly of a macrocyclic synthetic host, calyx[4]resorcinarene with four thiol groups (R4SH) were investigated [Faull, Gupta, 2003]. They demonstrated that the noncovalent chemical selectivity of SAMs of hosts calyx[4]resorcinarene extends to isomers of several different guest molecules. A selective Quinone-functionalised calix[4]arenes having carboxylic acid groups or thiol groups were prepared (Parket al., 2001). The sensors exhibited a selective affinity towards specific hard-metal ions in aqueous media.

Surface-plasmon waves (SPWs) are extremely sensitive to small changes in the refractive index near the sensor surface and the changes in the refractive index are proportional to the sample mass, so the adsorption of molecules on the metal film or conformational change in the adsorbed molecules can be detected accurately (Boussaad et al., 2000; Zacher, Wischerhoff, 2002; Georgiadis et al., 2000; Sarkar, Somasundaran, 2003; Chah et al., 2002). In these works, a chromogenic calix[4]arene molecule was immobilized onto modified SAM gold surface of an SPR sensor chip for heavy metals detection. Two functionalized procedures were proposed. The first is based on the oxidation of calixarene-SAM alcohol group to quinone. In the second procedure, pyridine was made to protect the oxygen donor group and to catalyze the reaction between SAM and the calixarene alcohol group.

2. EXPERIMENTAL

2.1 SPR Material and Principle

Surface Plasmon Resonance Spectrometer BIO-SUPLAR 2 (Analytical µ-Systems, Germany) produced by Biacore company was used. It is based on the Kretschmann type prism and GaAs solid-state laser (λ= 670nm).

The SPR detection-principle is constituted by measurement of the intensity of the reflected light. At the SPR-angle, a sharp decrease or 'dip' of intensity is measured. The position of the SPR-angle depends on the refractive index in the substance with a low-refractive index close to the sensing surface. The refractive index near the sensor surface changes, because of ions binding to the surface. As a result, the SPR-angle will change according to the amount of bound ions.

During a binding analysis, SPR changes occur as a solution is passed over the surface of a sensor chip. To perform an analysis, one interactant is captured on a sensor surface. The sensor surface forms one wall of a flow cell. A sample containing the other interactant(s) is injected onto this surface in a precisely controlled flow. Fixed wavelength light in a fan-shaped form is directed at the sensor surface and molecular binding events are detected as changes in the particular angle where SPR creates extinction of light. This change is measured continuously to form a sensorgram (Fig. 1), which provides a complete record of the progress of association or dissociation of the interactants.

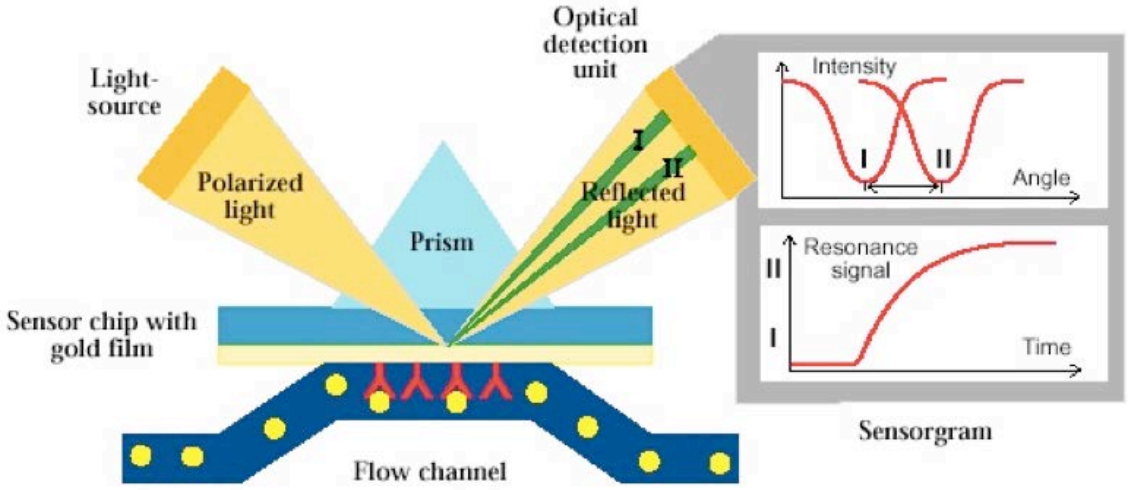


Figure 1 SPR sensing principle

2.2 Synthesis of the chromogenic amide derivative calix[4]arene

The synthesis of tetra-O-substituted calix[4]arene derivative was performed by the reaction sequence depicted in Figure 2. The treatment of p-tetrakis(phenylazo)calix[4]arene with tertiary acetamide (α-chloro-N,N-diethylacetamide) in the presence of CaH_2 as base gave p-tetrakisphenylazocalix[4]arene tetra-amide derivative in cone conformation (Halouani et al., 2002).

