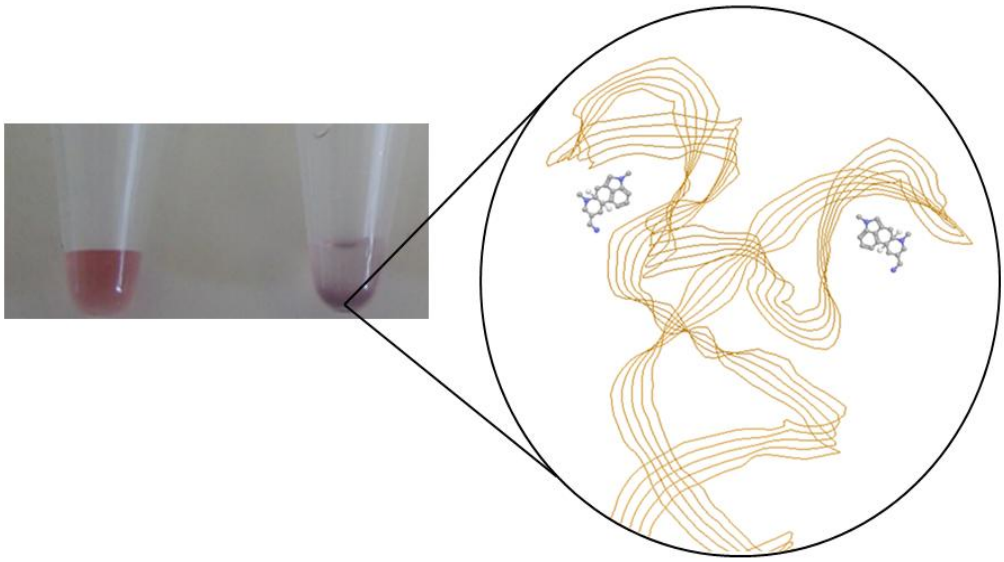
Thesis

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Wetenschappen aan de Universiteit Antwerpen

Dissertation for the degree of Doctor in Sciences at the University of Antwerp

Development of a biosensor for ergot alkaloids



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Antwerpen 2016

sphere

This study was funded by the Federal Public Service of Health, Food Chain Safety and Environment (FOD) project Ergot RF6204. This PhD thesis was supported by a Dehousse grant and by the Institute for Agricultural and Fisheries Research (ILVO).

Acknowledgements

Regarding this PhD project, the first person to be acknowledged is Dr. Johan Robbens, who wrote this topic, which was accepted by the Federal Public Service of Health, Food Chain Safety and Environment (FOD). I am very grateful to Dr. Johan Robbens for having initiated projects about biosensing technologies and gathering different skills in his team, which revealed to be a fruitful research. I also thank Prof. Ronny Blust for allowing the realization of this work in the field of biosensing technologies, which was uncommon for a laboratory of physiology and ecotoxicology. Thanks to this support, it was possible to work on a passionating and innovative project, opening new horizons in the very recent context of biotechnologies, and more especially the use of biological ligands in sensors.

Regarding my past as a scientist, and more particularly as a chemist, the use of ligands to bind to pollutants was already one of my favorite topics, as I worked on ligands used for the extraction of lanthanides used as models for the extraction of radioactive elements from radioactive effluents during my Master in Chemistry at the University of Burgundy, under the supervision of Prof. Michel Meyer (University of Burgundy, France). There, I had the opportunity to synthesize chemical ligands binding to lanthanides, study the complexes formed and finally graft them onto a silica columns allowing the extraction of lanthanides from solutions. These ligands were then used by the Commission à l'Energie Atomique to remove radioactive elements from radioactive effluents. Although I enjoyed working on this project, I wished this process would serve other applications than supporting nuclear energy industry and would rather be used for environmental purposes such as the decontamination of radioactively polluted sites. This different point of view about the use of the ligands synthesized, and in a more general way about the use of radioactivity, made me renounce doing a PhD there. However, the study was very interesting and I particularly enjoyed working on Europium element. There, I also learnt a lot about ligands and I always thought to come back one day to the synthesis and use of new ligands for environmental purposes.

Then, I started my PhD at the University of Antwerp with Dr. Johan Robbens, who brought me towards the very recent use of synthetic DNA ligands, named aptamers, which can be specifically selected for any compound of interest and used as sensing tools in biosensors. As the use of biological ligands was new for me, I had to learn about the techniques of molecular biology, such as DNA selection and amplification, and molecular cloning. Fortunately, Dr. Jaytry Mehta, molecular biologist and mathematician, was there in the team of Dr. Johan Robbens, together with Dr. Bieke Van Dorst, bioengineer, to help me with this part. I am especially thankful to Dr. Jaytry Mehta, who friendly taught me a lot about aptamers and all the practical work in the laboratory. I really appreciated our constructive talks, which helped me to have a better understanding of the phenomenons underlying the results we obtained. It was always a pleasure to work with you, Jaytry. Bieke Van Dorst is also a person I appreciated a lot and I have to thank many times for at least all the

publications we wrote together. Unfortunately, I arrived in the laboratory when Bieke was ending her PhD, and we did not have so much time to spend together in the laboratory, neither to run all the experiments we wished.
Throughout this innovative project involving chemistry and biology, I needed the help of quite a few scientists. Along the way, I met interesting people, who have really played an important role in my PhD.
First, Prof. Sarah De Saeger is to be thanked for having organized the Ergot FOD project, having allowed me working with ergot alkaloids and having provided me the standards as well as natural ergot contaminated samples. In this same team of the Ergot FOD project, I thank Prof. Peter Dubruel for his interesting suggestions about organic synthesis.
Regarding the problems that arised in this project, I am very grateful to Prof. Bert Maes, who nicely guided me to find a way to obtain the ergot alkaloid template, on which the study was based. Dr. Tom Raws is to be thanked for having done the little-risky chemical cleavage of metergoline. Then, I thank Prof. Erik Goormaghtigh, who helped me with the infrared analysis of ergot-coated material. Also, I thank Prof. Wouter Herrebout for having made possible the infrared analysis in his laboratory. I also thank the team of Prof. Lemiere and Norbert Hancke for having analyzed by mass spectrometry my synthesis products.
Prof. François Van Hove is acknowledged for his interest in this topic and for having evaluated the results obtained as a jury member of the FOD project. I especially thank Prof. François Van Hove for having elucidated the mechanism underlying the results obtained with the surface plasmon resonance analysis, and improving the quality of the article that was published about the selection of aptamers for ergot alkaloids.
I have to thank Dr. Larysa Darchuck for having done a Raman analysis of the complexes of DNA and ergot alkaloids. Unfortunately, due to the complexity of the results, this analysis could not be completed as Larysa's contract was already ending. I really wished we could proceed further with this analysis, and maybe write an article about it.
Then, I thank Prof. Adrian Covaci and Lic. Walid Maho for having done the mass spectrometry analysis of the samples in the study of extraction of ergot alkaloids from naturally ergot contaminated samples by DNA-supported silica.
I also thank all the jury members for the reviewing of this thesis and their comments and suggestions in the redaction of this thesis.
Then, I thank the members of the SPHERE (Laboratory of Systemic Physiological and Ecotoxicological Research) laboratory, alias EBT, who made it a good working atmosphere.
The SPHERE laboratory, of what it became in a more personnal view, "my sphere of innovation in sensing technology and extraction systems", was a pleasant environment for the realization of this PhD project. I sincerely hope that this present thesis will be representative of the work accomplished and informative enough for the reader to enter the huge world of small molecules.
And finally, I have to thank my great family for always having supported me in my projects.

Table of Content

List of abbreviations

AFM: atomic force microscopy
APTS: 3-aminopropyltriethoxysilane
ATP: adenosine tri-phosphate
bp: base pair
CE: capillary-electrophoresis
cfu: colony forming unit
CTS: 3-chloropropyltrimethoxysilane
DABCYL: 4-((4-(dimethylamino)phenyl)azo)benzoic acid
DDS: dichlorodimethylsilane
DMF: dimethylformamide
DNA: deoxyribonucleic acid
dNTP: deoxyrobunucleotide triphosphate
ds: double-stranded
EDTA: ethylenediamine tetraacetic acid
EFM: electromotive force
EFSA: European food safety authority
FOD: Federale Overheidsdienst
FRET: fluorescence resonance energy transfer
FTIR-ATR: Fourier transform infrared attenuated total reflectance
GC-MS: gas-chromatrography mass-spectrometry
hCG: human chorionic gonadotropin
HIV: human immunodeficiency virus
HPLC: high-pressure liquid-chromatography
IDT: Integrated DNA Technology
ILVO: Instituut voor Landbouw en Visserij Onderzoek
ITC: isothermal titration calorimetry
IUPAC: International Union of Pure and Applied Chemistry
LC-MS/MS: liquid-chromatography tandem mass-spectrometry
LC-QTOF-MS: liquid chromatography quadrupole time-of-flight mass-spectrometry
LNA: locked nucleic acid
LOD: limit of detection
LOQ: limit of quantification
LSD: lysergic acid diethylamide
MIP: molecularly imprinted polymer
NMR: nuclear magnetic resonance
NHS: N-hydroxysuccinimide
OTA: ochratoxin A
PCR: polymerase chain reaction

PEG: polyethylene glycol
PNA: peptide nucleic acid
POC: point-of-care
ppm: part per million
QD: quantum dots
QCM: quartz crystal microbalance
RET: resonance energy transfer
RNA: ribonucleic acid
rpm: round per minute
RT: room temperature
RU: resonance unit
SAM: self-assembled monolayer
ScFv: single-chain antibody fragment
SELEX: systematic evolution of ligands by exponential enrichment
SPHERE: laboratory of Systemic Physiological and Ecotoxicological Research
SPR: surface plasmon resonance
ss: single-stranded
TAE: Tris/Acetate/EDTA
TCEP: tris(2-carboxyethyl)phosphine
TLC: thin layer chromatography
Tm: melting temperature
Tris: tris(hydroxymethyl)aminomethane
UPLC: ultra performance liquid chromatography
USD: United States Dollar
UV-Vis: ultraviolet-visible
VIB: Vlaams Instituut voor Biotechnologie

Introduction

1. Definition and history of ergot

Ergot is the name commonly used to describe a cereal crop contamination by fungi members of the *Clavipitaceae* family, such as the *Claviceps* species (*Claviceps purpurea*, *Claviceps fusiformis*, *Claviceps sorghi*, *Claviceps africana, Claviceps sorghicola*…), *Neotyphodium* species and *Epichloe* species [1-9]. Several cereal crops and grasses can be parasited by these fungi; the most likely to be contaminated being rye (*Secale cereale*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), fescue grass (*Festuca rubra*) and bluegrass (*Poa pratensis*) [1-9]. Ergot poisoning or ergotism is a disease due to the ingestion of ergot contaminated cereal crops, which can be lethal to humans and animals [10-13]. During millenaries, hundreds of thousand people died because of ergotism and epidemics occurred periodically in all continents. The first authenticated reports about ergot were written in China in 1100 BC, in which the fungal contamination is described, as well as the oxytocic properties of ergot alkaloids, used in obstetrics [10-11]. Some documented epidemics of gangrenous ergotism were reported in 944 in France when twenty thousand persons died and then fifty years later, forty thousand persons died after consumption of contaminated bread [11]. In Germany, epidemics were reported several times during the sixteenth century where another form of ergotism was characterized by hallucinations and convulsions [13]. Finally, epidemics occurred periodically in different continents until nowadays. The last important epidemics in humans occurred in India (1975), in Russia (1995) in Australia (1996) and in Ethiopia (2001) [14-17]. A general spread of ergot contamination has been observed, mainly due to globalization and the intensive culture of hybrid and genetically modified species of cereal crops, which were shown to be less resistant to ergot contamination [18-20]. However, ergot contamination is better controlled and epidemics are less likely to occur nowadays thanks to changes in farming practices, including deep plowing buries, post-harvest field burning, rotations with non-host plants and seed cleaning [1, 15]. Though, ergot contamination in the wild nature remains an important veterinary problem, particularly in cattle, horse, sheep, pig and chicken [15, 21-26]. The effects of ergot poisoning can differ depending on the composition of the fungal structures, which can produce more than forty ergot alkaloids in various proportions. Two forms of ergotism have been reported: gangrenous ergotism and convulsive ergotism [10-13]. The initial symptoms of gangrenous and convulsive forms are described to be similar but after a short period of vague illness, the symptoms separate into two distinct patterns. In the case of gangrenous ergotism, ischaemia starts in the limbs, followed by an intense burning sensation, called Holy Fire or St Anthony's Fire, and gangrene can occur. Abortion of pregnant women can occur as well. In the case of convulsive ergotism, the symptoms were characterized by painful involuntary flexion or extension of the muscles in the limbs, especially the hands, hallucination, fever and epileptic seizures, with a mortality rate of 10-20% [10]. The complexity of the content of ergot

sclerotia remained during a long time a "chemical mess" and it took sometimes a lifetime for scientists to isolate ergot alkaloids from natural extract of sclerotia and understand their mechanism of action [27]. The French pharmaceutical chemist Charles Tanret can be quoted for having isolated some ergot alkaloids [13]. In the 1900's, Henry Dale, George Barger and Arthur Ewins isolated some ergots alkaloids and characterized them [10]. The other main group of scientists to be quoted is the team of Arthur Stoll and Albert Hofmann, regarding the isolation, characterization of ergot alkaloids and the elaboration of new synthetic ergot alkaloids [13]. In 1943, Arthur Stoll and Albert Hofmann demonstrated that ergotoxine was a mixture of three closely related ergot alkaloids: ergocornine, ergocristine and ergokryptine [27]. As the biological effects of some ergot alkaloids were better understood, some of them were used for pharmaceutical purposes. For instance, ergotamine is used as a medicinal drug against migraine. An important research effort was made until now to isolate, separate and characterize ergot alkaloids, as well as to unravel their mechanism of action [11, 27].

In the first part of this review, the main species of fungi and plants producing ergot alkaloids will be described. Then, an overview of the economic impact of ergot will be presented. Finally, the different techniques used for the detection of ergot alkaloids nowadays will be discussed.

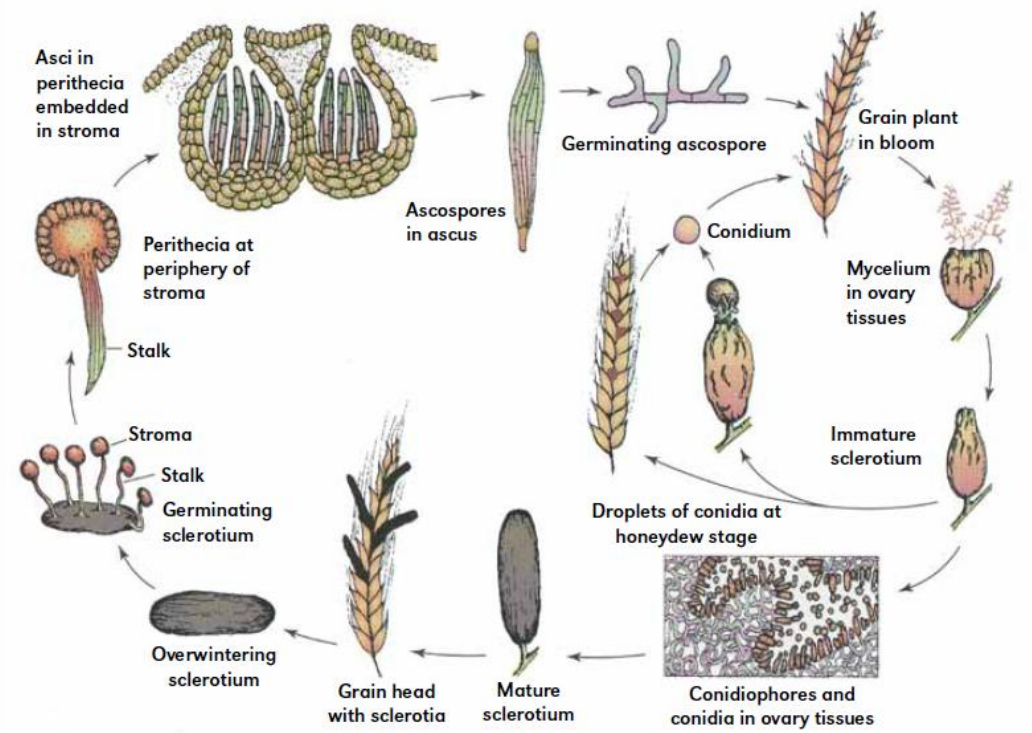
2. Fungi and plant species producing ergot alkaloids

2.1. Main species of the genus *Claviceps*

The presence of ergot akaloids in food and feed is due to the contamination of cereal crops by fungi, which produce these mycotoxins. Most of these fungi belong to the *Clavipitaceae* family. There are several distinct species of ergot fungi parasiting grain crops and grasses of economic importance: *Claviceps purpurea*, *C. africana*, *C. fusiformis*, *C. paspali*, *C. cyperi*, *C. gigantea* and *Sphacelia sorghi* [28]. The species of the genus *Claviceps* have evolved to adapt to different host species and climates, resulting in various features and morphologies. For instance, *Claviceps purpurea* is mainly present in Northern countries and usually develops onto rye or wheat; while *Claviceps africana* is more likely to be found in warm countries such as Africa or South America and preferably contaminate sorghum or grasses [5]. *Claviceps paspali* is another species originating from South America, and is only known to colonize grasses of the genus *Paspalum*. Also, important variations can be seen within the same species. For instance, *Claviceps purpurea* species was divided in three groups (G1, G2 and G3) based on habitat association, sclerotia and conidia morphology, as well as alkaloid production [29]. All the fungi of the genus *Claviceps* have in common the production of sclerotia in the host florets or inflorescence, and form hard compact mass of fungal tissues containing ergot alkaloids. The ergot sclerotia usually contain 30-40% of fatty acids and up to 2% of ergot alkaloids [30]. The others components of sclerotia are free amino acids, ergothionine, uracil, guanidine, free aromatic and heterocyclic amines (tyramine, histamine,

agmatine) and alkylamines [30]. The ergot fungi have two stages of parasitic development: the sclerotial stage and the sphacelial stage [3, 30]. When a spore of the fungus contaminates a floret of cereal cro, it replaces the seed in the ovary of the plant, mimicking a pollen grain growing onto the plant, and forms a compact fungal mass, or sclerotium, containing the ergot alkaloids (**Figure 1**). During winter time, the sclerotium falls onto the ground and remains dormant until spring season. Then, it germinates, forms fruiting bodies and finally ejects spores, which can contaminate other cereal crops and grasses. Then, the sclerotium falls onto the ground and remains dormant during winter-time. During spring or rainy season, it germinates by forming stromata which release ascospores, which can contaminate other florets. During this sphacelial stage, there is no production of ergot alkaloids in the fungal structures. *Claviceps* species can also form sticky honeydew, attracting insects, which are responsible for secondary spread of the conidia [31].

Figure 1. *The life cycle of Claviceps species on small grain cereals and grasses [3].*



Claviceps purpurea

Claviceps purpurea is one of the most abundant species of the genus *Claviceps*. It can infect more than 400 different cereal crops and grasses, and it is widely spread around the world, especially in the Northern hemisphere, such as Europe and North America [5, 32-37]. *Claviceps purpurea* could adapt to different host plants and climates, and this species was divided into three groups: G1 group associated with terrestrial grasses, G2 group associated with wet and shady environments, and G3 in salt marsh habitats [29]. *Claviceps purpurea* is

an important cereal crop infecting fungus, significantly reducing seed yield [5]. The species from the subfamily Pooidea are very susceptible to ergot contamination; such as the species *Agrosis*, *Avena*, *Festuca*, *Lolium*, *Poa*, *Secale* and *Triticum*. During the Middle-Ages, rye (*Secale cereale*) and wheat (*Triticum* species) were regularly concerned by the contamination by *Claviceps purpurea*, which led to important epidemics killing thousands of people when bread was prepared with contaminated flour [10, 13]. Nowadays, with the better understanding of ergot contamination, cereal grains are usually sorted and epidemics of ergotism are considered less likely to occur in developed countries. However, the high level of pollution, especially the presence of metal ions in soils, encouraging the development of fungi and the introduction of hybrid cereal crops in the fields, which were shown to be less resistant to fungi contamination, are supposed to be responsible for a recent increase of contamination by the different ergot species [1]. For instance, it was observed that transgenic wheat *Triticum aestivum* lines expressing the wheat Pm3b gene against the fungus powdery mildew *Blumeria graminis f.sp. tritici.* had a 40-fold increase of infection by *Claviceps purpurea* compared to the control lines in the field [18]. It was also observed that male sterile rye was more likely to infection by *Claviceps purpurea* than common rye, probably due to the fact that florets of male sterile rye remain open for a longer time, allowing the inoculum a greater access to the unpollinated ovary of rye [19].