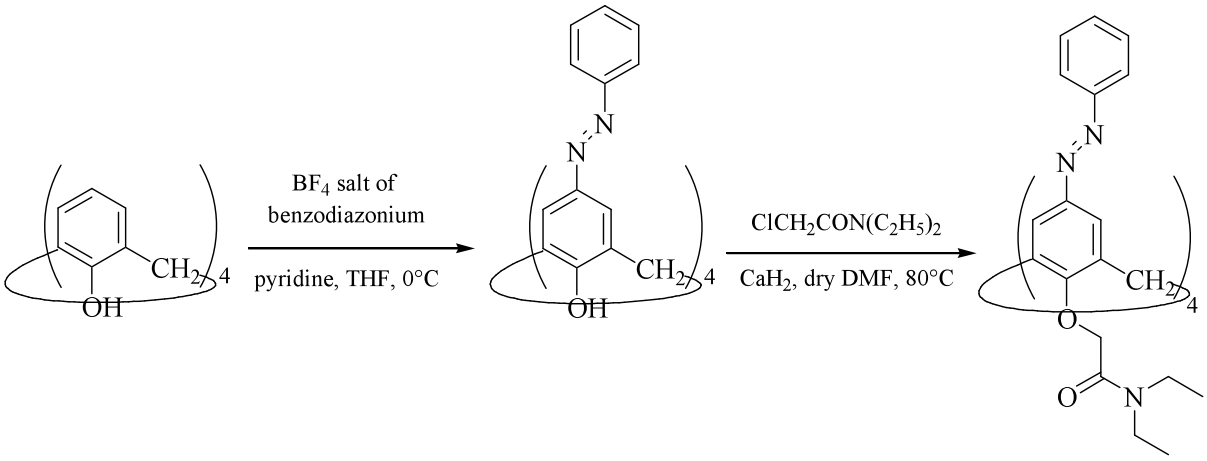


Figure 2 Reaction sequence for the synthesis of p-tetrakisphenylazocalix[4]arene tetra-amide derivative.

2.3 Reagents and samples preparation

Several samples necessary to elaborate the sensor were prepared. First, 10^{-2} M of cysteamine solution was prepared in which a sensor was immersed. Calix[4]arene solution was prepared by dissolved 5mg of calix[4]arene powder in 2ml of chloroform. Calix[4]arene was deposited on the sensor by dip-coating technique. This technique makes it possible to have homogeneous layers. PCC or pyridinium chlorochromate, known as *Corey's Reagent* (Corey and Suggs, 1975), was prepared. To 22ml of 6M HCl 12g of CrO3 were added. After ultrasonic mixing for 10min., 9,5g of pyridine were added to the homogeneous solution. Ultrasonic mixing over 5min. and cooling to 0 °C. A dilute sulfochromic oxidant solution and aqueous solutions containing 0µM to 1µM of the metal ions Cu^{+2}, Co^{+2}, Cd^{+2} and Mg^{+2} were prepared. Phosphate buffer solution PBS was prepared at pH = 8.

2.4 Immobilization of calix[4]arene on the Self-autoassembled monolyer of cysteamine onto gold surface

A principle scheme for the functionalization of calix[4]arene-SAM onto a gold surface for ions detection is presented in Fig. 3:

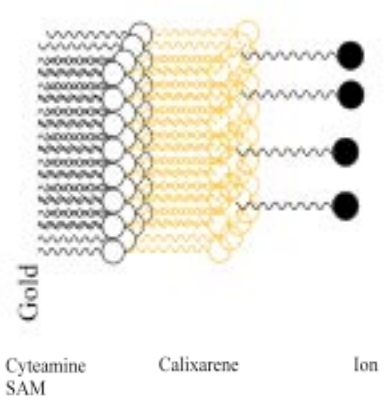


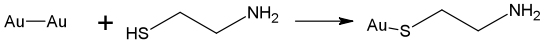
Figure 3 SAM-Calix[4]arene scheme for ion detection.

To functionalize a SPR the sensor, two procedures were proposed:

First procedure steps:

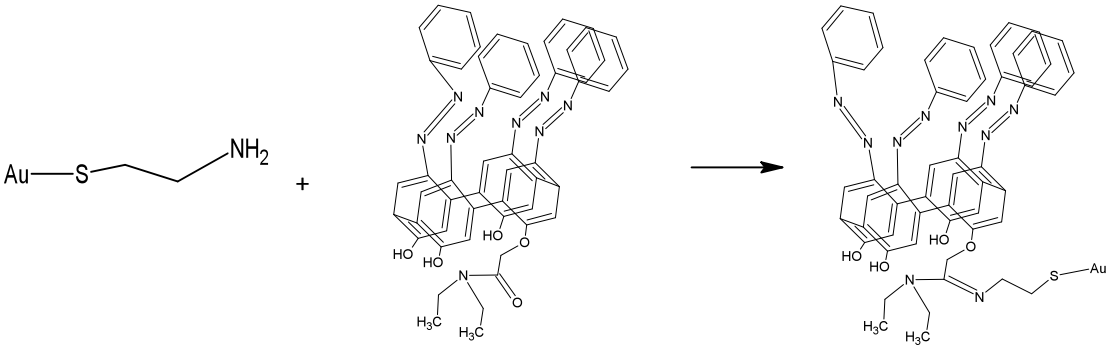
In the first procedure, five stages are necessary:

1. In SPR the adhesion between the gold layer and glass is only physical. Pirhana was not made to clean the gold surface. Accordingly, acetone and ethanol were used.
2. Grafting of SAM on the gold surface: The sensor is immersed in 15mM of cysteamine during two hours.