On a structural aspect, the black sclerotia of *Claviceps purpurea* can easily be distinguished from the grains due to the long shape of the sclerotia and the high contrast in color with the grain, typically light yellow (**Figure 2**). Due to the contrasting color of the sclerotium and the fact that it contains compounds which can be lethal to humans and animals, *Claviceps purpurea* was suggested to be an aposematic fungus [38]. The dark color can be interpretated to be a warning signal to prevent animals from eating the contaminated plants, providing to the plant an enhanced resistance to insects and herbivores [38]. For instance, it was observed that cattle shows reluctance to eating contaminated grains [39, 40]. A certain symbiosis can exist between *Claviceps purpurea* and the host plant; the plant can benefit from this enhanced resistance to herbivores, while the fungus can benefit from nutrients and photosynthetic resources. However, a high level of contamination of cereal crops by *Claviceps purpurea* can lead to a serious decrease in the seed production yield of the plant and a depletion of it. Regarding the toxicity of *Claviceps purpurea* sclerotia, it has been shown that a concentration of more than 0.1% of ergot sclerotia in feed could be harmful to animals [39]. The black sclerotia of *Claviceps purpurea* can contain more than 40 biologically active molecules, called ergot alkaloids. The content of ergot alkaloids produced by *Claviceps purpurea* tends to be rich in ergopeptines; such as ergocristine, ergotamine, ergosine, ergocornine and ergokryptine. According to the common ergot alkaloids pattern in *Claviceps purpurea* sclerotia, the main ergot alakoid found is usually ergocristine representing 35% of the total ergot alkaloid content, ergotamine 25%, ergosine 20% [41-43]. Although the composition of sclerotia differs depending on geographical and climatic conditions, these three ergotoxins are usually found in more important proportion than the

others. Ergocornine usually represents about 10% of the ergot content and ergometrine (also called ergonovine) up to 10% as well [42].

Figure 2. *Photographs of Claviceps purpurea. Left: Claviceps purpurea contaminating oats (Avena sativa). Right: Claviceps purpurea contaminating fescue grass (Festuca perrenium).*

Claviceps africana, sorghum ergot

Claviceps africana is a second widely spread fungus species, which is mainly found in the Southern hemisphere, in countries such as South America, Africa, India and Australia [5]; as *Claviceps africana* develops preferably in warm or sub-tropical climates. The principal hosts for *Claviceps africana* include *Sorghum bicolor Moench* and other sorghum species as well as their hybrid derivatives [20, 44, 45]. The life cycle of *Claviceps africana* is similar to the one of *Claviceps purpurea*. However, the fungal structures of *Claviceps africana* are different in appearance, as they have a rounded shape light yellow on the top and orange at its basis, and produce sticky honeydew, which disseminates the spores of the fungus (**Figure 3**). The sphacelium of *Claviceps africana* is the initial structure of the fungus, which can exude sticky honeydew, a mixture of conidia and liquid mixture. The sclerotia formed as dormant fungal structure do not produce honeydew. However, both the sphacelium and sclerotium of *Claviceps africana* contain ergot alkaloids. The ergot alkaloid content of *Claviceps africana* differs a lot from *Claviceps purpurea*. Instead of ergopeptines, the ergot alkaloid pattern of samples of *Claviceps africana* usually contains a preponderant amount of dihydroergosine (80%), dihydroelymoclavine and festuclavine [46, 47]. Due to the fact that the spores of *Claviceps africana* can be transported through long distances by the wind, the fungus rapidely spreads through large geographic areas. This fungus is originally better adapted to warmer countries but recently reached more temperate countries. Historically speaking, local and occasional outbreaks of *Claviceps africana* were observed in the 1960s. Then, a high incidence of ergot occurred in male sterile sorghum breeding lines in Nigeria during

1963-1965 [48, 49]. In the 1990s, *Claviceps africana* spread to Southern African region, associated with the expansion of the regional F1 hybrid seed production programme [48, 50]. This was followed by a sudden worldwide spread of *Claviceps africana*. It was observed in Brazil in 1995; in South America and Australia in 1996; in Central and North America, as well as the Caribbean in 1997 [5, 20, 48]. The rapid spread of *Claviceps africana* worldwide was attributed to the use of hybrid lines of sorghum, the introduction of genetically modified species and globalization, as main factors [20, 48, 50, 51]. The presence of *Claviceps africana* on sorghum has an important economic impact, as it leads to significant seed loss. Moreover, the sticky honeydew can make seed harvest difficult or impossible. For instance, losses from 10% to 80% in India and about 25% in Zimbabwe were reported due to contamination by *Claviceps africana* [20].

Figure 3. *Photograph of sorghum contaminated by Claviceps africana (sorghum ergot) [20]*.

Claviceps cyperi

Claviceps cyperi can parazite grasses, such as nut sedge grass (*Cyperus esculentus*) [52]. The dark brown sclerotia of *Claviceps cyperi* produce peptide ergot alkaloids, such as ergokryptine, ergosine, ergocornine, ergocristine and ergotamine [24, 52]. Various outbreaks of ergotism were reported since 1996 in dairy cattle consuming maize silage and teff hay contaminated with ergotised nut sedge grass [24]. The typical syndroms observed in cattle infected by *Claviceps cyperi* are hyperthermia and aglactia [24].

Claviceps gigantea

Claviceps gigantea occurs on maize (*Zea mays*). This pathogen was observed in the region of Mexico. The sclerotia of *Claviceps gigantea* are, as suggests their name, of a big size. These sclerotia only contain small quantities (0.03% of sclerotia) of dihydro-derivatives of agroclavine and elymoclavine, which are of weak pharmacological importance compared to the unreduced ergot alkaloids [53]. Festuclavine and chanoclavine were also found, but no peptide alkaloids nor free lysergic acid [54]. Nevertheless, the presence of this pathogen can reduce the maize harvest yield by 50%, which is not neglectible for the food industry [55].

Claviceps paspali

This species originating from South America and has spread through America, Australia, Southern Africa and the Mediterranean region since 1950 [56]. Since then, *Claviceps paspali* occurs sporadically in these regions. This species colonizes grasses of the genus *Paspalum*, used in animal feed, such as *Paspalum dilatatum*. The presence of *Claviceps paspali* in feed can induce neurological disorder in grazing cattle, and can lead to death in case of unability to obtain water [57]. Ergot poisoning by *Claviceps paspali* can induce a "staggers" syndrome in sheep, horse and cattle; causing a degeneration in some brainstem nuclei and the spinal cord [23, 57-58]. The main ergot alkaloids produced by *Claviceps paspali* are lysergic acid, ergometrine and lysergic acid hydroxylamide [59].

Neotyphodium coenophialum

Neotyphidium coenophialum is a fungus of the *Clavipitaceae* family, which parasites grasses such as tall fescue. This fungus is widely spread around the world and especially in North America, Eurasia, Africa and Australia [25, 60]. Tall fescue is the most widely utilized perennial ryegrass in the world. It is for instance the most important forage in the USA, with 14 million ha. Tall fescue is regularly concerned by infection by *Neotyphodium coenophialum*. This clavicipataceous endophyte was originally identified as *Epichloë typhina,* later renamed *Acremonium coenophialum* and now known as *Neotyphodium coenophialum* [25]. The ingestion of contaminated fescue by livestock can cause a disease know as "fescue toxicosis", characterized by weight loss, gangrene of feet and ears, necrosis, elevated body temperature, reduced conception rates and aglactia. Two types of alkaloids are responsible for fescue toxicosis by *Neotyphodium coenophialum*: ergot alkaloids and lolines alkaloids. The ergot alkaloid content usually comprises 10 to 50% of ergovaline and other ergopeptines such as ergotamine, ergokryptine and ergocornine [25, 61]. These ergopeptine alkaloids are structurally similar to the biogenic amines serotonin, dopamine, adrenalin and noradrenalin and have affinity for their receptors. Interaction of the ergopeptines with these biogenic amine receptors alter the normal homeostatic mechanisms, resulting in hyperthermia or gangrenous ergotism [25, 62].

2.2. Superior plants producing ergot alkaloids

Lysergic acid derivatives can also be found in the seeds of the flowers of *Ipomoea violacea* (Morning Glory), *Argyreia nervosa*, *Rivea corymbosa* and a few other members of the *Convolvulacea*. The major active compound in these plants is ergine, or lysergic acid amide, but other ergot alkaloids such as ergometrine, lysergic acid alpha-hydroxyethylamide and also clavine alkaloids can also be found in small amounts.

Ipomoea violacea

Ipomoea vioalacea is an ornamental plant from the *Convolvulacea*, which also produces ergot alkaloids. *Ipomoea violacea* has been used by Mexican shamans during millenaries, but

its content was unknown until the 1960's. It was first difficult to believe that other organisms than fungi could also produce ergot alkaloids, until the ethno-botanist Richard Schultes sent some samples to Albert Hoffman in 1959, who analyzed them and confirmed the presence of ergot alkaloids [63]. This plant with funnel-shaped leaves and bright white, pink, purple or blue flowers contains in its black seeds some lysergic acid amide, chanoclavine, elymoclavine and lysergol [64]. It was determined that one seed usually contains approximately 0.01 mg of lysergic acid amide.

Argyreia nervosa

This plant, native from the Indian subcontinent, belongs to the family of *Convolvulacea* as well; and comprises some 30 species, which were introduced to numerous areas worldwide [65, 66]. The composition of the seeds of *Argyreia nervosa* contains the highest concentration of psychoactive compounds in the entire family [58]. One seed of *Argyreia nervosa* usually contains about 0.25 mg of lysergic acid amide, and some other clavine alkaloids [67].

3. Economic impact of ergot

Besides the food safety and health concerns, ergot also represents an economically devastating issue. By uptaking the carbon absorbed during photosynthesis, ergot delays grain maturity and causes consequently losses of production and devalorisation of grain quality. Moreover, in the case of *Claviceps africana*, the sugary exudates produced by the fungus makes harvesting and seed processing more difficult. Nowadays, ergot usually causes 5 to 10% yield loss in small grains [2]. According to the 2013 producer harvest samples collected and analyzed by the Grain Research Lab (GRL) at the Canadian Grain Commission, 18% of Canadian Western Red Spring wheat samples and 10.7% of the Canadian Western Amber durum samples received from across Western Canada were downgraded due to ergot [68]. In Europe, several recent studies show that the incidence of ergot in cereals nowadays is ranging around 50% in rye and triticale, and approximately 35% in wheat [69, 70]. The spread of ergot has been increasing a lot this last two decades. For instance, rye, wheat and barley were recorded to contain an average of 65%, 40% and 11% respectively in Lithuania between 1996 and 2000; whereas the incidence of ergot contamination was rare before 1990 [37]. Hybrid seed industries are particularly affected by the presence of ergot [18-20, 44, 45]. The susceptibility of male sterile plants to infection is due to to the fact that their florets remain open for a long period of time and allow the inoculum to easily access the unpollinated ovaries. For instance, losses of 10 to 80% of hybrid seed production in India were reported this last decade due to ergot infection [20]. Also, a widespread infection of cereal crops caused by *Claviceps africana* occurred in Brazil leading to the loss of 3 million USD for the hybrid seed industry in 1995. Losses in seed production fields ranging between 10% and 80% were reported in India and 10% to 25% in Zimbabwe in 1997 [20]. Texas, which

produces 95% of the United States hybrid sorghum (40 000 ha) was also seriously affected. In 1997, 45% of hybrid sorghum fields were parasited; in 2002, more than 80% and finally in 2004 approximately 90% [20]. A study on genetically modified wheat (*Triticum aestivum*) expressing the *Pm3b* gene against the fungus mildew (*Blumeria graminis*) has shown that it was less resistant to ergot infection by *Claviceps purpurea* when grown in the field [18]. The fact that hybrid cereal crops are more likely to be contaminated by ergot is also increasing the spread of the fungi to other species. The introduction of genetically modified cereal crops and globalization are supposed to be the main factors responsible for the recent increase of ergot contamination worldwide during the last two decades.

Ergot is also a problem for the production of forage grasses. Tall fescue is a grass of main economical importance, as it is the most utilized perennial grass in the world. However, tall fescue is regularly concerned by ergot infection, representing a risk for grazing cattle as it can result in weight loss, reduced fertility, higher body temperature and respiration, and in more severe cases a gangrene of the animals' limbs [71]. In the United States, more than 50% of the tall fescue pastures harbour plants were infected with *Neotyphodium coenophialum* and the economic losses due to livestock intoxication has been estimated to 600 million USD annually between the 1990's and nowadays [71, 72]. In Kentucky, 9% of loss in bluegrass (*Poa pratensis*) production was recorded in 1996 after seed cleaning to meet purity standards [7]. The use of fungicide was considered but would increase the costs of hybrid cereal production, which would not be profitable anymore.

4. Regulations concerning ergot

Nowadays, most regulations concerning ergot in food specify a recommended limit based on the percentage by weight, or number of ergot sclerotia in grain, rather than the ergot alkaloid concentration. These limitations are most often applied to animal feed. In general, feed containing more than 0.1% of ergot sclerotia is not suitable for livestock, but many countries have voluntarily developed higher standards. In Europe, ergot fungal structures (sclerotia) in food are limited to 0.5 g/kg for wheat and 1 g/kg (0.1%) for animal feed by the European directive 2002/32/EC [32]. In 2012, The European Food Safety Authority (EFSA) published an opinion on ergot as undesirable substance in animal feed [32]. In Canada and the USA, the tolerance level for ergot sclerotia in grain is 3000 µg/kg grain. In the United Kingdom the amount is limited to 0.001% ergot by weight for feed and a zero tolerance in food is required. Finally, Australia and New-Zealand have set a limit for ergot of 0.05% in food and 0.3% for feed. As an average estimation, a sorghum sample containing 0.3% of sclerotia will contain about 1 mg of ergot alkaloids/kg (1 ppm). All the regulations set so far do not specify limits for individual ergot alkaloids but concern the global amount of sclerotia. There can be an inaccurate estimation of harmful potential because of several factors. Indeed, the amount of toxins is not proportional to the weight of fungus sclerotia and depends a lot on the fungal stage of development and conditions of development.

Moreover, the harmful potential of ergot alkaloids can differ enormously depending on their chemical structure. The European Commission has recommended member states to consider monitoring the presence of the six main ergot alkaloids in cereals and cereal products intended for human consumption or animal feeding before July 2017 [73]. Ergoty cereals stocks can be either destroyed, diluted or undergo a clean-up procedure. The grains used for human or animal consumption are mechanically cleaned of ergot sclerotia. Several techniques are used including gravity separation, sieves and color-sorting systems, which were shown to be the most effective sorting systems, especially when the ergot bodies are the same size as the grains.

5. Main analytical and chemical techniques for the screening of ergot alkaloids

For food safety concerns, several techniques were developed to detect the presence of ergot alkaloids in food and feed. The detection of ergot alkaloids is possible by laboratory analysis techniques such as thin-layer chromatography, liquid chromatography, gas-chromatography, liquid-chromatography mass spectrometry (LC-MS), high-pressure liquid chromatography (HPLC) or by chemical methods [74]. The main techniques used for the analysis of ergot alkaloids in food and feed are presented in this section.

5.1. Thin-layer chromatography

Thin layer chromatography (TLC) followed by staining with colorimetric reagents can be performed as a preliminary test for ergot alkaloids. The sample to be tested is spotted on a TLC plate next to a standard (ergotamine for instance, or another ergot alkaloid). The separation of ergot alkaloids usually requires tertiary or quaternary eluents, such as toluene-ethyl acetate-diethylamine (70:20:10), toluene-chloroform-ethanol (28.5:57:14.5) or diisopropyl ether-tetrahydrofuran-diethylamine (70:15:15:0.1) [75]. After chromatography, the plate is sprayed with reagents, in order to reveal some coloration. For instance, Van Urk's reagent, consisting of p-dimethylbenzaldehyde (PDAB) in 15% H_2SO_4 and traces of $FeCl_3$, gives a blue color to indole alkaloids [76]. As ergot alkaloids possess an indole moiety (2-member ring), they can be spotted this way as a first estimation [77-79].

5.2. Liquid chromatography (LC)

High Pressure Liquid Chromatography (HPLC) can be used for the detection of ergot alkaloids with a good detection limit, but it requires an important sample preparation. Detection limits in the range of 5 µg/kg or approximately 50 ppb could be achieved using this technique [80, 81]. LC or HPLC coupled with fluorescence detection (LC-FLD and HPLC-FLD) are currently used for the routine analysis of the six main ergot alkaloids usually found in

food and feed: ergometrine, ergotamine, ergosine, α-ergocristine, ergokryptine, ergocornine; and their respective -inine isomers [82, 83].

5.3. Mass spectrometry techniques

Liquid Chromatography-Mass spectrometry (LC-MS) and Liquid chromatography -tandem mass spectrometry (LC-MS/MS) are the most commonly used techniques nowadays for the detection and quantification of ergot akaloids [41, 84-87], as well as for the discovery of new ergot alkaloids [88, 89]. The tandem mass spectrometry analysis allows a fragmentation of ergot alkaloids into lysergic acid and smaller fragments. The presence of the lysergic acid structure (m/z =223 or m/z = 208) is always observed in the fragmentation of ergot alkaloids in positive mode, making this technique very reliable and also very useful for the detection of new ergot alkaloids having an unknown molecular weight [89-92]. For routine analysis, LC-MS/MS is currently used to determine the presence of the six major ergot alkaloids quoted above and their epimers. Mass spectrometry technique can achieve a detection limit varying from 0.1 to 1 µg/kg depending on the analyte and the matrix [41], making it the most accurate technique for the detection of ergot alkaloids in food and feed samples. However, an important sample preparation is necessary. MS/MS without chromatographic separation can be used to identify ergot alkaloids as well, but there is no distinction between the epimeric ergot alkaloids [77]. Gas chromatography- Mass spectrometry (GC-MS) can also be used for determination of ergot alkaloids; but as ergopeptines decompose in a hot injector, only the peptide portion of ergot alkaloids can be analyzed, and epimers can not be differentiated.

5.4. Capillary zone electrophoresis

Capillary zone electrophoresis was also reported for the detection of ergot alkaloids and their epimers [93, 94], but it is less applied than the other techniques quoted in this paragraph for routine analysis, and the discovery of new ergot alkaloids is not possible with this technique. However, good resolution of racemic ergot alkaloid derivatives in their enantiomers was achieved in a background electrolyte containing either beta-cyclodextrin or its derivative, or gamma-cyclodextrin [94]. Also, a sensitive high performance capillary zone electrophoresis (HPCZE) method for the determination of ergovaline in the endophyte-infected fescue seed was reported [95]. With this method, detection and quantification of ergovaline at low micrograms per kilogram in the seeds were possible.

5.4. Affinity biosensors

Affinity techniques are based on a specific recognition element, such as an antibody or an aptamer. Immunoassay methods including Enzyme Linked Immunosorbent Assays (ELISA) are frequently used to determine the presence of ergot alkaloids in fescue grass forage [96-

98]. ELISAs have the advantages of being rapid and easy to operate but are less specific and less accurate than LC-FLD or LC-MS methods [89].