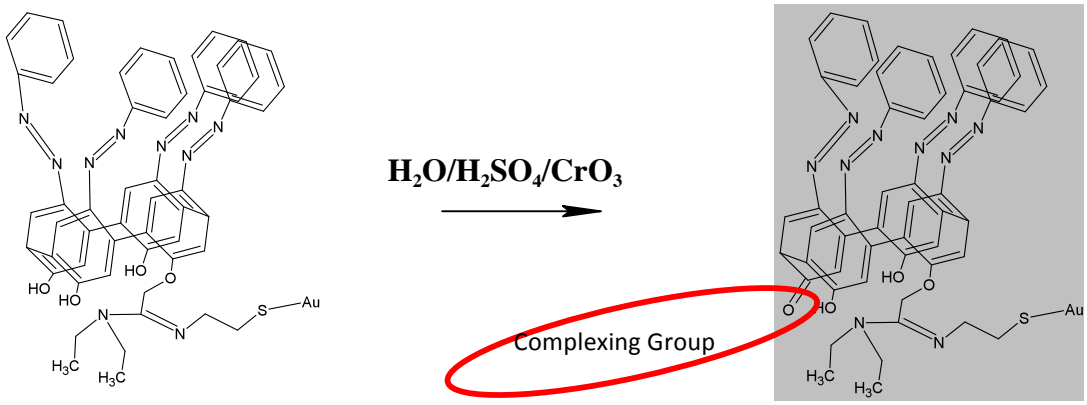


3. Cleaning of SAM by ethanol.
4. Deposition of calix[4]arene onto the SAM by spin coating method: An oxygen donor group of calixarene reacts with amine of SAM (Shervedani and

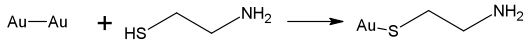
Mozaffari, 2005; Sunet al., 1998; Xia et al., 1999; Delvaux and Demoustier-Champagne, 2003):

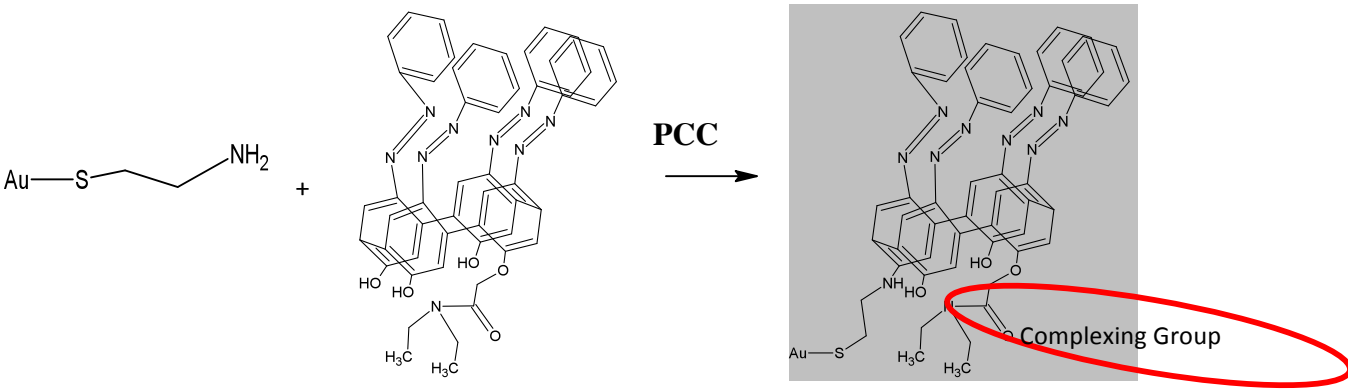


5. Activation of grafted surface by oxidation Cr_2O_3/H_2SO_4 in diluted water solution and formation of quinone (Hudlicky, 1990):



Second procedure steps:

1. In SPR the adhesion between the gold layer and glass is only physical. Pirhana was not made to clean the gold surface. Accordingly, acetone and ethanol were used,
2. Grafting of SAM on the gold surface: The sensor is plunged in 15mM of cysteamine solution for two hours: 
3. Cleaning of SAM with ethanol.
4. Deposition of calix[4]arene onto the SAM in PCC solution. PCC was made to protect the oxygen donor group and catalyze the reaction between SAM and calixarene alcohol group (Hunsen, 2005; Hunsen, 2005; Muzart, 1992).



3. RESULTS AND DISCUSSION

The sensor was characterized by electrochemical impedance spectroscopy EIS. All tests were made by SPR method. The influence of pH on ions detection was studied and optimized for each ion. The regeneration of the sensor was made by injection of 10mM EDTA.

3.1 Sensor characterisation by electrochemical impedance spectroscopy EIS

The EIS is a powerful technique for characterization and studying electrical and electrochemical properties of a large variety of systems.

All electrochemical measurements were carried out using VOLTALAB 40 analyzer (PGZ301 & VoltaMaster 4).

A three-electrode electrochemical cell was used with a chemical gold-electrode as the working electrode. A calomel-electrode was used as the reference electrode and a platinum-electrode was used as the auxiliary electrode. Measurements were used in buffer solution at adjusted pH of about 7.3.

Concerning the use of EIS to characterize thin films in contact with electrolyte solutions, three different contributions, bulk, interfacial and electrolytes may be determined (Macdonald, 1987). From an electrochemical point of view, when a metal is placed in contact with an electrolyte, a potential is generated due to the unequal distribution of charge across the interface. In addition, hydrated ions will not be able to approach indefinitely close to the interface. To explain such phenomena, Helmholts has proposed the well-known theory of the double layer. This interface behaves as widely described by Randles' equivalent circuit, (Fig. 4) (Macdonald, 1987; Hsu and Mansfeld, 2001; Ding et al., 2005). In such a model, the charge transfer resistance R1 is generally in parallel with the constant phase element CPE contained in the modified layer capacitance and the double layer capacitance and in series with the solution resistance Rs.:

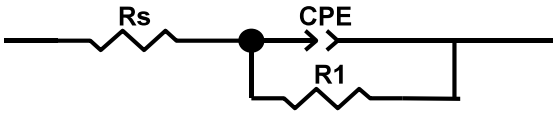


Figure 4 Equivalent circuit model, Rs, R1 and CPE represent the resistance of electrolyte, the charge transfer resistance, and constant phase element, respectively.