6. Conclusion

Several species of fungi and some superior plants are known to produce ergot alkaloids. The ergot producing fungi species are parasitic organisms which develop onto plants, mainly cereal crops and grasses; including the main cereal crops cultivated for food and feed such as wheat, rye, barley and sorghum. All ergot alkaloids have an epimer which is biologically active, and which can induce several abnormal symptoms. The symptoms can differ quite a lot depending on the ergot alkaloid present in the sample and and their severity as well. The presence of ergot alkaloids in food and feed represents an important risk of food poisoning for humans and animals. Factors such as pollution, globalization and the introduction of genetically modified cereal crops in cultures, which have been shown to be less resistant to ergot fungi species, are often cited to explain the recent global outbreak of ergot contamination of cereal crops. There is no legislation for ergot alkaloids in food and feed but many countries, such as the USA, UK, Europe, Canada and Australia have implemented maximum limits for ergot sclerotia allowed in cereals. A first screening can be done by TLC, however the result has to be confirmed by the use of instrumental procedures. For that, analytical methods, such as HPLC or mass spectrometry techniques (MS/MS, LC-MS/MS, GC-MS), are mainly used for the detection of ergot alkaloids, and they can provide an accurate detection of ergot alkaloids, both in quantitative and qualitative aspects. However, their use is not obvious and requires skilled personel, due to the complexity of the analytical equipments and the important sample preparation required. Therefore, new complementary high-throughput techniques were investigated for the fast and simple detection of ergot alkaloids. For instance, ELISAs represent a good complementary technique, which is now frequently used in food and feed industries. Then, aptamers should also provide in a near future another interesting fast and simple technique for the specific detection of ergot alkaloids.

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Chapter I: Aim and outline of this PhD study

1. Problem

The presence of ergot in food and feed remains a major food safety concern. As the grain clean-up usually allow a removal of 82% of ergot sclerotia only [1], there is still a risk that ergot sclerotia remain after the process. As seen previously, analytical techniques can be used for ergot screening in food and feed. However, most analytical techniques must be performed by skilled personnel in laboratories equiped with sophisticated instruments. Moreover, these analyses are usually expensive and time-consuming. Direct sensing of ergot alkaloids in flour production sites or even in fields is impossible in many cases. Also, it is unimaginable for developing countries to obtain these types of analyses performed because of the prohibitive price of the equipment. Therefore, there is a need for complementary techniques or tests that can ease the detection of ergot alkaloids in food and feed. This could be profitable to several levels in food risk assessment: cereal-crops production sites, food factories and regulatory and sanitary organizations. In order to compensate the lack of rapid tests for ergot alkaloids, this project aimed to develop a fast, cheap and mobile sensor for the detection of ergot alkaloids in food and feed. The key point in the construction of a sensor is to obtain a recognition element which can specifically recognize ergot alkaloids and to consequently translate the binding event into a signal that can be easily interpretated for the user. It is also important that other compounds do not interfere with the signal given by the binding of target molecule. Several recognition elements can be used in sensors, chemical ligands as well as biological ligands. Regarding the specificity of the recognition element towards the target molecule, biological ligands are usually more specific because they are bigger structures and they have a more complex interaction with the target molecules, which cannot be easily falsified by other compounds. The different types of recognition elements used in sensors are detailed in the first part of this chapter. Then, the selection and evaluation of binding affinity of the chosen type of biorecognition element, nucleic acid aptamers, will be presented in this chapter.

2. Choice of the type of recognition element for ergot alkaloids

2.1. Ligands used in sensing and extraction systems

The use of chemical and biological ligands for sensing applications can complete and sometimes replace the use of expensive analytical equipments. This also allows to avoid time-consuming analyses as well, which moreover require skilled personnel. Various types of ligands are used in sensors and extraction systems. The first ligands used in sensing and extraction systems were chemical ligands, inspired by the observation of natural complexes

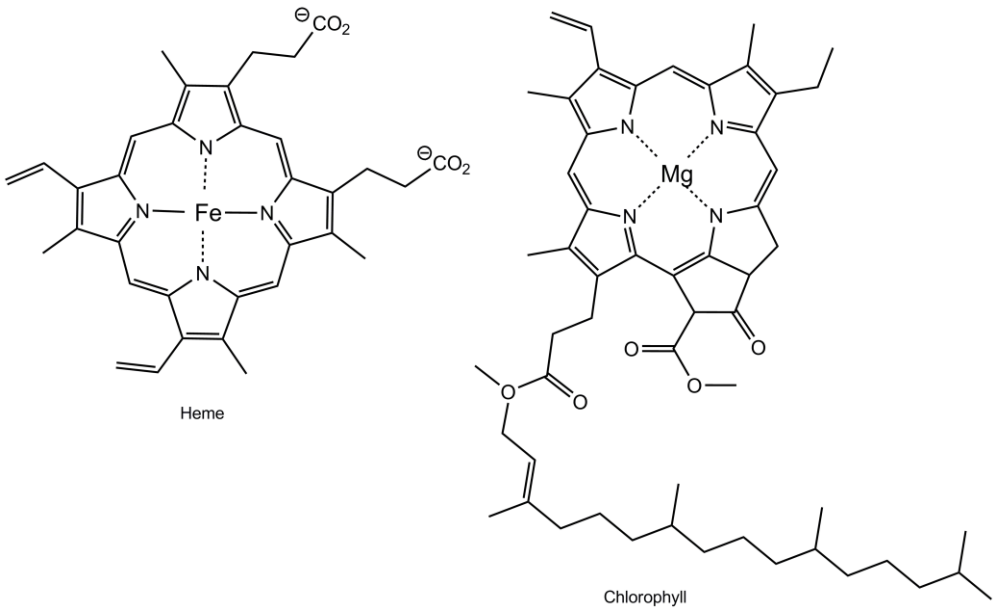
that occur in nature. In coordination chemistry, a ligand is an ion or molecule which binds to a target compound to form a coordination complex. The formation of complexes is based on shape complementarity and intermolecular interactions, such as ionic interaction, hydrogen bonding, pi-stacking, Van der Waals forces, hydrophobic effects… Ligands can be chemical structures or biological macromolecules. The main difference between these two types of ligands is that biological structures are in a general manner much bigger in size than chemical ligands, offering a more important interaction surface, and thus making biological recognition elements possibly more specific than chemical ligands. The different types of ligands are presented in this section; and the choice of the ligand for this study is discussed.

2.1.1. Chemical ligands

a) Macrocycles

In the nature, macrocyclic structures can specifically bind to metallic ions, such as iron, magnesium, calcium, potassium, etc… As an example, the heme of the haemoglobin in red blood cells is known to firmly bind to iron (**Figure 4**), which transports oxygen and carbon dioxide. Many other examples could be cited, such as the green pigment in plants, chlorophyll, which is based on a macrocyclic structure complexing magnesium (**Figure 4**).

Figure 4. *Examples of natural complexes of macrocyclic ligands and metals: chemical structures of heme and chlorophyll molecules.*



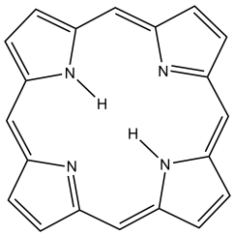
This observation led scientists to synthesize and study macrocycles for the sensing and the extraction of ions, chemicals and pollutants. Chemically speaking, a macrocyle can be defined as a "cyclic molecule with one or more donor ligating atoms, in a hetero atom (N, O or S) containing ring which has at least nine atoms" [2]. In recent years, an important research effort was devoted to the synthesis of ligands that can specifically bind to target

molecules, and inducing a concommitant spectroscopic response, such as a color change or variation in fluorescence emission or intensity [2]. Macrocyclic ligands were classified into several groups depending on the hetero atoms contained in their cycle: polyoxamacrocycles contain atoms of oxygen and are also called crown-ethers, polyazamacrocycles contain atoms of nitrogen, polythiamacrocycles contain atoms of sulfur, and finally polyphosphamacrocycles contain atoms of the element phosphorus. Many macrocyclic structures were synthesized in order to be used as chelators of metals or organic compounds [3, 4]. A few examples of macrocycles and their applications is given below:

- Porphyrins

Porphyrins are a class of naturally occuring macrocyclic compounds, which have been extensively studied and applied in analytical chemistry. The porphyrin molecule contains four pyrrole rings linked via methine bridges (**Figure 5**), which is the base of the heme and the chlorophyll structures. Prophyrins form very stable complexes with metals such as copper, zing, manganese, cobalt, lithium… Porphyrins are very often used in sensing systems for the determination of metal ions, having numerous applications in analytical chemistry, such as: the detection of trace cyanide in waste waters, the determination of nitrite concentration for water quality assessment, the titration of cobalt ions, the screening of toxic organohalides in aqueous samples, or the determination of nitric oxide in biological samples [5].

Figure 5. *Chemical structure of porphyrin macrocycle system.*

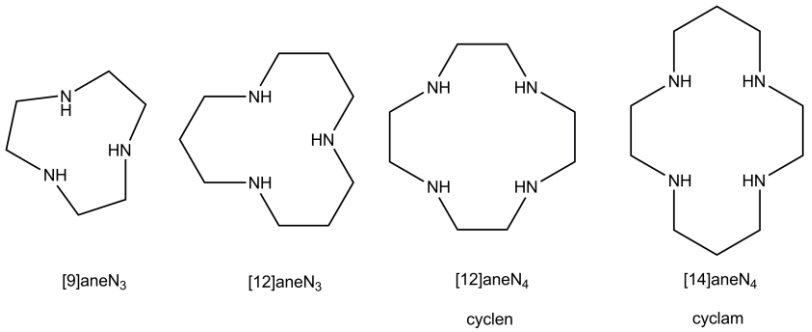


- Polyazamacrocycles

Polyazamacrocycles are cyclic structures which contain nitrogen donor groups **(Figure 6)**. Pendant arms can be attached to polyazamacrocycles, in order to enhance metal immobilization, or to allow the fixation of the macrocyclic ligand onto solid supports. Functionalized polyazamacrocycles were reported to form complexes with: transition metals, such as nickel, copper, chromium, iron and manganese; lanthanides and actinides. Thus, the range of applications of polyazamacrocyclic chemistry is very broad. Polyazamacrocycles are used for instance for the extraction of uranium and transuranic elements from radiocatively contaminaed waste waters [6]. These ligands also have applications in the medical field, and they are also used as magnetic resonance imaging contrast agents [7]. It was shown that complexes of polyazamacrocycles and gadolinium

allow to greatly enhancing the contrast between normal and diseased tissues. Finally, polyazamacrocycle complexes with transition metals were reported to act as antibacterial agents against *Escherichia coli*, several *Streptococcus* strains and *Staphylococcus aureus* [8].

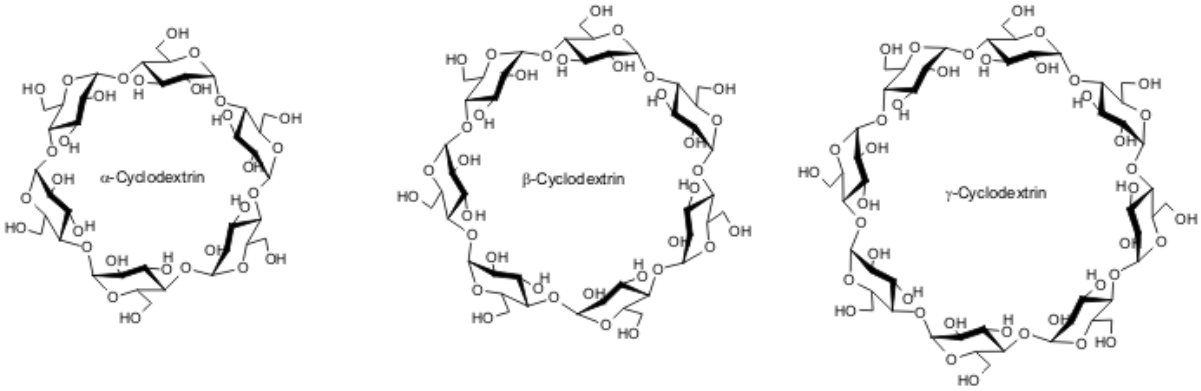
Figure 6. *Chemical structures of polyazamacrocyles.*



- Cyclodextrins

Cyclodextrins are cyclic oligosaccharides of glucose (**Figure 7**), containing a relatively hydrophobic central cavity and hydrophilic outer surface [3, 9]. The cavity provides a lipophilic microenvironment into which drug molecules can be included. An important pharmaceutical application of cyclodextrins is to enhance drug solubility in aqueous solutions. Cyclodextrins make good excipients, which often replace cosolvents, surfactants or lipids as formulation adjuncts [9, 10].

Figure 7. *Chemical structures of cyclodextrins having 6 to 8 units (from left to right).*



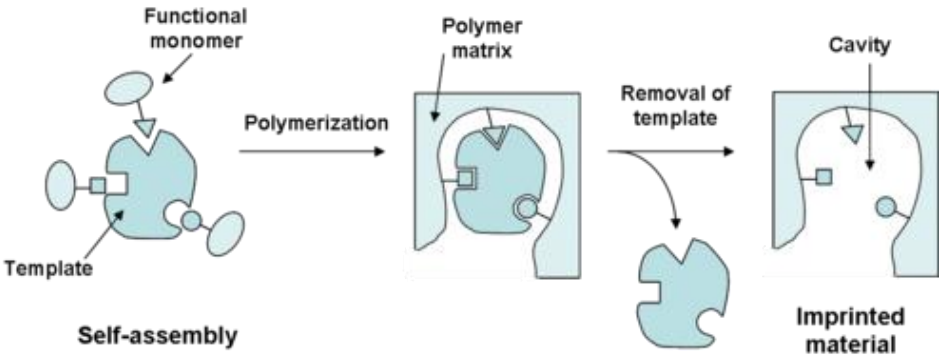
For the recognition of ions or organic structures, chemical ligands such as macrocycles can be used; but the specificity is rarely limited to one compound or one family of compounds. Therefore, other types of ligands were developed to obtain a better specificity.

b) Molecularly imprinted polymers (MIPs)

Molecularly imprinted polymers or MIPs are another class of chemical ligands which have appeared the last two decades, and which have shown a good ability to bind to a given

target molecule. MIPs are polymers formed in the presence of a given target molecule; which is removed after the process (**Figure 8**). Once solidified, MIPs keep cavities having the shape of the target molecule in their structure. MIPs can be seen as molecular moulds, which moreover have a chemical affinity with the target compound. Molecular imprinting technology is the technique used to design artificial receptors with a predetermined selectivity and specificity for a given analyte, in which functional monomers having intermolecular interactions with the analyte are incorporated in the polymer matrix **(Figure 8)**; driving the molecular recognition phenomena [11]. Thus, the resultant polymer matrix can recognize and bind selectively the given analyte. MIPs are robust ligands, which can have a high selectivity and specificity. Thus, MIPs can very well be used as recognition elements for the sensing or the extraction of various compounds, such as organic pollutants, toxins, amino acids or proteins [11-13]. The team of De Saeger *et al.* has recently developed a MIP for ergot alkaloids, which could successfully extract 56% to 71% of six main ergot alkaloids and their epimers in barley samples [14, 15].

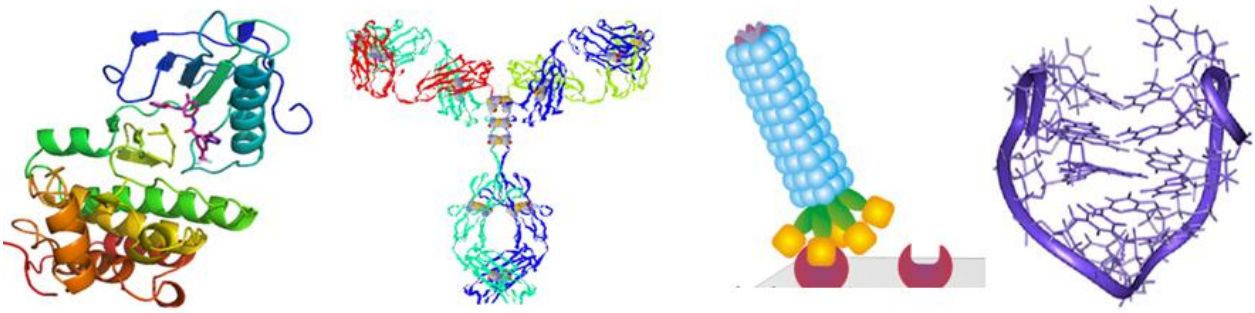
Figure 8. *Scheme of molecular imprinting. A functional monomer is self-assembled with a template molecule, and embedded in a polymer matrix by a polymerization procedure. The template molecule is removed afterwards, leaving a cavity in the polymerized material.*



2.1.2. Biological ligands

Besides chemical ligands, biological structures can also be very well used as recognition elements. Biological ligands are usually elaborated and complex structures, which can sometimes offer a better specificity than chemical ligands. Biological recognition elements are usually based on proteinic structures, such as antibodies, enzymes and receptors; or nucleic acid structures, such as aptamers. These huge biological structures can interact very specifically with the target molecules. Some examples of biological recognition elements are given in **Figure 9**, with the schematic representation of an antibody, a receptor and a nucleic acid aptamer. The different biological structures used as recognition elements of sensing systems are detailed in this section.

Figure 9. *Schematic representation of some biological ligands. From left to right: Receptor complexing an inhibitor [16], antibody, general representation of a phage complexing its target molecule [13], and DNA aptamer binding to its target molecule, the protein thrombin.*



a) Enzymes

Enzymes were the first biological structures to be used as recognition elements integrated in sensors when Professor Leland C. Clark published an article in which the catalytic enzyme glucose-oxidase was integrated in an electrochemical sensor for the sensing of glucose [17]. In this paper, he explains how to "make electrochemical sensors more intelligent by adding enzyme transducers". In this sensor, the enzyme glucose-oxidase was placed close to the electrodes measuring the concentration of dioxygen. In presence of dioxygen, the glucose-oxidase turns glucose into gluconolactone and hydrogen peroxide. The decrease of the concentration of dioxygen, proportional to the concentration of glucose, could be monitored by this electrochemical system. This was the first biosensor to be launched on the market in 1975, which turned to be very successful; as glucose-oxidase biosensors have been manufactured by several companies around the world until nowadays [18]. Several types of glucose-oxidase sensors were developed, either monitoring the concentration of dioxygen or hydrogen peroxide in the medium, the second ones being the most accurate. Glucose-oxidase biosensors became particularly useful to diabetic patients, who were then able to monitor themselves the concentration of glucose in their blood thanks to a small portable device, instead of permanently going to the hospital for routine check-ups. Since then, many biosensors using various enzymes were developed for the detection of a broad variety of compounds for medical purposes, food quality control, or the detection of heavy metals or organic pollutants [19]. Besides glucose-oxidase, horseradish peroxidase and alkaline phosphatase are enzymes notably used in biosensors. However, enzymes suffer from a lack of stability and susceptibility to degradation by environmental factors or chemical agents. Moreover, the purification of enzymes can be laborious and costly.

b) Receptors

Receptors are natural ligands which can initiate a cellular response upon binding to their target molecule; and can therefore have a use as recognition elements in sensors [20]. Receptors are usually not specific to one molecule, but a wide range of molecules having chemical similarities. Receptor biosensors can therefore be very useful for drug discovery for instance [21]. For example, the detection of cholinergic agents was rendered possible by using an electrochemical biosensor using an acetylcholine receptor [22]. Another example was reported by the team of Aristotelous *et al.*, who developed a G-protein biosensor using surface plasmon resonance [23]. Based on the fact that G-protein coupled receptors are the primary target of currently marketed drugs, this biosensor allowed the fast screening of drugs interacting with this type of receptors. A real-time the interaction G-protein coupled receptors and the ligands tested could be obtained this way. However, in a general manner, receptor sensors have a few disadvantages, such as their unstability, susceptibility to degradation and laborious purification. Receptors can only be used effectively within a limited period of time; making them hardly usable for routine analysis.

c) Antibodies

Antibodies are the most frequently used recognition elements in biosensing systems. Antibodies offer a wide range of possibilities for sensor development as it is often feasible to obtain very specific and sensitive antibodies towards certain targets; especially virus markers, hormones and various immunogenic compounds. With the notable exception of the glucose sensor, most of the rapid detection systems employ antibodies as recognition elements [20, 24, 25]. Several types of antibodies can be produced: polyclonal, monoclonal and recombinant antibodies. Polyclonal antibodies involve the exploitation of the immune system of animals, such as rabbits or goats [26]. Monoclonal antibodies are generated through hybridoma technology, and murine hosts (mice for instance) are commonly selected for immunization [26]. Hybridoma technology is the production of hybrid cell lines by fusing an antibody-forming cell produced by an animal with a myeloma cell (cancer cell) that can grow indefinitely in culture [27]. Finally recombinant antibodies are generated through the use of phage display technology and the biopanning of antibody repertoires (libraries) against a target of interest [26]. Antibodies are very often used in marketed sensors. As an example of an antibody-based sensor, home pregnancy test kits are based on monoclonal antibodies specific to human chorionic gonadotropin (hCG), which is an hormone produced by fertilized egg in humans. In this type of test, a colorimetric reaction occurs in presence of hCG hormone, which involves an enzyme linked to the antibody that can recognize the hormone. This technique, also called enzyme-linked immunosorbent assay (ELISA), is one of the most frequently encountered, as it can give a rapid and specific semi-quantitative signal in a portable and convenient disposable device. Many other tests for clinical diagnostics have been developed using ELISA technique until nowadays [28, 29]. Antibodies were also

produced for ergot alkaloids [30, 31]. The ELISA test developed by the team of Shelby *et al.* was reported to detect a level of 10 ng/g of ergonovine in spiked wheat samples. The high performance provided by antibody detection is the main reason for their important use in diagnostics and for the detection of contaminants. However, antibodies also have a few disadvantages. Firstly, the proteinic structure of antibodies is likely to be damaged by chemicals or environmental factors such as heat. Also, certain target molecules can be very toxic without being immunogenic, which represents a limitation to the use of antibodies. Moreover, antibody production often requires the use of animals, which can cause ethical problems.

d) Phages

Phages are DNA viruses lacking their own metabolic machinery and use bacterial hosts for their multiplication. Phages identify the bacterial receptors via their tail spike proteins and bind to definite receptors on bacterial surface in order to infuse their genetic material inside the bacteria [32]. Based on the life cycle, phages were classified into two categories: lytic and lysogenic. Lytic or virulent phages multiply within the bacterial host, and subsequently release their assembled particles through lysis of the host cell. On the contrary, lysogenic or temperate phages integrate their genetic material into the host chromosome leading to their replication together with the host bacterium for generations [33]. Most phages recognize their host very specifically, and this can be exploited for bacterial identification for instance [34]. Moreover, it is possible to fuse a gene encoding for the peptide or protein of interest to the phage surface protein encoding gene(s) resulting in the expression of the hybrid protein on the surface of the phage; which is the underlying principle of phage display [13, 35]. A comprehensive review by Smith and Petrenko summarizes the theory and technical details of phage display technology [36]. Platforms based on filamentous phages constitute the most-widely used bacteriophage-based display tools due to several advantages; such as the possibility of insertion of large DNA fragments without disrupting phage packaging and their non-lytic multiplication which allows the production of high concentrations of phages in bacteria [33]. Peptides, cellular proteins, target specific antibodies or antibody fragments such as single chain variable fragments (ScFv), antigen binding fragment (Fab), *etc.* have successfully been expressed on the surface of phages for different targets; leading to applications in molecular imaging and biosensor development [34, 36, 37]. Then, phage display also found some applications in the discovery of the mechanism of action of pollutants [38]; as the proteins displayed by phages that bind to a target compound can be identified and compared to the protein sequences in human genome for instance. A few biosensors were developed using phages as recognition component for the detection of pathogens such as *Escherichia coli*, *Staphylococcus aureus* and spores of *Bacillus anthrasis* [32]. A good sensitivity is usually obtained with phage-based biosensors, usually ranging from 10^2 to 10^4 cfu or less of bacteria [34]. However, phages

have been less used so far for sensing purposes, as phages can lack stability, and can potentially be unsafe to use.

d) Nucleic acids and aptamers

Lately, another type of biological ligands, nucleic acids, gave interest to scientists in terms of sensing tools since their technology was better understood and technically feasible. Nucleic acids can be used in two different ways as sensing tools, either as hybridization probes, either as "chemical antibodies", in which only their shape and not their sequence is involved in the recognition process. Firstly, nucleic acids can hybridize to a complementary sequence. Therefore, nucleic acids can be used to specifically recognize a given target sequence. The detection of specific DNA sequences of pathogens or microorganisms is then possible with nucleic acid probes [39, 40]. DNA hybridization methodology is an emerging and very promising discipline, which has potential applications in areas such as genetics, pathology or microbiology [39-41]. There are numerous examples of commercial tests based on DNA hybridization used for the detection of pathogens in clinical and environmental samples, such as *Escherichia coli*, *Mycobacterium tuberculosis, Staphylococcus aureus*, HIV, hepatitis C, *etc*… [42, 43]. Moreover, it is also possible to detect point mutations in DNA by using DNA probes as well, which has potential applications in oncology for instance [44]. DNA hybridization probes were shown to be very accurate, and were able to detect as low as one single base mismatch in a given target DNA sequence [44]. Since the 1990s, it was shown that nucleic acids could also be used to recognize other molecules than nucleic acids, such as small organic compounds or proteins [45-47]. The mode of binding in this case is the same as for the formation of a complex instead of hybridization, which is comparable to the interaction that can occur with antibodies and antigens [48]. These small nucleic acid fragments, able to bind to a target molecule and form a complex, were named aptamers; based on the Latin word *aptus* meaning "to fit" [45, 47, 49]. Aptamers are short single-stranded DNA (ssDNA) or RNA with a specific and complex three-dimensional shape characterized by stems, loops, bulges, hairpins, pseudoknots, triplexes, and/or quadruplexes. The terminology aptamer also include small peptides [50], but they are usually made of nucleic acids. Binding between aptamer and target molecule is provided by different intermolecular interactions like electrostatic interactions between charged groups, stacking of aromatic structures contained in organic compounds and the nucleobases, hydrogen bonds, and the complementary in three-dimensional shape [51]. Aptamers can be developed for a vast variety of possible targets ranging from small organic molecules to proteins and even complex structures like cells or viruses [13, 52, 53]. Nucleic acids have a number of advantages over antibodies as sensing probes. Firstly, RNA and DNA libraries can be produced by chemical synthesis. Thus, the production of nucleic acid probes can be done on a large scale and is animal-friendly, as opposite to the production of antibodies. Another advantage of oligonucleotide-based probes and aptamers over protein-based antibodies is their stability at elevated temperatures and chemical stability. Nucleic acids can recover

their native conformation, whereas antibodies easily undergo irreversible denaturation [54]. Moreover, the levels of sensitivity obtained with RNA or DNA aptamers can be very high, and sometimes better than what can be obtained with antibodies. Indeed, the dissociation constants (Kd) of aptamers usually span from micromolar (µM) to nanomolar (nM) range against various targets, and in some case in picomolar range (pM) [55]. Furthermore, aptamers are capable of excellent target discrimination. For instance, aptamers have been reported to exhibit greater than 10,000-fold binding affinity for theophylline over caffeine, which differ from one another in structure by only a single methyl group [56]. Finally, aptamers can be generated for a broad range of compounds and offer new possibilities for non-immunogenic compounds or strongly toxic compounds for which antibody production would be difficult or even impossible [57, 58]. Because of their efficiency and robustness, aptamers are expected to play a dominant role in sensing technology in the future [58, 59]. Due to their interesting features, aptamers were chosen as recognition elements in this study.

2.2. Selection of DNA aptamers for ergot alkaloids

DNA aptamers are generated by incubating a ssDNA library with the target molecule and selecting the DNA sequences having the highest affinity for it. The methodology used for the selection procedure is named SELEX (Systematic Evolution of Ligands by EXponential enrichment), an iterative selection process in which the DNA aptamers are successively incubated with the target molecules and eluted from them; then the DNA sequences obtained are recovered and amplified in order to be used for the following selection cycles, resulting in a competition between the different binding elements [48, 60, 61]. When the recovery rate of aptamers eluted after a cycle of selection reaches the amount used for the selection, the pool has finished evolving; meaning that the best binders were selected from the DNA library. Several modifications can be brought to the SELEX procedure in order to improve the selectivity and affinity of the aptamers, or to facilitate the partitioning of DNA sequences bound from the rest of the library [62]. For instance, it is possible to improve the specificity of the DNA aptamer towards a certain target molecule by using molecules close in structure to the target molecule in a counter-selection. In the most elaborated techniques, atomic force microscopy (AFM) can be used to only keep the aptamers having a strong affinity for the target molecule (AFM-SELEX). Capillary electrophoresis SELEX (CE-SELEX) can alternatively be used in order to improve the partitioning of the bound and unbound DNA sequences. Many different SELEX formats can be used to select the best binding DNA aptamers, which are finally separated from the rest of the library and eluted from the target molecules. In most of the cases, it is necessary to immobilize the target molecule onto a support to perform a selection of aptamers. The immobilization of the target molecule is therefore the first step in the process. Covalent couplings of the target molecule are usually preferred for their stability, but other types of fixation are also possible; such as the immobilization of biotin-functionalized molecules onto (strept)avidin supports [63]. The

chemical structure of the target molecule is driving the chemistry of the immobilization step. If the target molecule is not reactive enough, it is then necessary to obtain a chemically reactive derivative or a biotinylated derivative for instance. In this study, it was firstly necessary to choose which ergot alkaloids would be the most judicious to work on, as ergot alkaloids represent a broad family of compounds. The chemical structures of ergot alkaloids and the coupling reactions of ergot alkaloids onto supports prior to the selection procedure are detailed in **Chapter II**. Once a support coated with the target molecule is obtained, it is then possible to easily separate the nucleic acid fragments bound to the target molecule from the rest of the library. The aptamers bound to the target molecule are eluted, amplified, purified and used for the following selection cycles. The amplification of aptamers is done by polymerase chain reaction (PCR). PCR is a technique of molecular biology developed in the 1980s, which allows to generate a high number of copies of DNA [64]. In the case of aptamers, which are short nucleic acid fragments, it is usually necessary to optimize the PCR conditions, in order to avoid the formation of by-products; which is one of the main problems encountered in aptamer selection [65]. At the end of each selection cycle, the selected aptamers are cloned [66]. Molecular cloning allows to obtain sufficient amounts of several aptamers, which can be sequenced afterwards. Then, the sequenced aptamers are analyzed and characterized. The secondary structures of the selected aptamers can be determined by using softwares such as Mfold [67] or OligoAnalyzer (Integrated DNA Technology) [68], which can calculate the lowest energy levels of the different secondary structures formed by the different aptamers depending on their internal hydrogen binding between the bases A with T, and C with G. Then, it is possible to also determine the tridimensional structure of the aptamers by using RNA1,2,3 software for instance [69]. The **Chapter III** describes the selection of aptamers for ergot alkaloids and their characterization.

2.3. Monitoring the formation of complexes between ergot alkaloids and DNA aptamers

After the selection and characterization of aptamer sequences, it is important to compare the binding strength of the different selected aptamers and to analyze the complexes formed with the target molecule. The interaction between DNA aptamers and the target molecules can be determined by different analytical techniques, such as surface plasmon resonance (SPR), quartz crystal microbalance (QCM) or fluorometry, in order to quantify the association and dissociation constants of the complexes of aptamers and target molecules. Surface plasmon resonance is an optical technique providing information which can be directly linked to the amount of material bound to a chip [70-73]. The principle consists of the production of a plasmon, or free electrons, into a gold chip. A light beam strikes the surface flow cell at the certain angle, the one for which the light is totally reflected. Under those conditions, an electromagnetic component of the light, the evanescent wave, makes

the free electrons oscillate onto the gold chip. In case that some material is bound onto the chip, the refractive index changes and the incident angle (SPR) changes proportionally to the mass adsorbed onto the chip. The gold surface is generally coated with a functionalized dextran matrix, onto which either the ligand, either the target molecule, is immobilized. The other one is passed through a flow channel onto the chip. Depending on the mass bound onto the surface, the SPR angle is converted into resonance units, which allows the evaluation of the association and dissociation constants of the complexes of ligands and target molecules. Besides SPR technique, Quartz Crystal Microbalance (QCM) is another frequently used technique, which can also lead to the determination of association and dissociation constants of complexes [73-75]. QCM technique relies on the properties of certain materials to produce electricity when pressurized, or the opposite effect to change their shape and oscillate when a voltage is applied by some electrodes. Typically, gold layers are evaporated onto both sides of a quartz crystal to form electrodes, and an alternative electric field is applied. As molecules adsorb onto one of the electrodes; the oscillation frequency of the quartz crystal decreases. The kinetics of adsorption can be defined by recording the oscillation frequency as a function of time. Finally, other types of analyses can be performed to characterize complexes, such as fluorometry, UV-Vis absorption or circular dichroism [76]. With these techniques, the ligand is immobilized onto a support and is incubated with the labelled target molecule. The target molecule is then eluted from the ligand and the concentration is measured by a fluorometer or spectrophotometer. These techniques allow the determination of the same parameters as SPR or QCM but via an indirect way. Usually, only one of these techniques is used to compare the affinity of the different aptamers obtained after the selection procedure. In this study, surface plasmon resonance was used for the determination of the binding parameters of the complexes of aptamers and ergot alkaloids. The surface plasmon resonance analysis of the selected aptamers is described in **Chapter IV**. Depending on their binding properties, the best selected aptamers can be used for the construction of sensors and various types of assays.

3. Production of an aptamer-based optical test for ergot alkaloids

The terminology biosensor is used to describe an integrated system combining a biological recognition element linked to a transduction technique, in order to produce a signal in presence of the target compound to be detected. There are several definitions to describe biosensors. The IUPAC definition for a biosensor was defined as such: a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element that is retained in direct spatial contact with a transducing element [77]. Depending on the transduction technique used, the different biosensors were classified as such: optical, electrochemical, mass, magnetic, calorimetric and micromechanical [77, 78]. This last decade, several assays and biosensors

were constructed using aptamers as recognition elements. The fact that aptamers can undergo significant conformational change upon binding to their target molecule allows the generation of optical signals by labelling aptamers with fluorophores, such as fluorescin isothiocyanate, rhodamine, coumarin, cyanin, *etc*… [79]. For instance, it is possible to construct molecular beacons by labelling the aptamer by a fluorophore on one part of it and a quencher at the opposite side. The binding of the target molecule to the aptamer can then result in decreasing the distance between the fluorophore and the quencher, and then reducing the fluorescence of the aptamer, which can be measured by a spectrophotometer [80, 81]. It is also possible to generate colorimetric reactions in the visible range with aptamers, by linking the aptamers to metallic nanoparticles or polymers [82-84]. This way, no apparatus is required for the interpretation of the result and this allows the construction of paper tests or dipsticks. Optical tests mainly provide a qualitative to semi-quantitative responses. They have the advantages of being easy to use, handy and cheap. However, for more accurate quantitative analysis, several other types of sensors are more suitable, such as electrochemical devices or SPR and QCM transducers for instance [85-87]. For the construction of electrochemical aptasensors, DNA aptamers can be covalently linked to a redox markers, in order to produce a redox reaction when a conformational change of the aptamer occur upon binding to the target molecule. Alternatively, the construction of mass-based aptasensors, such as QCM or SPR aptasensors, there is no need for labels. Several types of biosensors can then be constructed with aptamers, and they can be seen as complementary to each other. For instance, for rapid tests with a yes/no answer, optical sensors seem to be well-suited. Then, the use of more elaborate sensors, such as electrochemical or mass-based transducer, can offer the possibility of giving an accurate quantitative measurement of the amount of target molecule in the sample. In the present study, it was chosen to develop an optical aptasensor for ergot alkaloids, which could provide a qualitative or semi-quantitative detection of ergot alkaloids. The production of an aptamer-based sensing system is presented in the **Chapter V**. Other transduction techniques can be used with the selected aptamers to construct other types of sensors using the same aptamers for the detection of ergot alkaloids.

4. Other applications with the aptamers selected for ergot alkaloids

Until now, aptamers were mainly used in sensing systems, but there are also a few other different applications envisaged with aptamers, such as their use a therapeutic agents or drug delivery tools [85]. Very recently, aptamers started to be used in extraction systems as well [88, 89]. Therefore, it was interesting to evaluate if the DNA aptamers selected for ergot alkaloids could be also suited for the extraction of ergot alkaloids in ergot contaminated samples. This could have a potential use for food clean-up for instance. The development of

a specific extraction system for ergot alkaloids based on DNA aptamers is presented in the **Chapter VI** of this thesis.

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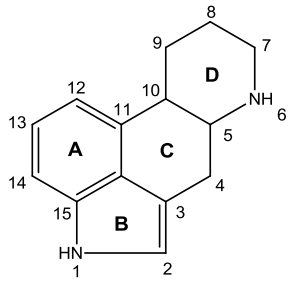
Chapter II: Chemistry of Ergot alkaloids

1. Introduction

Ergot alkaloids represent a wide class of biologically active compounds. Nowadays, more than 40 ergot alkaloids and their epimers are known. All ergot alkaloids are based on a four-member ring named ergoline (**Figure 10**), which is responsible for their biological activity; as ergoline interacts with adrenergic, dopaminergic and serotinergic receptors in humans and animals [1]. Ergoline and its derivatives especially have a high biological activity on the central nervous system and cardiovascular system. The substitution of the ergoline ring system modulates the pharmacological activity of ergot alkaloids, making them have a very broad spectrum of biological activity. Some highly toxic ergot alkaloids can induce a lethal disease known as ergotism; some other possess interesting medicinal properties and are used for medical purposes; and finally a few of them, such as lysergic acid and lysergic acid diethylamide, are listed as drugs of abuse, which are prohibited in many countries. Among the ergot alkaloids having medicinal properties, ergometrine (also called ergonovine) and its semi-synthetic derivative methylergonovine maleate are used for the prevention and treatment of excessive uterine bleeding following birth; and also to initiate delivery. However, they are replaced in some cases by the natural hormone oxytocin to avoid side-effects including nausea and hypertension [1, 2]. Other ergot alkaloids, such as ergotamine tartrate and dihydroergotamine are known to be powerful agents against migraine [3-5]. Also, some semi-synthetic ergot alkaloids, such as bromocryptine, lisuride and pergolide mesylate, have shown to be effective for the treatment of Parkinson's disease [6, 7]. The wide and complex family of ergot alkaloids has been classified into sub-categories: lysergic acid and its simple amides, clavines and ergopeptines [6]. Different species of fungi and plants can produce similar ergot alkaloids as secondary metabolites, and a mixture of various proportions of several ergot alkaloids is usually found in ergot alkaloid producers. However, it was observed that lysergic acid and its simple amides are usually observed in the superior plants producing ergot alkaloids; while more elaborated ergopeptines are commonly found in the fungi sclerotia of *Claviceps* species. In this chapter, the chemical structure of ergot alkaloids will be described. Then, the choice of the target molecules for this project among the ergot alkaloid family will be discussed. The synthesis and characterization of a template ergot alkaloid will also be presented. Finally, the chemical immobilization of ergot alkaloids onto a support prior to the selection of DNA aptamers will be shown, with the covalent coupling of ergot alkaloids onto magnetic beads. It was chosen to present the work on the template molecules in a separate chapter; as this part, which is only based on chemical techniques, requires a different knowledge than the work on DNA that will be presented afterwards.