The EIS measurements were used to characterize the functionalized electrode. Fig. 5 shows the faradaic impedance spectra presented as Nyquist plots (Zim vs. Zre) upon the assembly of the three layers on the electrode:

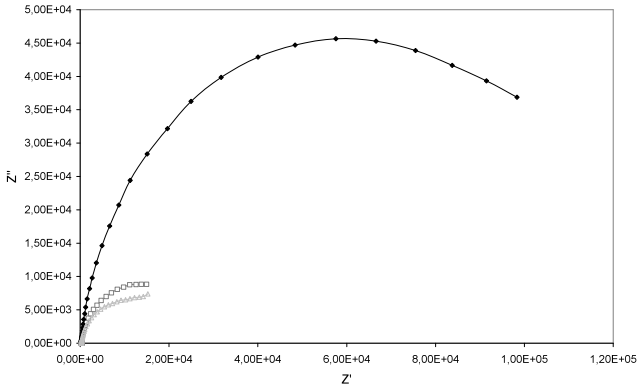


Figure 5 EIS measurement of sensor in buffer solution:

a) Gold b) SAM c) Calixa[4]rene-SAM

The bare Au electrode exhibits an almost straight line (curve a) that is characteristic for a mass diffusional limiting electron-transfer process. In the case of gold electron-transfer, the resistance is about 8869Ω. Assembly of the cysteamine monolayer on the electrode surface (curve b) generates a layer on the electrode that introduces a barrier to the interfacial electron-transfer. This is reflected by the appearance of the semicircle part on the spectrum, corresponding to a charge transfer resistance of R1=24613Ω. The deposition of calix[4]arene onto the SAM cysteamine layer results in an increase of the electron-transfer resistance to R1=60052Ω (curve c). The covalent attachment of each layer increases the charge transfer resistance.

3.2 pH Optimization

The SPR method was used for pH optimization and test-sensors. PH detection of the sensor was optimized for each ion at 1µM concentration. The pH of buffer solution was adjusted by using 0.1M of HCl or NaOH solution. Fig. 6 shows that ion detection is made in basic solution at a pH range between 7 and 8 (7.3 for copper, 7.8 for Cd^{+2} and Mg^{+2}, and 8 for cadmium). This is in agreement with proton/ion exchange (Norlin et al., 2002; Profumo et al., 2006). At low pH, quinone protonated and ion complexation does not take place. At pH>7, oxygen was deprotenated making the sensor surface become negatively charged. Thus, the incorporation of ion by the calix[4]arene-SAM can also be favored by electrostatic interactions (Freire and Kubota, 2004). Such coupling of favorable characteristics is most likely the key to achieving the remarkable sensitivity to ions of the proposed sensor.

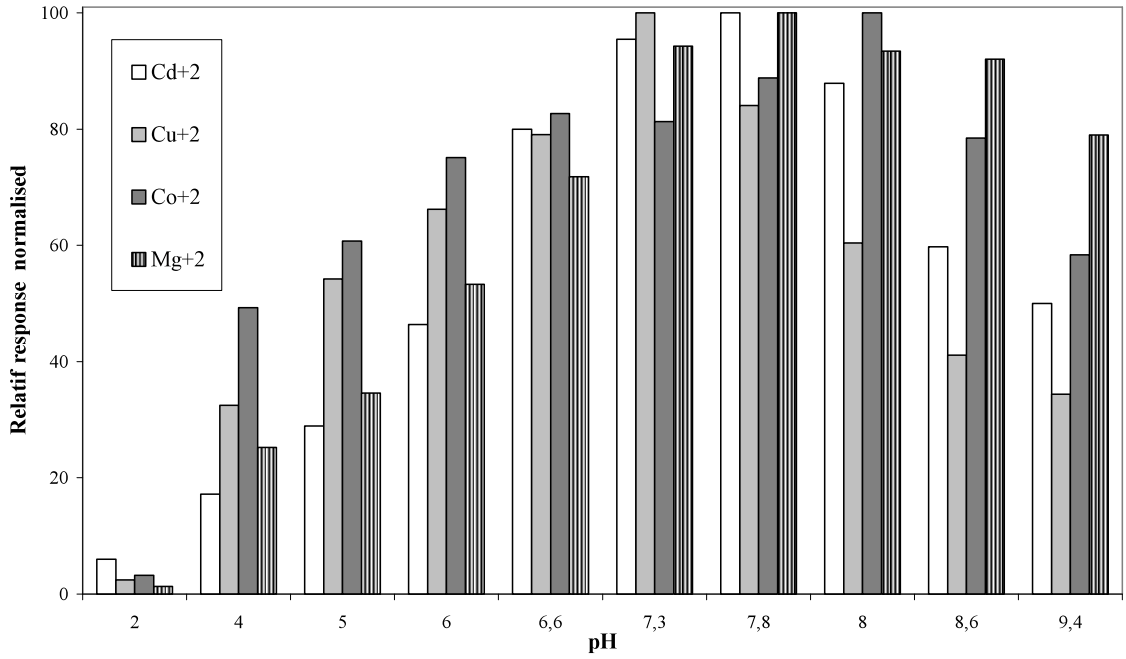


Figure 6 Optimization of pH for each ion.

3.3 Sensor detection

For all ions, we observed that the output refractive index increases with increasing ion concentration. As shown in (Halouani et al., 2002) metal ions are coordinated with the oxygen atoms of the amide group and complexation of ions takes place.