Figure 10. *Chemical structure of ergoline.*



2. Classification of ergot alkaloids

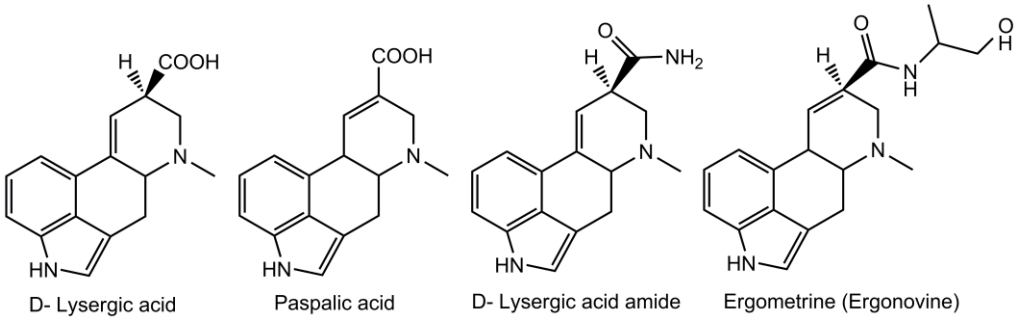
All ergot alkaloids derive from a four-member ring called ergoline, in which the four cycles of ergoline have been assigned a letter from A to D (**Figure 10**). In more elaborated ergot alkaloids structures, substitutions occur on the cycle D on position C8, which is a chirality centre. Usually, only one of the epimers is pharmacologically active. Epimerization of the group on position C8 usually occurs in protic solvents. The common structural feature of ergot alkaloids is the ergoline ring which is methylated on the N-6 nitrogen atom, substituted on C-8 and possess a C-8, C-9 or C-9, C-10 double bond [8, 9]. The ring D can be unsaturated on position 8-9 or 9-10 and the corresponding ergot alkaloids are called 8-ergolenes or 9-ergolenes [10]. Based on their complexity, ergot alkaloids have been classified into three main categories: lysergic acid and its simple amides, clavines and ergopeptines [6, 8, 10].

2.1. Lysergic acid and its simple amides

D-Lysergic acid and paspalic acid are derived from the substitution of ergoline on position C8 by a carboxylic acid group and by a methyl group on position N6 (**Figure 11**). They are the natural precursors of ergot alkaloids. D-lysergic acid can be found in large quantities in the seed of *Ipomoea violacea* for instance. D-lysergic acid has also been reported in small amounts (9 mg/L) together with isolysergic acid in broth of saprophytically grown *Claviceps* strains [10]. However, in the development of sclerotia of *Claviceps* species, this precursor is rapidly converted into more elaborated ergot alkaloids. The molecule of lysergic acid possesses two centres of asymmetry, or chirality, on positions C5 and C8 (**Figure 11**). The active form of lysergic acid was assigned the configuration 8R: 5R, and all the natural pharmacologically active ergot alkaloids are derivative of this structure, which has the full chemical name of D-6-methyl-9,10-ergoline-8beta-carboxylic acid [11]. D-lysergic acid can also be obtained by the hydrolysis of peptide ergot alkaloids in basic medium [10]. Paspalic acid (**Figure 11**) was isolated in relatively large amounts (620 mg/L) from saprophytic cultures of *Claviceps paspali*, which also contain notable amounts of lysergic acid amide [10]. Another simple amide of lysergic acid, ergometrine (**Figure 11**), is also found in high

proportion in young sclerotia of *Claviceps* species, and then converted into more complex peptide ergot alkaloids in mature sclerotia [11, 12].

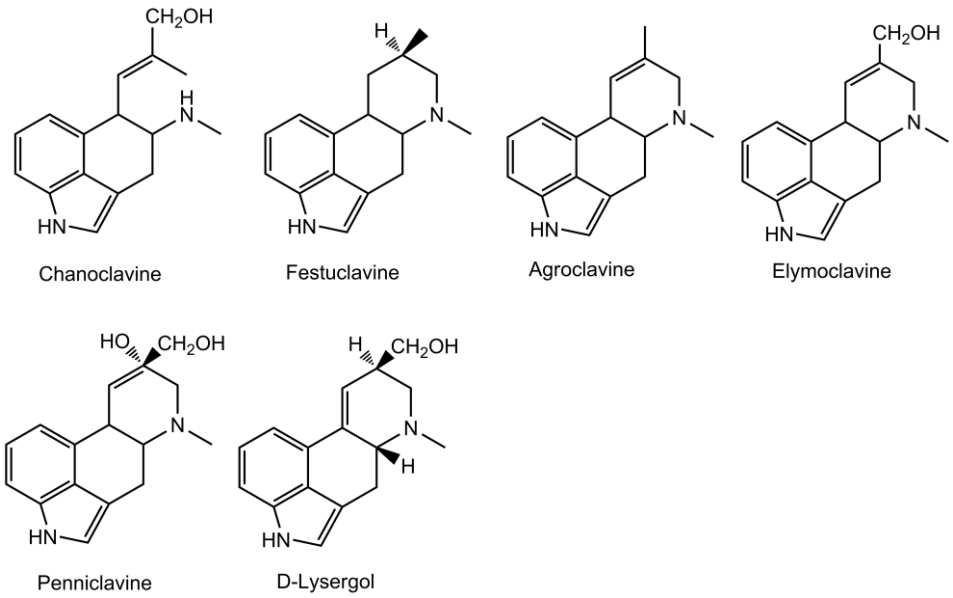
Figure 11. *Chemical structures of lysergic acid and its simple amides and paspalic acid.*



2.2. Clavines

The clavine alkaloids are hydroxyl- and dehydro- derivatives of 6,8-dimethyl-ergolene [10]. The chanoclavines, in which the ring D is open, are also members of this group. Some clavine ergot alkaloids, such as agroclavine, chanoclavine, elymoclavine, lysergol (**Figure 12**); are usually produced in the seeds of higher plants, such as *Ipomoea violacea* or *Rivea corymbosa* [13]. Agroclavine and elymoclavine are also produced in fungal structures as precursors of more elaborated ergot alkaloids in fungi. For instance, *Claviceps fusiformis* produces elymoclavine and *Claviceps africana* produces dihydro-ergot alkaloids derived from festuclavine such as dihydroelymoclavine, in which the ring D is saturated [14]. Other clavine alkaloids can be mentioned, such as festuclavine (**Figure 12**), produced in *Claviceps africana* and *Aspergillus* species; and penniclavine (**Figure 12**) produced in Clavipitaceous fungi species [15].

Figure 12. *Chemical structures of clavine ergot alkaloids.*



2.3. Peptide ergot alkaloids

The category of peptide ergot alkaloids contains the most elaborate ergot alkaloids structures, it is also the most abundant category in fungi. Superior plants do not produce these complex structures, which are only found in *Claviceps* species. The class of peptide ergot alkaloids is subdivided into two main types, ergopeptines and ergopeptams (**Figure 13**). Classical ergopeptines consist of a lysergic acid molecule linked by an amide bond to a cyclol-structured tripeptide (**Figure 13**) [10, 11]. Ergopeptines are the most widely spread natural peptide ergot alkaloids. The second type of peptide ergot alkaloids, the ergopeptams, has a non-cyclol-lactam group instead of a cyclol (**Figure 13**). Ergopeptams are rare compared to ergopeptines, and only three ergopeptams were found in ergot sclerotia: ergocristame, ergokryptame and ergocorname. In fungi of the genus *Claviceps*, ergopeptines typically represent 70 to 90% of the ergot alkaloid content of the sclerotia. This group has been subdivided into three groups depending on the amino-acid I of the cyclol group: the ergotamine group, ergotoxin group and ergoxine group. An overview of the three groups of ergopeptines is given in **Table 1** [10]. A fourth group, the β-ergoannam group, is produced by saprophytic cultures, but does not naturally occur in the nature. The main ergopeptines produced by *Claviceps purpurea*, which are of a major food safety concern, are: ergotamine, ergosine, ergocornine, ergokryptine and ergocristine (**Table 1**, **Figure 14**) [16]. Ergovaline is another peptide ergot alkaloid produced by *Claviceps* species and *Neotyphodium* species, and which is also involved in livestock toxicoses caused by ingestion of endophyte-infected grasses [16-18].

Figure 13. *General chemical structure of ergopeptines and ergopeptams, with the amino-acids I, II and III highlighted in respectively, green, blue and pink areas [11].*

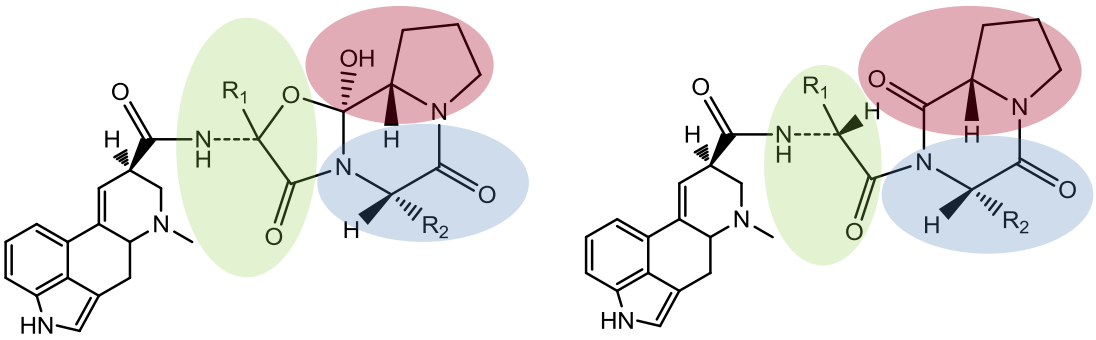
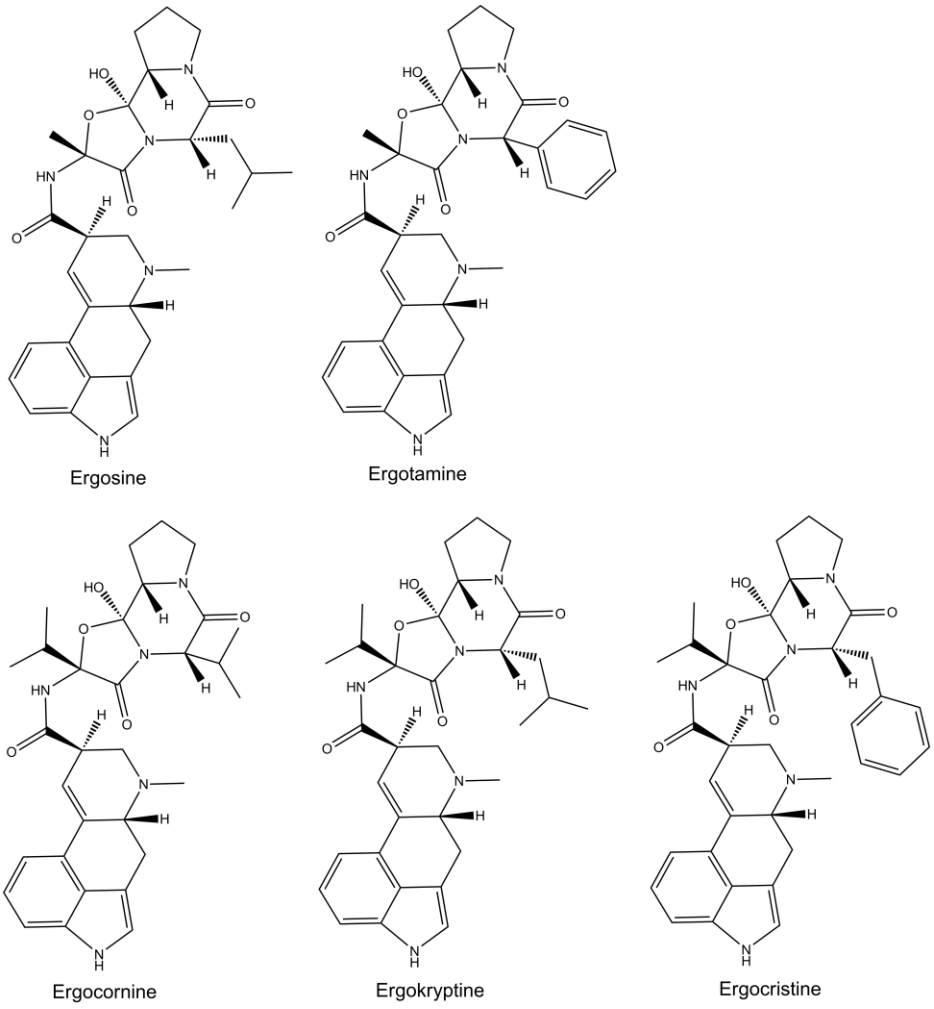


Table 1. *Classification of ergopeptines [10].*

R2 / L-amino-acid II R1 / L-amino-acid I	CH_2-C_6H_5 **Phenylalanine**	CH-$(CH_3)_2$ **Valine**	CH_2-$CH(CH_3)_2$ **Leucine**	$CH(CH_3)CH_2$-CH_3 **Isoleucine**	CH_2-CH_2-$CH(CH_3)_2$ **Homoleucine**	CH2-CH3 **α -Aminobutyric acid**
CH_3 **Alanine**	Ergotamine	Ergovaline	Ergosine		Ergohexine	
$CH(CH_3)_2$ **Valine**	Ergocristine	Ergocornine	α-Ergokryptine	β-Ergokryptine	Ergoheptine	Ergobutyrine
CH_2-CH_3 **Aminobutyric acid**	Ergostine	Ergonine	α-Ergoptine			Ergobutine

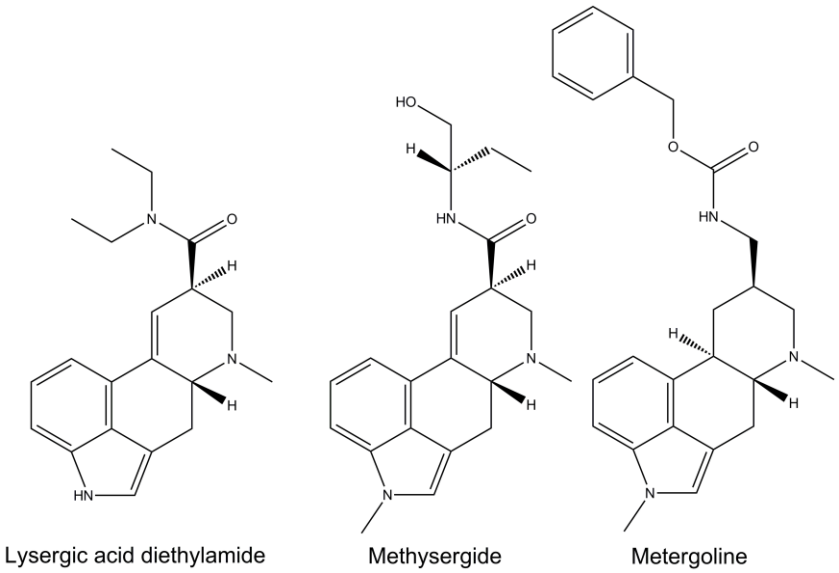
Figure 14. *Chemical structures of the main ergopeptines found in Claviceps species: ergosine, ergotamine, ergocornine, ergokryptine and ergocristine.*



2.4. Synthetic ergot alkaloids

Due to their biological activity, ergot alkaloids are also a source of pharmaceutical compounds. A few natural ergot alkaloids are used for medical purposes, such as ergometrine and ergotamine. But, many other synthetic ergot alkaloids derivatives were prepared until nowadays. One common change is the elimination of the double-bond between C9-C10 by catalytic hydrogenation. Dihydro-ergotamine, dihydro-ergocristine and dihydro-ergokryptine are obtained this way [10]. Dihydroergotamine is prescribed to treat hypotension and to prevent migraine. Dihydroergocristine is used to treat vascular disorders and Alzheimer's disease [19]. Methysergide (**Figure 15**) is another semi-synthetic ergot alkaloid indicated for the treatment of vascular headache [6]. Methysergide is either obtained via synthesis from lysergic acid, or by methylation of ergometrine. Metergoline (**Figure 15**) is another example of semi-synthetic ergot alkaloid, which is known as an anti-serotonin agent. The highly hallucinogenic lysergic acid diethylamide (LSD) (**Figure 15**) was synthesized by Hoffman in 1938, who was a researcher at Sandoz pharmaceutical company and who was working on the elaboration of new medical compounds deriving from ergot. The company tried to find a therapeutical utility for LSD but it was observed that numerous patients experienced as many adverse reactions as beneficial ones, and this compound became no longer in use for therapy.

Figure 15. *Chemical structures of some semi-synthetic ergot alkaloids: lysergic acid diethylamide, methysergide and metergoline.*



3. Experimental part I: Synthesis of a template ergot alkaloid

As ergot alkaloids represent a wide family of compounds, it was necessary to choose which ergot alkaloid(s) to work on. As the composition of ergot alkaloids can vary enormously from one sample to another depending on several factors such as: the strain of the fungus, the host-plant and stage of development of the fungus; it would be interesting to be able to recognize several ergot alkaloids. According to the survey of Diana Di Mavungu et al. [20] submitted to the EFSA, ergometrine is the most frequently occurring ergot alkaloid, especially in the samples with low concentrations. In the most heavily contaminated samples, ergosine is one of the most encountered ergot alkaloid. In rye feed samples, ergotamine were the most abundant ergot alkaloid; and in wheat samples, ergotamine and ergokryptine were found in major quantities [20, 21]. In other feed samples, such as barley, ergokryptine and ergocornine were the most abundant ergot alkaloids [21]. Due to the broad variety of target compounds, it was chosen to work on different ergot alkaloids, the aim being to obtain both very specific and general recognition elements for ergot alkaloids. Then, one ergopeptine was selected for this study. As most ergopeptines are only available in minute amounts, very expensive and moreover almost chemically inert compounds (except ergometrine), only ergocornine was selected for this project. Then, other ergot alkaloids were taken into consideration, such as metergoline; which is a commercially available ergot alkaloids and which has chemically reactive groups [22]. Finally, it was also chosen to work on a structure as close as possible to the one of the ergot alkaloid precursor ergoline. As the use of lysergic acid and similar compounds is very restricted by the law, it was decided to perform a chemical cleavage of metergoline in order to obtain a smaller ergot alkaloid, lysergamine, having the ergoline structure and a reactive primary amine moiety [23]. For the selection of aptamers, it is necessary to first immobilize the target molecule onto a support. The most commonly used supports are nitrocellulose membrane filtration systems, silica gel columns, sepharose or agarose columns, and magnetic micrometer-sized beads [24]. Magnetic beads are more and more used as solid supports as they possess several advantages: the partitioning of the bound aptamers from the rest of the library can easily be done by applying a magnet and removing the supernatant containing the unbound fraction of the library, only small quantities of target molecule and DNA library are required to perform the aptamer selection [25]. However, the characterization of magnetic beads coated with the target molecules is restricted to a few techniques only, due to the fact that the analysis has to be limited to the surface of the magnetic beads. In the literature, infrared spectroscopy and Raman spectroscopy have been reported for the characterization of magnetic beads after synthesis [26-29]. However, no direct characterization of the surface of magnetic beads was reported after coating with small organic compounds. Therefore, it was investigated if infrared spectroscopy could be employed for the analysis of the coating of magnetic beads with small organic molecules.

3.1. Materials and method

In order to obtain a more reactive ergot alkaloid having a chemical structure as close as possible as ergot alkaloids precursor, ergoline; metergoline was transformed into lysergamine (**Figure 16**) by hydrogenolysis in a Parr apparatus [23]. In a flask, 50 mg of 10% Pd/C (Sigma Aldrich, Belgium) were suspended in 15 mL of methanol and 250 mg of metergoline were added after dissolution in 2 mL of methanol. While stirring, the mixture was sparged with a slow stream of hydrogen gas (2.06 Bar) for 16 h at room temperature (RT). The reaction mixture was filtered through celite and the solvent was evaporated under reduced pressure by using a rotary evaporator. As an oil was obtained, ethyl acetate was added to the product and subsequently evaporated, resulting in a white to light yellow powder of lysergamine. The product resulting from metergoline cleavage was characterized by mass spectrometry in positive mode. The sample was prepared by diluting 2 µL of the reaction mixture in 198 µL of acetonitrile. For the analysis, 5 µL of this solution were injected in a 0.2 mL/min flow of acetonitrile with 0.1% formic acid on an Acquity UPLC system (Waters, Elstree, UK). The mass spectra were recorded using electrospray ionization in positive ion mode using a Xevo TQ MS system (Waters, Elstree, UK).

3.2. Mass spectrometry analysis of the product of the reaction

The reaction product of the cleavage of metergoline, lysergamine, was analyzed by mass spectrometry. The protonated molecule at m/z 270.24 (m/z calculated 270.19) was observed in the mass spectrum (**Figure 17**), confirming that lysergamine was obtained by hydrogenolysis of the benzyloxycarbonyl group of metergoline. The absence of peak at m/z 404 g/mol showed that the reaction was complete. The reaction product, lysergamine, contains a methylated ergoline structure bearing a primary amine (**Figure 16**).

Figure 16. *Scheme of the cleavage reaction of metergoline into lysergamine using palladium supported on activated carbon and dihydrogen.*

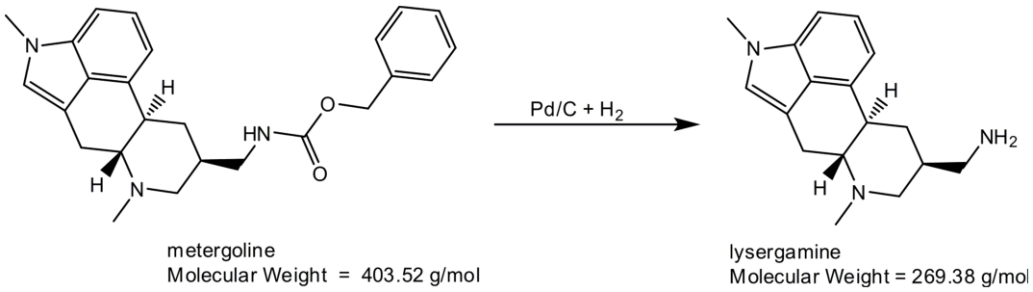
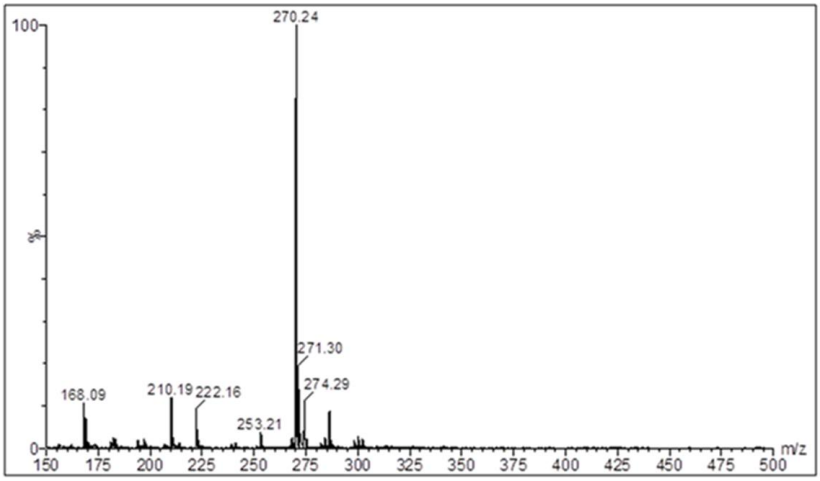


Figure 17. *Mass spectrometry analysis in positive mode of the reaction product obtained from the cleavage of metergoline into lysergamine.*



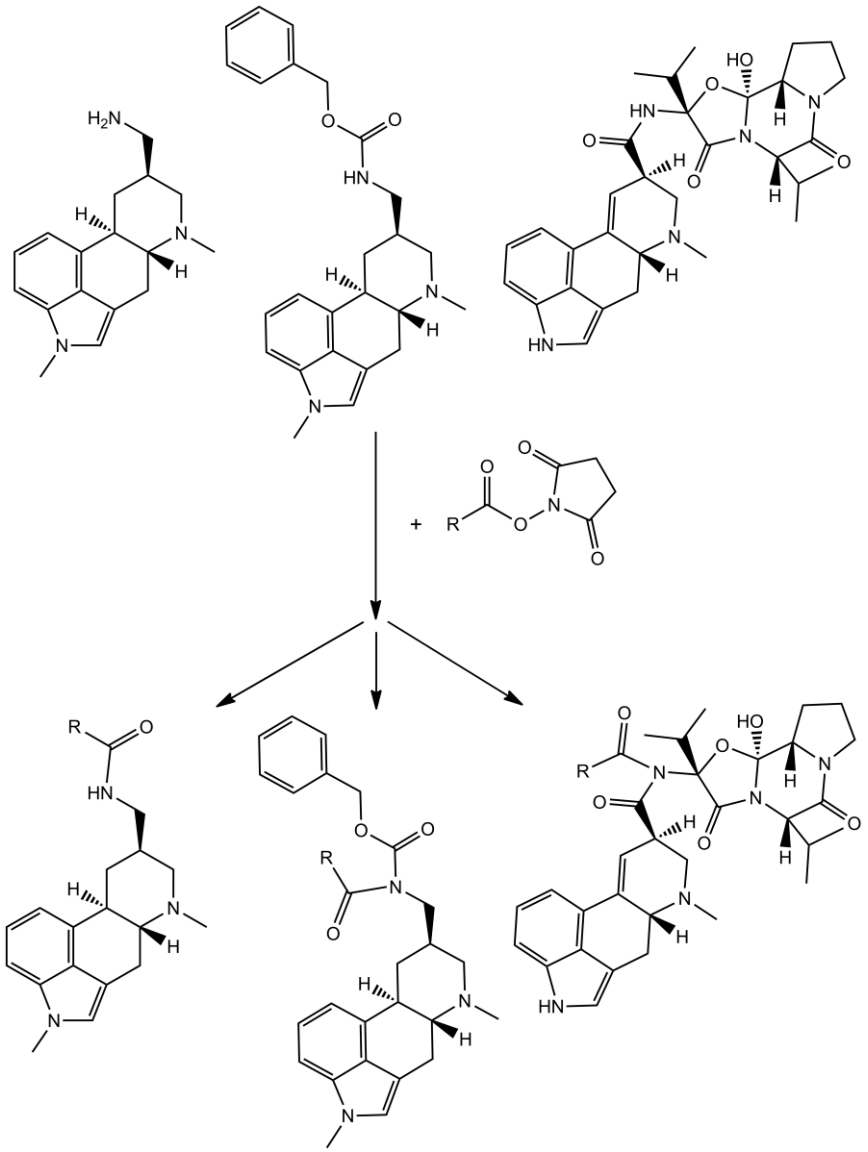
4. Experimental Part II: Coating of magnetic beads with ergot alkaloids

4.1. Materials and methods

The three selected ergot alkaloids: lysergamine, metergoline and ergocornine; were immobilized onto NHS-activated magnetic beads, as shown on **Figure 18**.

In three different vials, suspensions of NHS-activated magnetic beads (Invitrogen, Merelbeke, Belgium) were prepared by washing a quantity of 2×10^9 magnetic beads with 0.3 mL of dry DMF and placing them in 0.5 mL of dry DMF. Then, 5 mg of lysergamine were added in the first vial, after dissolution in 0.4 mL of dry DMF. In the second vial, 6 mg of metergoline in 0.5 mL of dry DMF were placed. In the third vial, 5 mg of ergocornine in 0.3 mL of dry DMF were added. The mixtures were stirred for 24 h at RT. By applying a magnet, the supernatants were removed and a quench solution of 2 µL of ethanolamine in 0.6 mL of dry DMF was added to each vial and the mixtures were stirred for 5 h at RT. After applying a magnet, the supernatants were discarded; the magnetic beads were then washed five times with 0.3 mL of DMF and finally placed in 0.4 mL of DMF. Quenched magnetic beads were prepared by adding a solution of 5 µL ethanolamine in 0.6 mL of dry DMF to a suspension of 2×10^9 NHS-functionalized magnetic beads in 0.3 mL of dry DMF. The mixture was stirred for 4 h at RT. By applying a magnet, the supernatant was discarded and the ethanolamine coated magnetic beads were washed five times with 0.3 mL of DMF; and were finally placed in 0.4 mL of DMF.

Figure 18. *Scheme of the coating of NHS-activated magnetic beads (with R the rest of magnetic bead) with lysergamine (left), metergoline (middle), and ergocornine (right).*



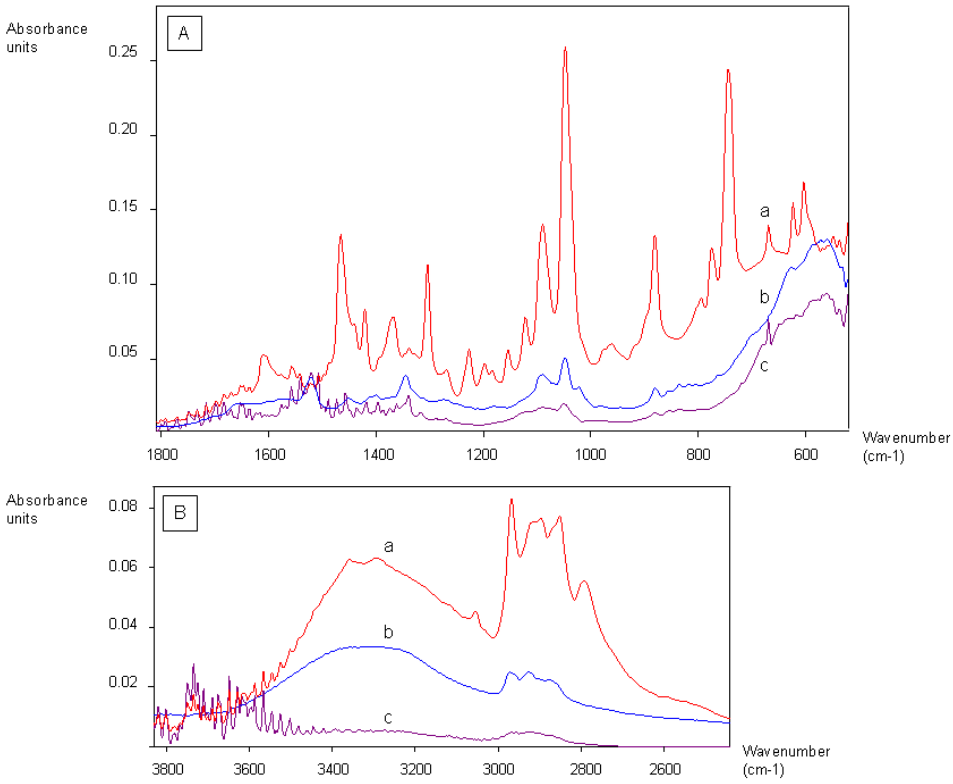
For the infrared analysis, a Fourier-Transform Attenuated Total Reflection Infrared spectroscopy (FTIR-ATR) apparatus Bruker Equinox 55 spectrometer (Brüker, Germany) was used either with a nickel chip or a diamond chip. Diamond chips offer the advantage of being flat; while nickel chips are curved, rendering the coating of the chip more difficult and requiring a more important volume of solution to be analyzed. In this analysis, 80 µL of the coated beads solutions were taken and washed several times with pure ethanol, and finally placed in 10 µL of pure ethanol. The concentrated solutions of coated beads were placed onto the infrared chip and the solvent was evaporated using a very slow stream of nitrogen gas to leave a layer of magnetic beads on the surface of the infrared apparatus chip. This step was repeated twice in order to obtain a solid layer of dried magnetic beads on the infrared chip. It is very important at this stage to cover the surface completely in order to

obtain a significant signal with FTIR-ATR spectroscopy. The process was repeated until the chip was completely covered by a solid layer of magnetic beads. Also, solutions of reagents were evaporated onto the infrared chip and analyzed, in order to compare the spectra.

4.2. Results and discussion

The resulting ergot alkaloid coated magnetic beads were analyzed by infrared spectroscopy and compared to the reagents of the chemical reaction in order to determine if the expected covalent binding occured. **Figure 19** shows the infrared analysis of the coupling between lysergamine and NHS-activated magnetic beads.

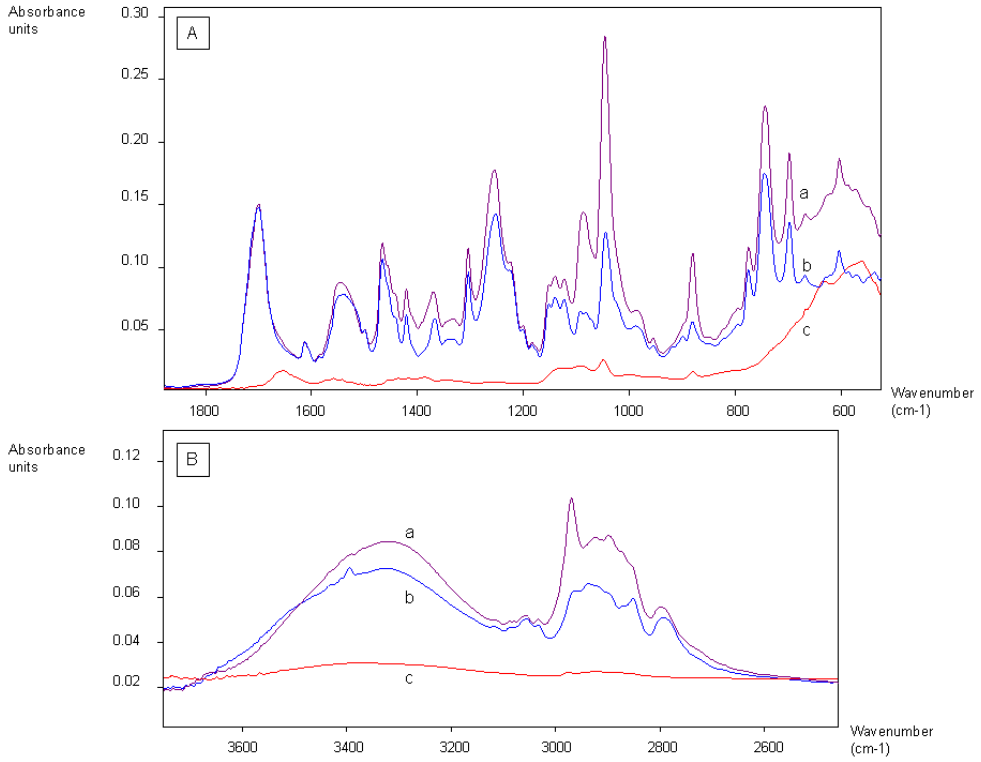
Figure 19. *Infrared spectra of the coating of NHS-functionalized magnetic beads with lysergamine. (**A**) Range from 600 cm−1 to 1800 cm−1 (**B**) Range from 2600 cm^{-1} to 3800 cm^{-1}. (**a**) Lysergamine (**b**) Ethanolamine quenched NHS-activated magnetic beads (**c**) Lysergamine coated NHS-activated magnetic beads.*



The infrared spectra of lysergamine (**a**), lysergamine coated magnetic beads (**c**) and ethanolamine coated magnetic beads (**b**) were analyzed and compared (**Figure 19**). This chemical reaction was characterized by the loss of the peaks of the primary amine (ν_{Max} cm^{-1} 3298 and 3355) and the formation of an amide bond (ν_{Max} cm^{-1} 1679). The stretching of the C–N peak at ν_{Max} cm^{-1} 1089 increased in the spectrum of the lysergamine coated beads, due to the formation of the amide bond. The characteristic peaks of lysergamine were found in the spectrum of lysergamine coated beads at ν_{Max} cm^{-1}: 2968, 2896, 1451, 1422, 1389, 1304,

1049, 880 and 679. The covalent binding of lysergamine occurred on the primary amine which is opposite to the methylated ergoline part. It was then possible to conclude that the methylated ergoline skeleton was situated on the outer part of the magnetic beads, consequently being accessible to the aptamer library in a further step of the selection procedure. **Figure 20** shows the infrared spectra obtained from the coating of NHS-functionalized magnetic beads by metergoline.

Figure 20. *Infrared spectra of the coating of NHS-functionalized magnetic beads with metergoline. (**A**) Range from 600 cm^{-1} to 1800 cm^{-1} (**B**) Range from 2600 cm^{-1} to 3600 cm^{-1}. (**a**) Metergoline coated NHS magnetic beads (**b**) Metergoline (**c**) Ethanolamine quenched NHS magnetic beads.*



This reaction was characterized by the loss of the N-H stretching peak of the carbamate group at ν_{Max} cm^{-1} 3400 and an increase of C–N stretching peak at ν_{Max} cm^{-1} 1090 due to the new bond formed between the magnetic beads and metergoline, were observed. The characteristic absorption peaks of metergoline were also found in the spectrum of metergoline coated magnetic beads. It was thus possible to determine that the molecules of metergoline were attached to the magnetic beads by the centre of the molecules, and that both the ergoline skeleton and the other cyclic part were exposed to the DNA library for the selection procedure. The FTIR-ATR spectra of the reaction between NHS-functionalized magnetic beads and ergocornine are shown in **Figures 21 and 22**, with the measurements of the following items: **(A)** ethanolamine quenched NHS-functionalized magnetic beads **(B)**

ergocornine solution before reaction **(C)** supernatant of the reaction without the magnetic beads **(D)** ergocornine functionalized NHS-activated magnetic beads. The absorption peaks of magnetic beads are mainly visible below 1200 cm^{-1} due to the rigidity of the material; and the characteristic peaks of ergocornine and ethanolamine are mainly visible beyond 1200 cm^{-1}. In the spectrum **(A)**, corresponding to ethanolamine quenched magnetic beads, the main absorption peaks were found at ν_{Max} cm^{-1}: 3400, 3200, 2930, 1737, 1379, 1208, 1133, 1071, 996 and 955. The peak at ν_{Max} cm^{-1} 3400 was attributed to the N-H stretch of ethanolamine and the peak at 3200 cm^{-1} was attributed to the -OH stretch of ethanolamine. The C=O stretch was determined to be at ν_{Max} 1737 cm^{-1}. The main absorption peaks of the magnetic beads **(A)** were found in the spectrum of the ergocornine coated magnetic beads at ν_{Max} cm^{-1} 1132, 1071, 1000 and 958. The characteristic absorption peaks of ergocornine **(B and C)** were found in the spectrum of ergocornine coated magnetic beads **(D)** at ν_{Max} cm^{-1} 3400 (NH), 3210 (OH), 1656 (CO), 1561, 1436, 1345 and 1390. As the reaction occurred on the non-fully substituted amide moiety of ergocornine, the absorption peak at ν_{Max} cm^{-1} 1724 was shifted to ν_{Max} cm^{-1} 1656; with the other peaks of substituted amides of ergocornine. It could be deduced from the infrared spectra that ergocornine was attached to the magnetic beads from its central amide moiety. The three types of ergot alkaloid coated magnetic beads were kept for the selection of DNA. It was observed that lysergamine and metergoline coated magnetic beads were stable and could be kept for several months at 4°C, while ergocornine coated magnetic beads were less stable and had to be freshly prepared before use.

Figure 21. *Infrared spectra of the coating of NHS-functionalized magnetic beads with ergocornine (ranging from 1000 to 4000 cm^{-1}). **(A)** ethanolamine quenched NHS-functionalized magnetic beads **(B)** ergocornine solution before reaction **(C)** supernatant of the reaction* without the magnetic beads **(D)** ergocornine functionalized NHS-activated magnetic beads.