For the oxidation procedure, the detection limit reached is lower than 10^{-5} µM for the most heavy metals used. Detection limits reached are 2 10^{-6} µM, 3 10^{-6} µM, 4 10^{-6} µM, and 6 10^{-6} µM for Cu^{+2}, Co+2, Cd^{+2}, and Mg+ ions, respectively (Fig. 7):

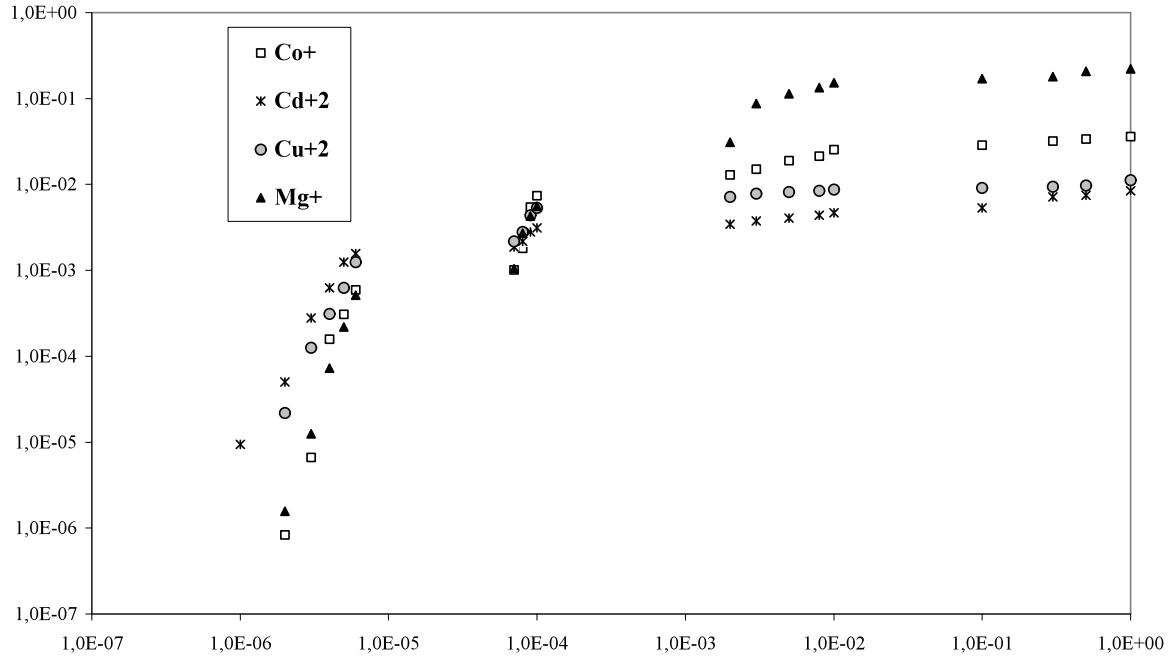


Figure 7 Calibration curve of SPR calix[4]arene SAM sensor for different zones of heavy metals detection: Oxidation procedure.

Four zones of detection were observed between 0 and 1µM.: A high concentration zone from 0.1 to 1µM (Fig. 8a), a second zone between 10^{-3} and 10^{-2} µM (Fig. 8b), a third zone between 10^{-4} and 10^{-5} µM (Fig. 8c) and a low concentration zone from 10^{-5} to 10^{-6} µM (Fig. 8d). In Figures 8a), b), c), and d), a linear variation of signal was showed for each heavy metal in all detection zones. The slopes of lines represent sensor-sensitivities. A recapitulative table which gives sensor-sensitivity for each zone was drawn (Tab. 1). As low sensitivities as 5.9 10^{-2}, 7.6 10^{-3}, 2.5 10^{-3} and 3.1 10^{-3} μM^{-1} for Mg^{+2}, Co^{+2}, Cu^{+2} and Cd^{+2} in high concentration zones respectively were observed. In the range between 10^{-4} and 10^{-5} µM, high sensitivity values were observed for heavy metals detection in which detection limit borders were reached. As shown in Tab. 1, the sensitivities obtained are 151.46, 210, 103.99, and 43.75μM^{-1} for Mg^{+2}, Co^{+2}, Cu^{+2} and Cd^{+2}, respectively:

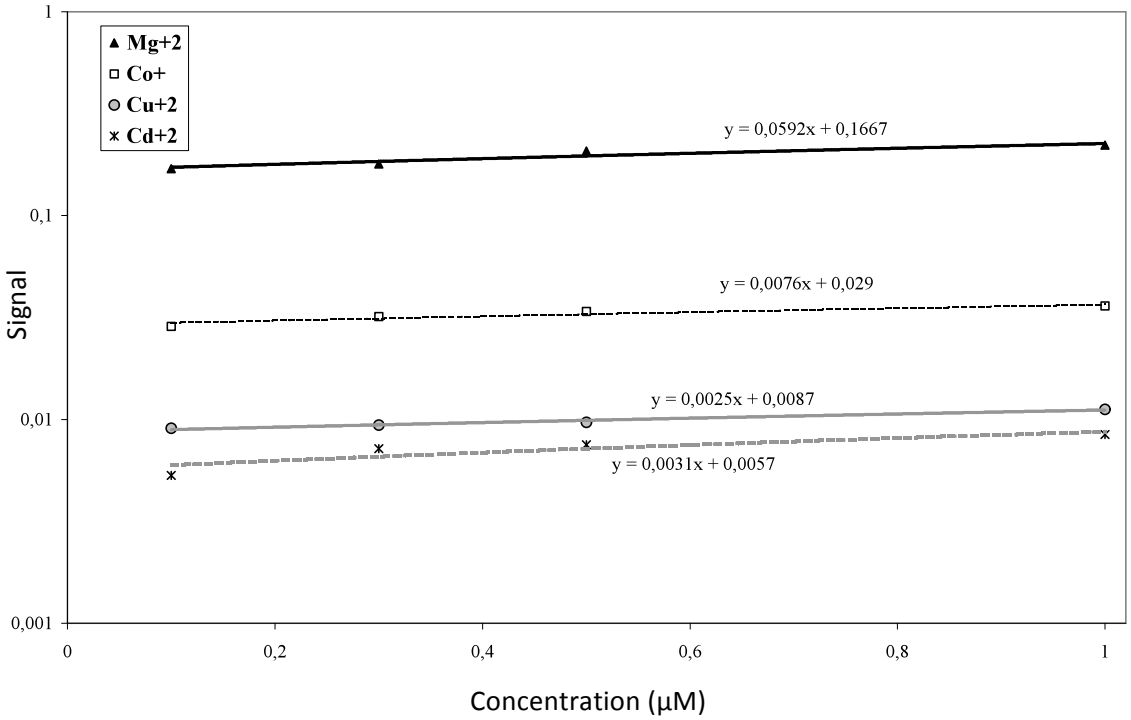


Figure 8 a) Behaviour of heavy metals adsorption on calixarene-SAM in different zones of detection Zone 1

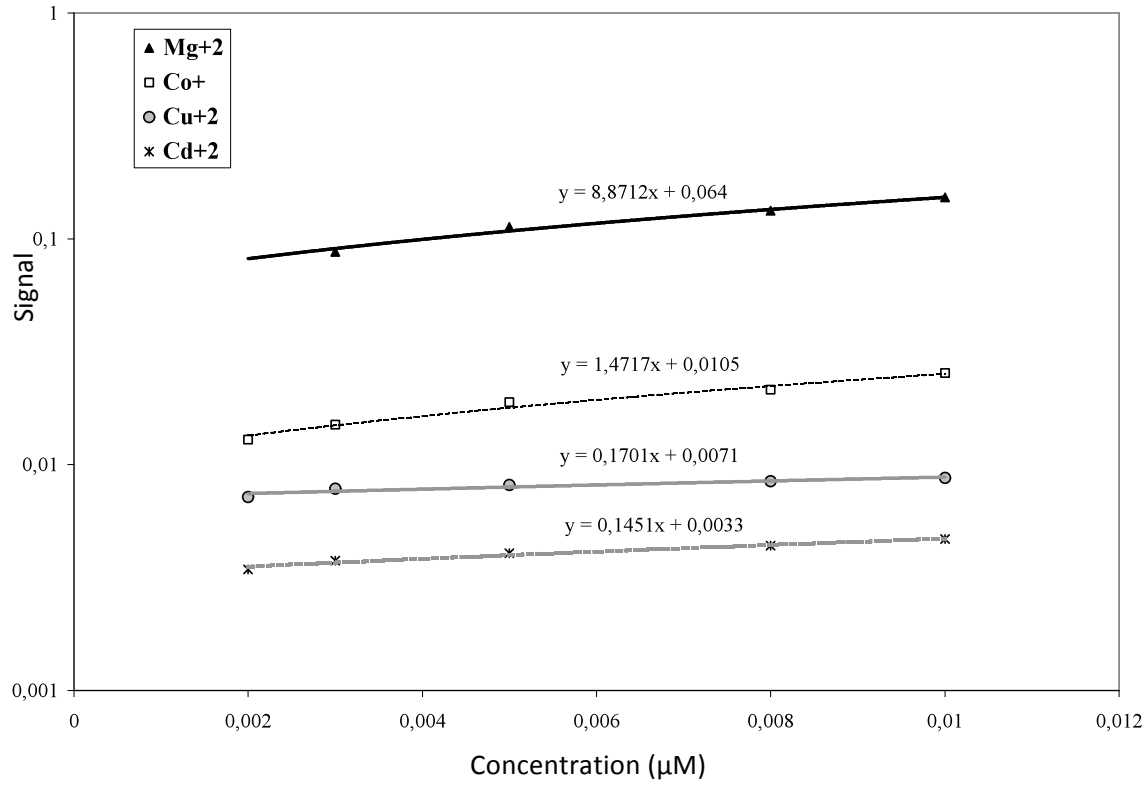


Figure 8 b) Zone 2

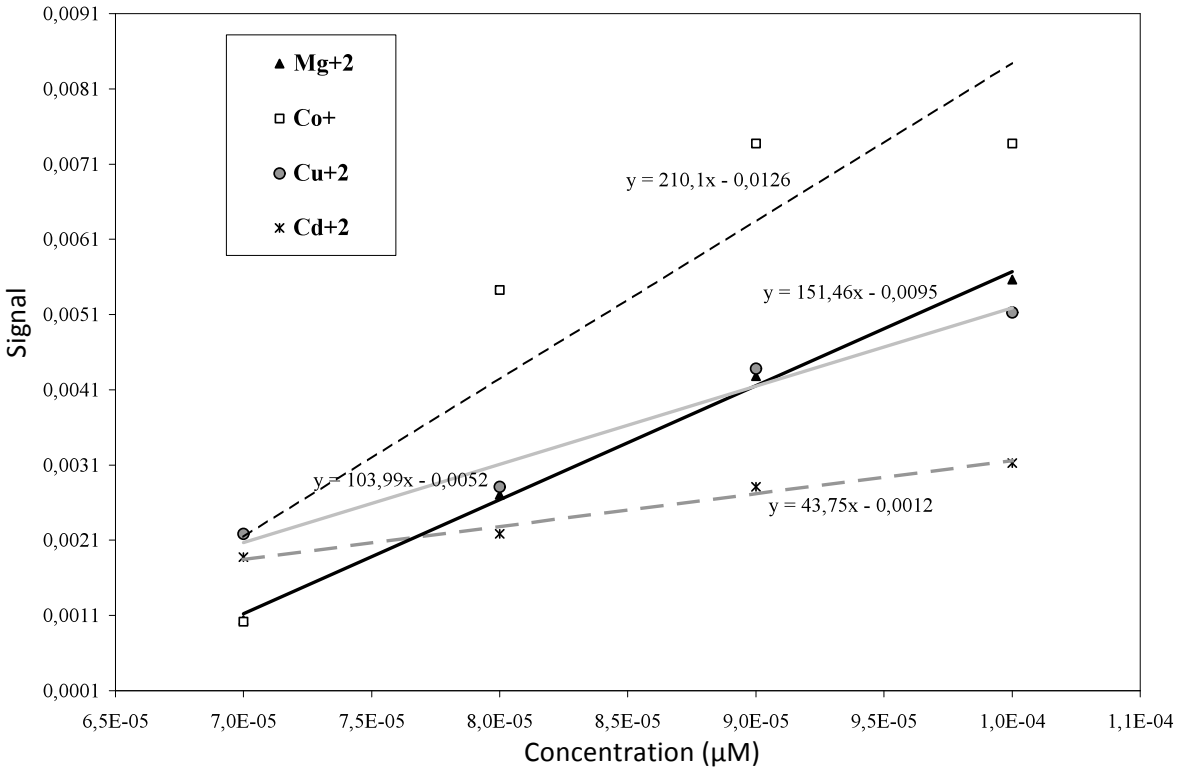


Figure 8 c) Zone 3

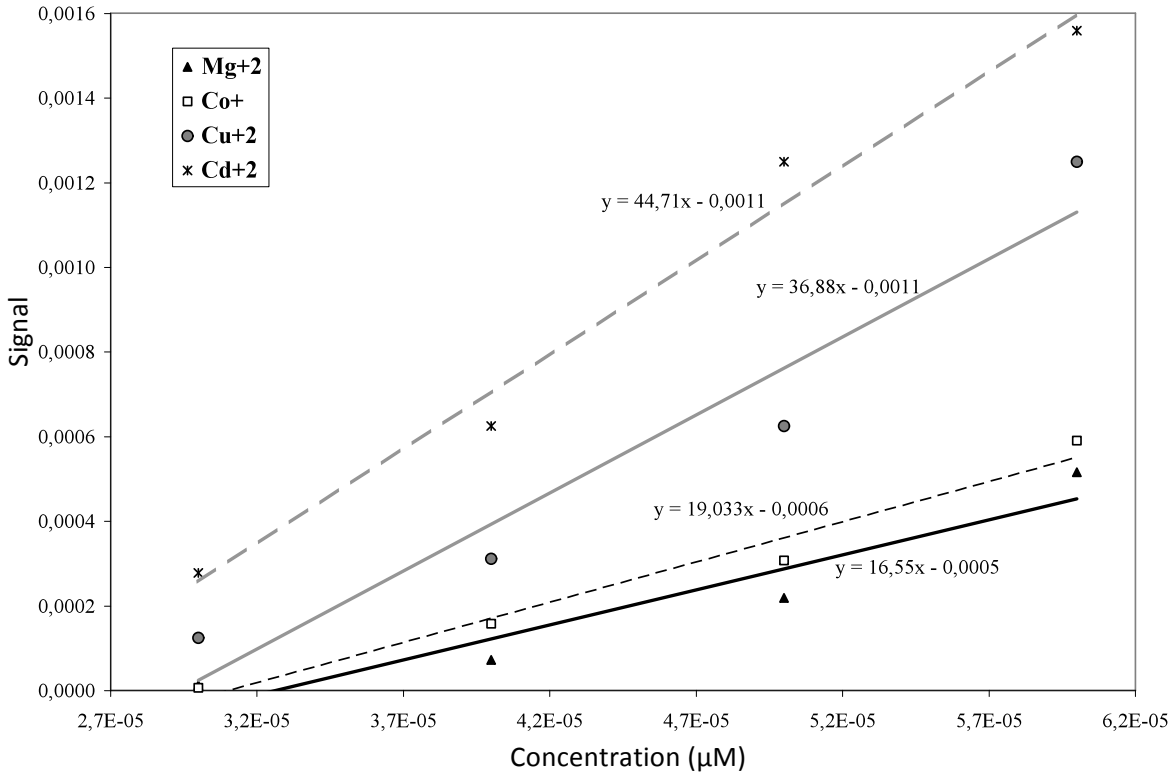


Figure 8 d) Zone 4

	Sensitivity (μM^{-1})			
	ZONE 1	**ZONE 2**	**ZONE 3**	**ZONE 4**
Mg^{+2}	0.0592	8,870	151,46	16,55
Co^{+2}	0.0076	1,470	210	19,033
Cu^{+2}	0.0025	0,170	103,99	36,88
Cd^{+2}	0.0031	0,145	43,75	44,71

Table 1 Sensor-sensitivities for the different zones: Oxidation procedure

In case of the second functionalization procedure where pyridine was made to protect oxygen donor group of calixarene (Fig. 9), copper and cadmium are only detected between 0 and 1µM. The detection-limit reached for Cd^{+2} is at about 10^{-5} µM and does not exceed 10^{-4} µM for copper. To determine the sensor-sensitivity, linear smoothing curves were plotted for each zone-detection. The same behavior was observed previously and a linear variation of a signal was shown for each heavy-metal in all detection-zones. Results obtained in Tab. 2 show different sensitivities of sensors for copper. The values obtained are 0.0024, 0.038, and 47.42μM^{-1} in the first, second, and third detection-zone, respectively. In case of the Cd^{+2} ion, the sensitivity values obtained are 0.0047, 0.60 to 4.12 and 20.42μM^{-1} for high concentration, second, third, and low concentration-zone, respectively.