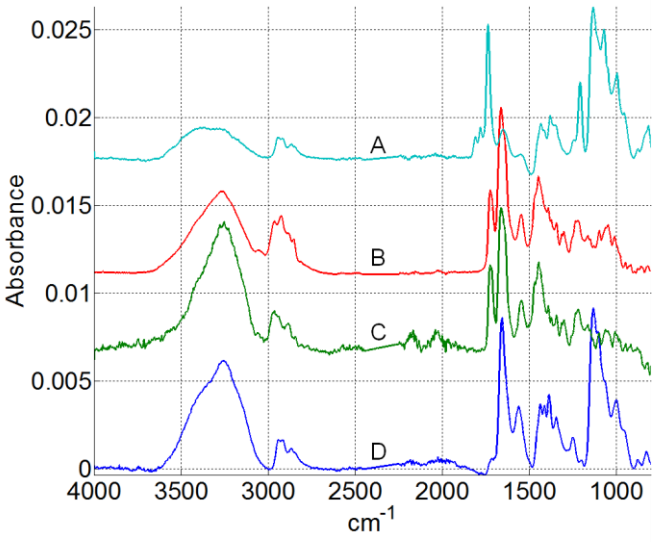
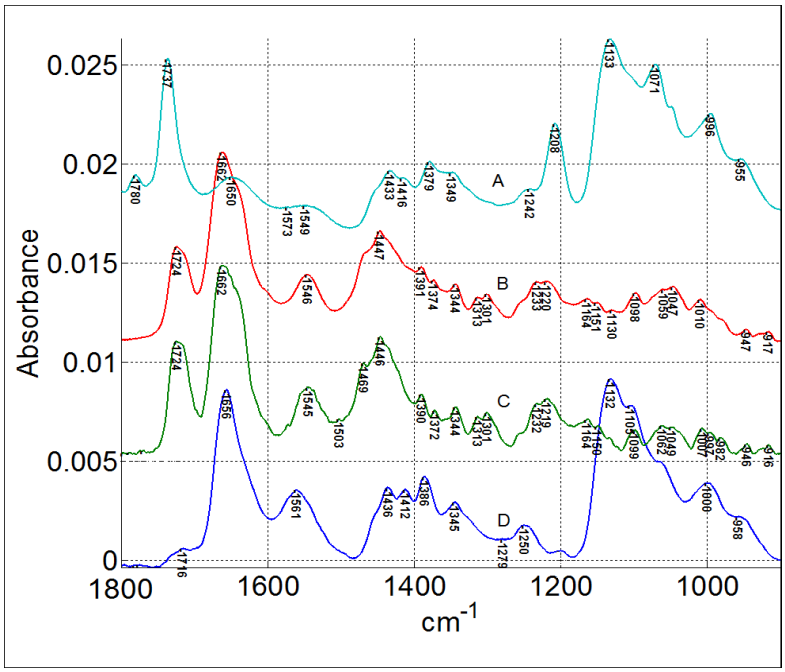


Figure 22. *Infrared spectra of the coating of NHS-functionalized magnetic beads with ergocornine (ranging from 1000 to 1800 cm^{-1}). **(A)** ethanolamine quenched NHS-functionalized magnetic beads **(B)** ergocornine solution before reaction **(C)** supernatant of the reaction without the magnetic beads **(D)** ergocornine functionalized NHS-activated magnetic beads.*



5. Conclusion

Ergot alkaloids can have various chemical structures, sizes and different chemical reactivities. Ergopeptines are very often encountered in ergot contaminated food and feed. However, ergopeptines are very elaborate chemical structures, which are less chemically reactive compared to smaller ergot alkaloids. Also, the composition of ergot in food and feed can vary a lot, and may or may not include certain ergot alkaloids. In order to select specific recognition elements which can either recognize one or several ergot alkaloids, several ergot alkaloids were studied: ergocornine, metergoline and its cleavage reaction product, lysergamine. The three ergot alkaloids were immobilized onto magnetic beads for the selection of DNA aptamers. The ergot alkaloid coated magnetic beads were successfully characterized by infrared spectroscopy technique, which enabled to determine the covalent bonds formed.

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Chapter III: Selection of DNA aptamers for ergot alkaloids

1. Introduction

In living organisms, proteins are mainly involved in the molecular recognition for metabolic purposes, while ribonucleic (RNA) and deoxyribonucleic acids (DNA) have mainly an informative role as supports for the genetic code. To a certain extent, sugars can play a complementary role for the recognition of certain targets together with proteins, but rarely nucleic acid structures. However, it has been shown that certain molecules can bind to nucleic acids, in a reversible or irreversible way; and also that some compounds, defined as mutagenic, teratogenic or carcinogenic, can strongly bind to nucleic acids and can have a causality effect on them and their translation into proteins [1-3]. Thus, nucleic acids can be seen as potential binders to target molecules, even if it is not their natural function in living organisms. Proteinic structures were first used in biosensors, such as enzymes or antibodies [4-6]. Receptors were also used but their production can be quite complicate. Moreover receptors are very sensitive to degradation and they sometimes lack specificity, as they may bind to molecules having a very different chemical structure [5]. Antibodies were then used instead, thanks to their efficiency in specifically recognizing targets with a high sensitivity. The pregnancy test kit available on the market, which can detect the presence of human chorionic gonadotropin (hCG) in urine, is probably the most popular marketed antibody-based biosensor [7]. Antibodies are very often used in biosensors and sensing assays; however, besides the fact that the production of antibodies is not always animal-friendly, the proteinic structure of antibodies is sensitive to natural degradation and conditions of use of sensors [8, 9]. Proteases or chemicals affecting proteins can be present in biological samples for instance, and temperature changes can affect them as well. For such reasons, some interest went lately towards the use of nucleic acid structures instead of proteins. These last two decades, nucleic acids have gained a lot of interest in the development of biosensors, due to their advantageous features. The affinity and specificity towards target molecules is usually comparable to those of antibodies, and sometimes even better [10]. Moreover, nucleic acids are more stable than proteinic structures and offer many possibilities of modification with new functional groups without affecting their activity [11, 12]. Then, their synthetic production is animal-friendly, accurate and reproducible [10-12]. Nucleic acids can be used in two different ways, either to hybridize to a complementary nucleic acid sequence, or to recognize a target molecule by chemical affinity. In this latter case, the nucleic acid structure is not used for its coding, but for the types of bases contained in its sequence and its shape; and is called aptamer [13]. Although the terminology aptamer can be used for small peptides as well, it is usually used to describe these synthetic fragments of single-stranded nucleic acids evolved *in vitro* towards a target

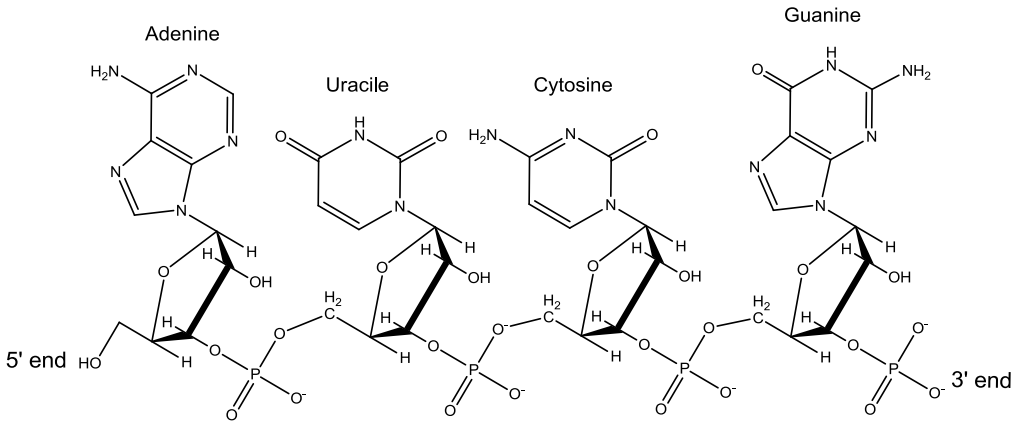
molecule [13, 14]. In this chapter, an overview of the different types of nucleic acids used in biosensors is given. Then, sensors based on hybridization probes and aptamers will be presented. Finally, the experimental part describes how DNA aptamers were selected for ergot alkaloids, using an *in vitro* selection procedure; including amplification, purification, cloning, sequencing and characterization of the selected aptamers.

2. Chemical structures of nucleic acids

2.1. RNA

In RNA chemical structure, the ribose and phosphate backbones support the four following bases: adenine, guanine, uracile and cytosine (**Figure 23**). RNA is known to fold on itself by following the base pairing properties of nucleic acids. Ribonucleic acid (RNA) is naturally found as single-stranded in living organisms, such as ribosomal, messenger or transfer RNA; which offers a greater diversity of shape compared to the structures of double-stranded nucleic acids. For this reason, the first nucleic acid aptamers reported were formerly made of RNA. The main disadvantage of using RNA is its instability towards enzymes, such as RNAses, which can be abundant in biological samples [10, 15]. Modified RNA aptamers were then introduced to overcome their sensitivity to degradation [10]. Also, DNA aptamers started to be more and more used for recognition purposes compared to RNA; as they benefit from a better stability.

Figure 23. *Chemical structure of RNA.*

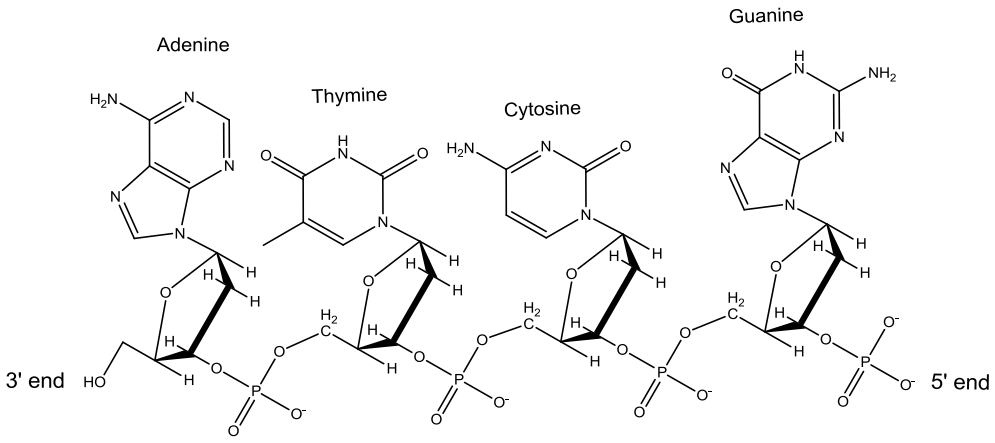


2.2. DNA

The chemical structure of DNA is very close to RNA, but the base thymine replaces uracile (**Figure 24**), and the phosphate backbone is linked to deoxyribose instead of ribose, making it less likely to degrade [15]. Chemically speaking, the properties of DNA are similar to the ones of RNA, and single-stranded DNA can fold on itself the same way as RNA. As DNA has a

better stability than RNA, the use of ssDNA aptamers in sensors became of a greater importance.

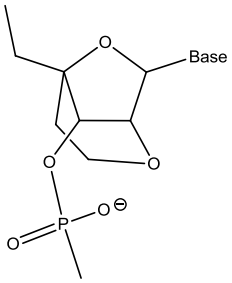
Figure 24. *Chemical structure of DNA.*



2.3. Other types of synthetic nucleic acids

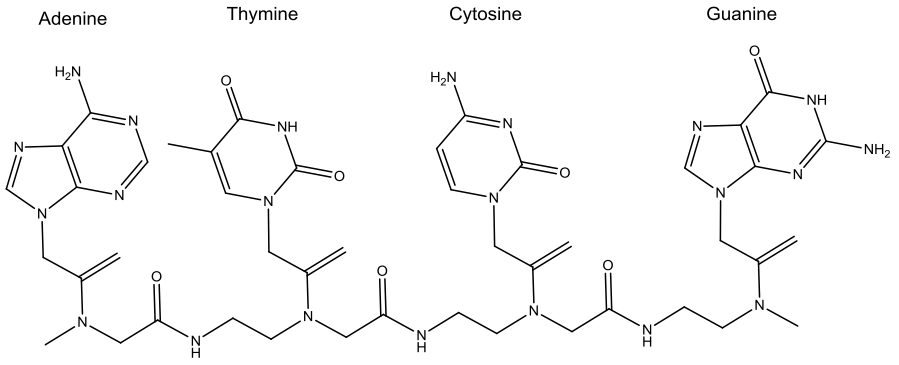
In order to enhance the chemical stability of DNA or to avoid the phosphate backbone, which is negatively charged, some derivatives of DNA were synthesized, such as locked-DNA (LNA) or peptide DNA (PNA). LNA bases have a structural modification, a bridge linking the 2'-oxygen of ribose with the 4'-carbon (**Figure 25**); rendering them very stable with a high melting point [16].

Figure 25. *Chemical structure of locked nucleic acid (LNA).*



Peptide DNA, or PNA, are constructed on an N-(2-aminoethyl)glycine structure (**Figure 26**) instead of a phosphate backbone, this allows to avoid having a charged phosphate backbone for instance [16].

Figure 26. *Chemical structure of peptide nucleic acids (PNA).*



3. Hybridization assays with nucleic acids

In case that the target is a nucleic acid sequence, a nucleic acid probe is probably one of the best choice based on the fact that nucleic acid sequences can assemble, or hybridize, to a complementary sequence by Watson and Crick base pairing between the bases A with T (two hydrogen bonds) and C with G (three hydrogen bonds). This hybridization property of DNA was used in the first place for numerous assays to hybridize to a complementary target DNA sequence, such as a part of viral, microbial, or other pathogenic DNA. Hybridization assays are very useful in different areas such as genetics, pathology, environmental sensing and microbiology [17, 18]. With nucleic acid hybridization probes, it is possible to measure targets that are in large number of copies, such as ribosomal or messenger RNA; or to measure targets present in minute amounts, after a pre-enrichment step (culture) and/or an *in vitro* amplification step (PCR) [17, 18]. After cell lysis and amplification, the target sequence can then hybridize to a nucleic acid probe bearing a label, allowing the detection of the target DNA fragment with simple analytical methods. There is a broad variety of analytical designs of DNA hybridization assays, more or less elaborated, but most of them imply an optical detection technique such as fluorescence or chemiluminescence. Different types of labels can be used, such as fluorescent labels, luminescent labels, enzymes or combinations of them. For instance, molecular beacons can be elaborated by linking a fluorophore and a quencher at the both ends of the nucleic acid probe. The presence of the target nucleic acid sequence can be exploited to separate the fluorophore from the quencher, which can be measured using a fluorometer [19]. Combinations of labels cited above are often used. For instance, Shalev et al. [20] used en enzyme probe, alkaline phosphatase, linked to the nucleic acid hybridization probe in order to convert the non-fluorescent substrate 4-methylubelliferyl phosphate into highly fluorescent 4-methylumbelliferone (excitation wavelength 365 nm, emission wavelength 455 nm). Also, surface plasmon resonance, quartz crystal microbalance and electrochemical transducers are currently used for nucleic acid hybridization assays [21, 22]. These techniques based on DNA hybridization offer the advantage of being very accurate. Also, it was shown that they could be used to detect very small variations in DNA sequences such as one single mismatch.

Therefore, DNA probes have shown to be the recognition elements of choice for the detection of single nucleotide polymorphism [23-26].

4. Nucleic acid aptamers

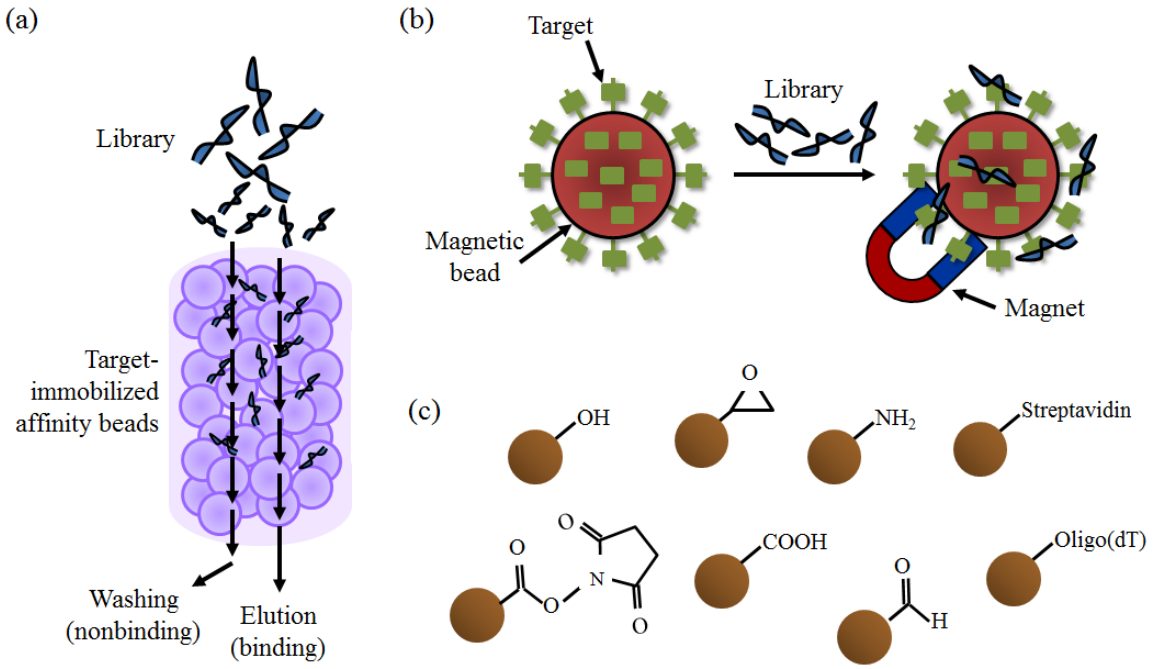
Nucleic acids can be used for other purposes than hybridization and can very well recognize molecules different from nucleic acids. Nucleic acid fragments which can recognize a target molecule based on molecular affinity, such as chemical affinity and complementarity of shape, are named aptamers. As these fragments of nucleic acids are not used for their coding, but for their shape and affinity towards the target molecule, the terminology aptamer was introduced by Ellington and Szostak in the 90's to describe them [13]. Nucleic acid aptamers are short fragments of RNA or single-stranded DNA, usually containing from 20 to 100 bases, which can specifically bind to a target molecule or family of compounds [13, 27-29]. The range of target compounds is extremely wide, going from atoms, molecules and even entire cells [12, 15, 30, 31]. The recognition of target molecules by nucleic acid aptamers is comparable to the recognition of antigens by antibodies. Aptamers and antibodies can both form stable complexes with their target molecules by shape complementarity and by chemical affinity. The main differences between aptamers and antibodies reside in their internal composition and ways of production. Antibodies are peptidic structures usually produced in animals whereas aptamers are nucleic acid structures which can be synthetically produced, and are therefore animal-friendly [31]. Depending on the target molecule, either aptamers or antibodies can be obtained and can compete in terms of sensitivity and specificity. Both of them can reach a sensitivity going to millimolar range to picomolar range in the best cases, and a specificity which can sometimes be reduced to one isomer or a broader range of related target compounds [30]. In terms of recognition, antibodies are well adapted for various proteins, hormones, viruses and bacteria or bacterial parts and in a broader view to any immunogenic compounds; whereas aptamers can be very well selected for non-immunogenic toxic compounds, various chemicals going from atoms to complex chemical structures and can be extended to the recognition of proteins and bigger edifices. Aptamers offer an interesting flexibility as their *in vitro* selection can be adjusted in order to improve their specificity by restricting the range of target related target compounds or alternatively to extend the specificity to a whole family of related chemical structures [32, 33].