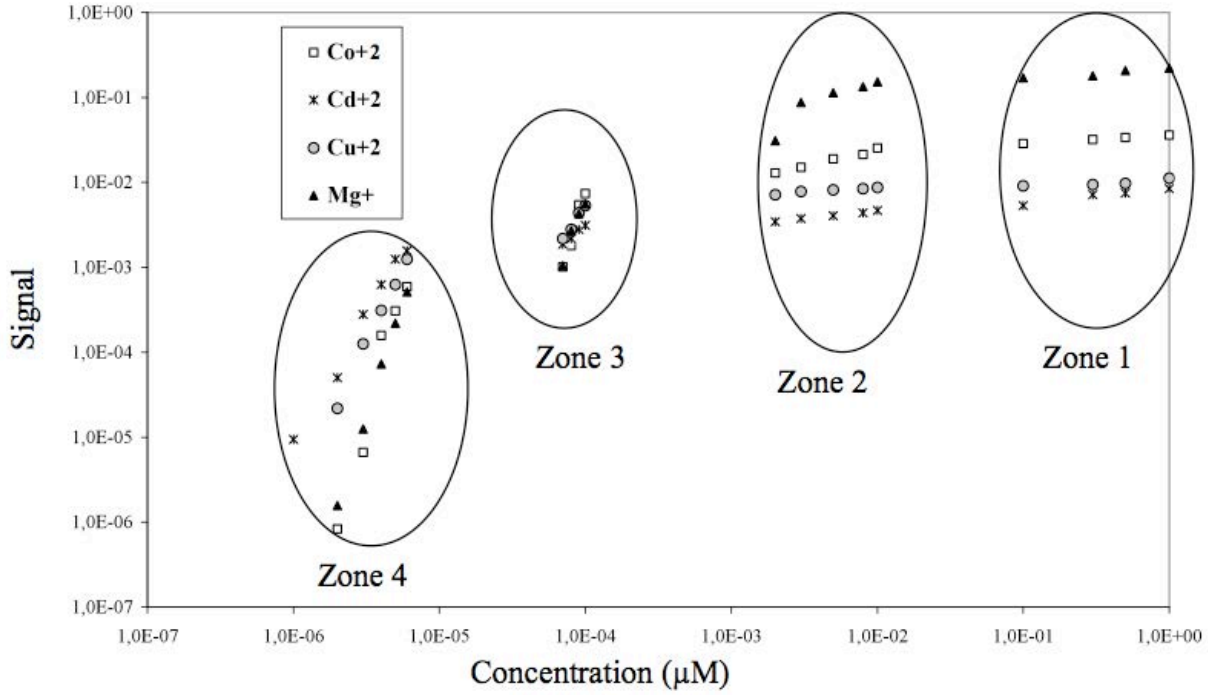


Figure 9 Calibration curve of SPR calix[4]arene SAM sensor for different zones of heavy metals detection: Second functionalization procedure

	Sensitivity (μM^{-1})			
	ZONE 1	**ZONE 2**	**ZONE 3**	**ZONE 4**
Cu^{+2}	0,0024	0,038	47,42	-
Cd^{+2}	0,0047	0,606	4,13	20,42

Table 1 Sensor-sensitivities for the different zones: Second procedure

Comparative results show that the second functionalization procedure is not adequate for heavy-metal detection and that the oxidation procedure is better, hence preferable for these types of ions.
We showed that signal is proportional to the capacity of adsorbed ions on the sensor-surface, which is in agreement with literature (Parsons et al., 1995; Karlsson, and Falt, 1997; Paarmann et al., 2002).
Different detection-limits observed for various heavy metals can be explained by several parameters: Adsorption potential and formation of various metal hydroxides which are not the same for different heavy-metals ion and oxygen deprotenation charging the sensor surface negatively (Halouani et al., 2002; Ma et al., 2001; Muñoz and Palmero, 2004; Farghaly, 2003). Accordingly, the incorporation of ions by the calix[4]arene-SAM can be favored by electrostatic interactions which directly influence the stability of complexes formed as well as its association binding (Freire and Kubota, 2004: Tsukube et al., 1997). Although there is an ionic force in aqueous media and interactions between host and guest such as π-stacking, dipolar-dipolar interactions have a strong influence on the stability of the complexes (Tsukube et al., 1997; Gong et al., 1998; Gocmen and Cakil, 1993; Rounaghi et al., 1997; Schneider, 1996; Bonas et al., 1998).

4. CONCLUSION

We demonstrated the possibility to use a new functionalized chromgenic calix[4]arene molecule onto SAM of cysteamine for the determination of ions in real samples. Such coupling of favorable characteristics is most likely the key to achieving the remarkable sensitivity to ions of the proposed sensors. We showed that ion detection depends on functionalized procedures and the pH of the solution. This work opens the way to sensors for selective ion detection and to the use of calix[4]arene in the field of biochemistry.

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