4.1. Systematic Evolution of Ligands by EXponential enrichment (SELEX)

Aptamers are selected out of libraries of nucleic acid by following an iterative selection procedure named SELEX, standing for Systematic Evolution of Ligands by EXponential enrichment [10, 34]. The SELEX procedure consists of cycles of steps of binding to the target molecule, recovery and amplification of potentially good aptamer ligands. The amplification of aptamers is done by using polymerase chain reaction (PCR) [10, 35, 36]. The selected

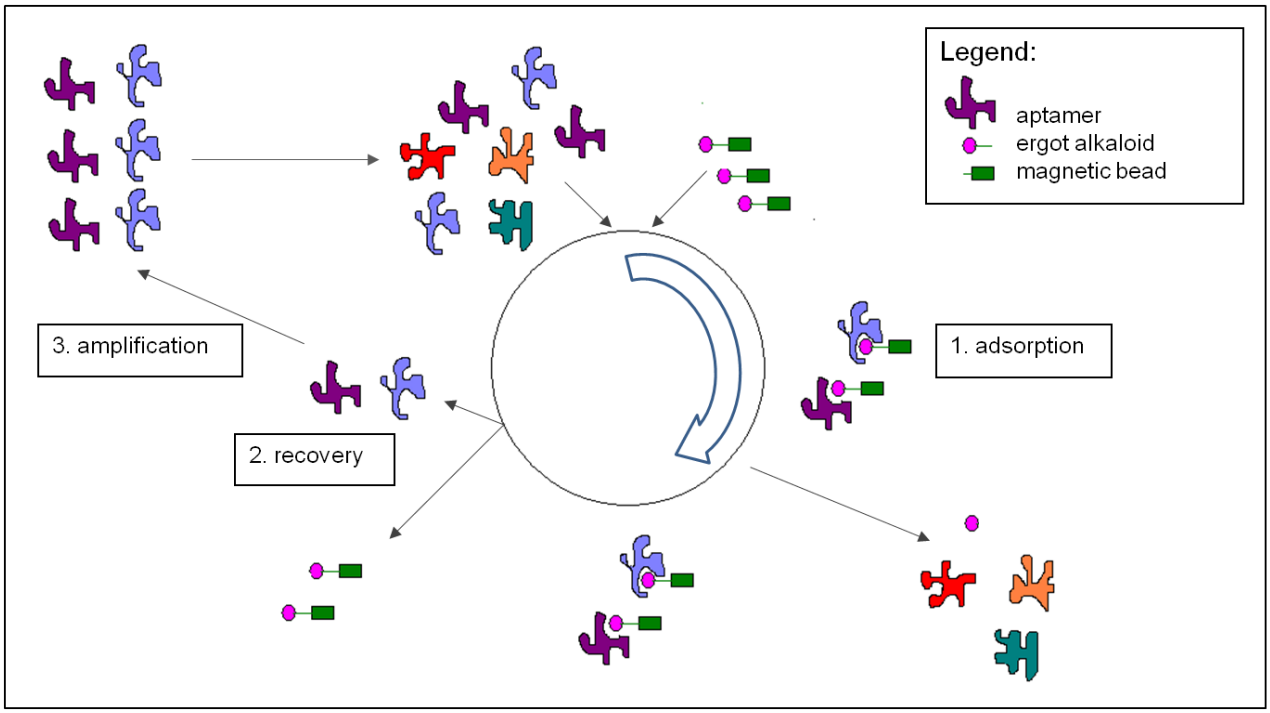
aptamers are then incubated and recovered several times with the target molecule until the recovery pool reaches the initial amount used for the selection, meaning that the best binding elements are selected out of the initial nucleic acid library [10, 13, 37-39]. The range of size of the aptamer library used is defined prior to the selection, and is usually going from 20 to 120 nucleotides. The advantage of using small libraries is the cheaper cost, but longer nucleic acid sequences allow the formation of more elaborate and complex secondary structures. In order to perform a selection of aptamers for a certain target molecule, it is usually necessary to first immobilize the target molecule onto a support in order to separate the binding nucleic acid sequences from the rest of the library. For that, several types of supports are currently used such as silica gel, microtitre plates, nitrocellulose filters or magnetic beads (**Figure 27**) [40].

Figure 27. ***(a)*** *A schematic illustration of the selection step from a library using an affinity column* ***(b)*** *The process of the selection step using magnetic beads* ***(c)*** *Several types of functional group-activated beads: alcohol, epoxy, amine, streptavidin, NHS, carboxylic acid, aldehyde and oligo-dT [40].*



In **Figure 28** is presented the general scheme of the selection procedure SELEX using magnetic beads, which was also used for ergot alkaloids; in which a single-stranded DNA library is incubated with magnetic beads coated with the molecule of interest in order to isolate the good binders called aptamers from the library, and amplify them afterwards. There are several advantages of using magnetic beads, such as the small amount of DNA library required and molecule of interest; the good partition between the bound and unbound DNA fragments due to the powerful magnetic separation, the possibility to covalently bind the molecule of interest and the obtention of a stable material.

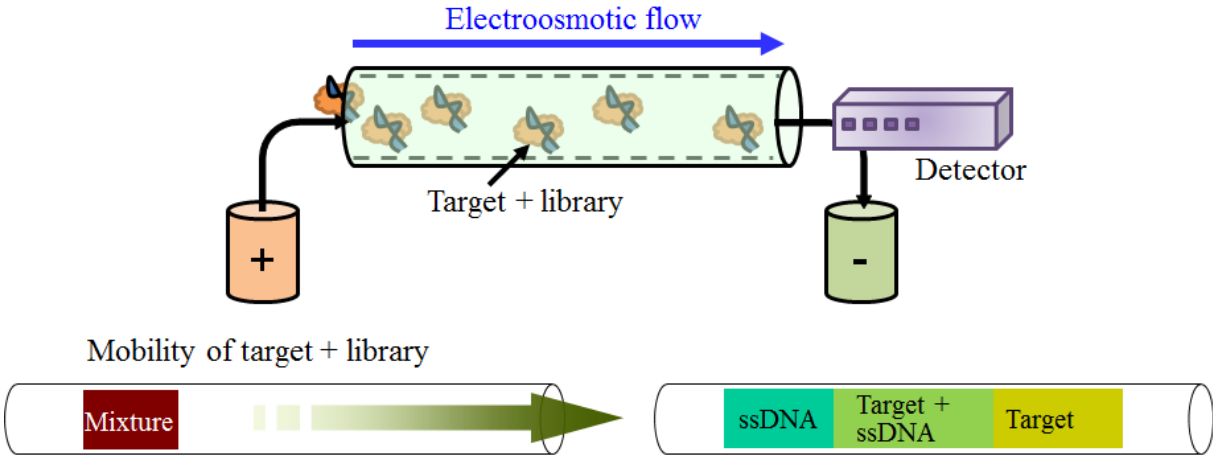
Figure 28. *Scheme of SELEX procedure (Systematic Evolution of Ligands by EXponential enrichment) for ergot alkaloids.* ***(1)*** *Adsorption. A random ssDNA library is incubated with the ergot alkaloid coated magnetic beads. By applying a magnetic stand, the fraction of ssDNA which is not bound to the ergot alkaloid coated magnetic beads is discarded.* ***(2)*** *Recovery. After washing steps, the fraction of ssDNA bound to the ergot alkaloid coated magnetic beads is eluted from the ergot alkaloid coated magnetic beads.*
(3) *Amplification. The selected ssDNA aptamers are amplified by polymerase chain reaction (PCR) in order to be used as input for the following selection cycle*.



Several modifications can be brought to the SELEX procedure in order to improve the aptamer binding efficiency [29, 30, 40]. For instance, capillary electrophoresis SELEX (CE-SELEX) allows the production of aptamers with fewer cycles of selections than typical SELEX. Capillary electrophoresis is an electrophoresis performed in a capillary tube usually containing silica, Teflon, or borosilicate glass; in which the different ionic species are separated by their charge, frictional forces and hydrodynamic radius under the influence of an electric field (**Figure 29**) [40]. The translational movement of the different ions is opposed by a retarding frictional force ($F_f = fv_{ep}$), which is proportional to the velocity of the ion, v_{ep}, and the friction coefficient f. The ion almost instantly reaches a steady state where the accelerating force equals the frictional force. The aptamers from the library that are bound to the target molecule have a different mobility than the target or the library, allowing a good partitioning of the potential interesting aptamers and the rest of the library. Another technique used to improve the aptamer selection is Atomic Force Microscopy SELEX (AFM-SELEX), allowing to select aptamers having strong affinity for the target compound. AFM technique can detect the adhesion or affinity force between a sample surface and a

cantilever. In this technique, aptamers selected from conventional SELEX procedure are tagged with fluorophores, producing more or less bright fluorescent spots observed by microscopy. The fluorescence intensity gives an indication about the binding strength of the aptamer to the target molecule, which can be exploited to select the best binding aptamers.

Figure 29. *Scheme of CE SELEX. Aptamers are selected based on the difference of mobility due to charge and mass [40].*



4.2. Polymerase Chain Reaction (PCR)

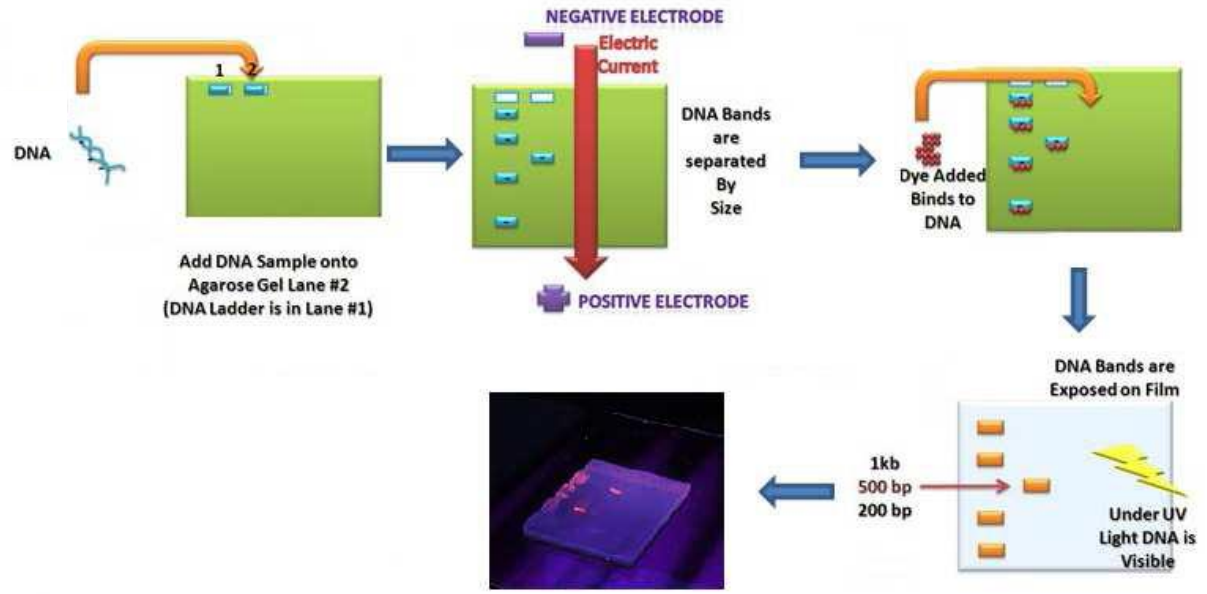
After the selection process, minute amounts of DNA binding to the target molecule (aptamers) are eluted. The eluted DNA sequences are amplified by PCR technique. PCR technique consists of enzymatic replication of DNA in a thermocycler, which can generate thousands to millions of copies of the DNA fragments [41, 42]. PCR technique, which was developed by Kary Mullis in the 1980's, allows the production of copies of a certain DNA sequence by using an enzyme, a DNA polymerase, a set of deoxyribonucleotide triphosphates (dNTPs), associated with primers and appropriate buffer. Primers are small DNA fragments complementary to the beginning and the end of the DNA sequence to be copied. The DNA library contains a random part flanked by two known common regions at the beginning and the end of their sequence, the primer region, enabling to carry out a general PCR for all the aptamers contained in the library. The choice of primer regions and primers is crucial for the selection of aptamers in order to ensure the feasibility of the PCR [43, 44]. The primer regions are designed to reduce primer-dimers formation and self-priming. A PCR is carried out in three distinct steps: denaturation step, annealing step and extension step, which are repeated several times in order to obtain sufficient amounts of DNA copies [45]. The first step is the DNA denaturation step that renders all the DNA fragments in the reaction single-stranded at a high temperature usually between 90°C to 97°C for 30 seconds. The second step is the primer annealing step, during which the PCR primers find their complementary targets and attach themselves to those sequences. Here, the choice of temperature is largely determined by the melting temperature (Tm) of the two

PCR primers. Again, the usual duration is 30 seconds. Finally, the last step in a PCR cycle is the extension step, during which the DNA polymerase is producing a complementary copy of the target DNA strand starting from the PCR primer sequence. The usual temperature of this step is 72°C, considered to be a good optimum temperature for thermal-stable polymerases [46]. The PCR product contains a mixture of aptamers and their copies, excess of primers and possible PCR by-products. After analysis, the PCR product is then extracted and purified.

4.3. Gel electrophoresis

The PCR products can be analyzed by using gel electrophoresis. Gel electrophoresis is a method of separation and analysis of charged molecules, which is currently employed for the separation and analysis of PCR products [47, 48]. Electrophoresis refers to the electromotive force (EMF) that is used to move charged molecules through a gel matrix. As nucleic acids are negatively charged, an electric field will make them move towards the anode, which is positively charged. They are placed in the wells of an agarose gel, which is placed into a buffer in the electrophoresis chamber. The electrical field is applied such as one end of the agarose gel is placed near the anode and the other end near the cathode (**Figure 30**). Depending on their charge and weight, the DNA fragments will move through the agarose matrix at different rates. As the charge to mass ratio (z) of the different DNA fragments is similar, the speed rate is largely determined by their weight; the smaller fragments moving faster [47, 48]. The DNA fragments are stained with fluorescent dyes such as ethidium bromide in order to make them visible. The DNA containing ethidium bromide in the agarose gel can be visualized by using a UV lamp (**Figure 30**).

Figure 30. *Scheme of agarose gel electrophoresis method used for the separation of DNA fragments having different lengths [48].*



A DNA ladder, consisting of a mixture of DNA fragments having a known length, is used in order to determine the size of the PCR products. Aptamers can be recovered either by extraction from agarose gel or by using a purification column. The advantage of extracting the DNA from agarose gel is that only the fraction containing the aptamer copies is taken, and all the other byproducts of the PCR can be discarded. This can play an important role in the aptamer selection, as more than 40 PCRs are usually required in order to generate enough potential aptamers during the selection procedure, besides the PCR reactions needed to copy the aptamers that will be sequenced

4.4. Cloning and sequencing of aptamers

The selection procedure generates a high number of different DNA aptamers, which have to be separated and amplified. Recombinant DNA technology allows the separation and the preparation of a large number of identical DNA molecules. The DNA fragments of interest are linked through standard 3'-5' phosphodiester bonds to a vector molecule, which can replicate when introduced in a host cell; and therefore produce a large number of copies [49]. Two types of vectors are commonly used: *Escherichia coli* vectors and bacteriophage λ vectors. Aptamer identification is usually done using *E. coli* plasmid vectors. These plasmid vectors are usually engineered to facilitate the cloning procedure. For instance, antibiotic resistance genes are usually inserted, such as ampicillin or kanamycin resistance genes. Each competent cell incorporates a single plasmid DNA molecule, which carries an antibiotic-resistance gene. When the treated cells are plated on a Petri dish of nutrient agar containing the antibiotic, only the rare transformed cells containing the antibiotic-resistance gene on the plasmid vector can survive. All the plasmids in such a colony of selected transformed cells are descended from the single plasmid taken up by the cell that established the colony [49]. Also, in order to facilitate the molecular cloning, a T-A cloning can be performed. T-A cloning is a technique which allows easy and quick cloning reaction, and which avoids the use of restriction enzymes. The technique relies on the ability of adenine (A) and thymine (T) (complementary base pairs) on different DNA fragments to hybridize and, in the presence of ligase, to become ligated together. The PCR is usually carried out with a thermostable *Taq* polymerase, which has a terminal transferase activity; which means that deoxyadenosine (dA) is preferentially added to the 3' termini of PCR product, leaving a single 3'-dA overhang. PCR products with dA overhangs can be directly cloned into a linearized plasmid vector with complementary dT overhangs at both ends [50-54]. This method, called T-A cloning, enables the rapid insertion of DNA into vectors. The recombinant DNA is inserted into host cells (*Escherichia coli*) by the process of transformation [53]. The vectors replicate with the bacteria, producing identical copies or clones of the insert DNA. The bacteria are grown on agar plates containing Luria-Broth medium. Colonies are picked, cultivated in liquid Luria-Broth medium and the plasmid vectors are extracted and purified. They are then sent to a

laboratory of genetics in order to be sequenced. This way, some of the different selected aptamers can be identified, allowing their characterization. Once the aptamer sequences are known, softwares such as Mfold or IDT Oligoanayzer can be used to determine the secondary structures of the selected aptamers and their inner properties [55, 56]. The tertiary structure of nucleic acids can also be determined, by using RNA1,2,3 or RNA 3D softwares for instance [57, 58].

5. Experimental Part: Selection of DNA aptamers for ergot alkaloids

5.1. Materials and Methods

5.1.1. Selection of aptamers using the SELEX procedure

Incubation of a library of synthetic single-stranded DNA with ergot alkaloid coated magnetic beads and recovery of binding sequences

Aptamers are generated out of a library, which consist of a pool of different DNA fragments having the same length; with a random region flanked by two fixed regions, which are used as primers for PCR reactions. The size of the DNA library usually ranges from 10 to 200 nucleotides [59]. Longer libraries allow a more important diversity of structure; however, the production costs are more important and it was shown that the efficiency of aptamer selection can decrease for long sequences [60]. In the case of very short libraries, a more important bias can occur during the amplification step [61]. For such reasons, an average library of 80 bases was used in this study, allowing a good diversity of shapes and motifs. The 80 base ssDNA library used was composed of 40 random nucleotides region flanked by two fixed primer regions of 20 bases, 5'-AGCAGCACAGAGGTCAGATG-N40-CCTATGCGTGCTACCGTGAA-3'. This library was chosen to also fulfil some requirements; such as reducing the possibility of primer dimer formation or self-priming of the fixed primer regions [62]. The selections for lysergamine, metergoline and ergocornine were carried out in parallel, a total of seven to ten selection cycles being usually necessary to generate highly binding aptamers. For the selection procedure, 80 µL of ergot alkaloid-coated magnetic beads solutions were placed in Eppendorf tubes and the solvent was removed and replaced by binding buffer (100 mM; 20 mM Tris-HCl; 5 mM KCl; 2 mM $MgCl_2$, $6H_2O$; 1 mM $CaCl_2$, $2H_2O$; pH adjusted to 7.6 with 1 M HCl), and finally placed in 300 µL of binding buffer. For the first round, 6 µL (15 µg) of 100 µM DNA library were placed in 200 µL of binding buffer and solutions of ergot alkaloid coated magnetic beads in binding buffer. After 20 min of incubation with gentle stirring of the mixture, a magnetic stand was applied and the supernatant was discarded. The beads were washed twice with the binding buffer. The ssDNA fraction bound to the ergot alkaloid coated magnetic beads was eluted with 3 ×

0.2 mL of elution buffer (3.5 M urea, 40 mM Tris-HCl, 10 mM EDTA, 0.02% Tween 20) by stirring gently (300 rpm) for 10 min at 80°C (Eppendorf Thermomixer, Belgium). Negative-selections with ethanolamine coated magnetic beads were applied starting from the second selection cycle, in order to remove the non-specifically bound aptamers. After each selection cycle, the recovered ssDNA was amplified by polymerase chain reaction (PCR) and used as input for the following selection cycle.

The eluted ssDNA was precipitated by adding 60 µL of 3 M sodium acetate (10% volume of elution buffer) and 1 mL of ice-cold ethanol. This solution was placed at −80°C for 1 h to ensure complete precipitation. The solution was centrifuged at 13,000 rpm at 4°C for 20 min in order to concentrate the DNA. The supernatant was discarded leaving approximately 50 µL of solution in the tube. Then, 1 mL of 70% ice-cold ethanol in water was added and the solution was centrifuged at 13,000 rpm at 4°C for 25 min. Again, the supernatant was discarded leaving 50 µL of solution in the tube and this latter step was repeated twice. The ethanol solution was evaporated in a speed vacuum device at 30°C for 10 min. The resulting DNA pellet was re-suspended in 15 µL of water (18.2 megohms/cm). The amount of ssDNA obtained from the selection was analyzed with a UV-Vis spectrophotometer (Nanodrop, Belgium) in order to determine the recovery rate.

5.1.2. Amplification of the selected ssDNA aptamers by polymerase chain reaction (PCR)

The ssDNA recovered from the selection procedure was amplified by PCR by using a thermostable *Taq* polymerase, PCR buffer, 10 mM dNTP solution and 25 mM $MgCl_2$ solution (Fermentas, Germany). The set of primers used for the PCR: 5'-AGCAGCACAGAGGTCAGATG-3' (forward primer) and 5'-TTCACGGTAGCACGCATAGG-3' (reverse primer), was provided by Eurogentec (Seraing, Belgium). In each vial, 6 µL of the ssDNA solution were placed in 20 µL water (18.2 megohms/cm). Then, the following solutions were added to the DNA template: 5 µL of PCR buffer, 3.5 µL of 10 mM dNTP, 11 µL of 25 mM $MgCl_2$, 0.5 µL of 100 µM solution of forward primer, 0.5 µL of 100 µM solution of reverse primer and 0.8 µL of *Taq* polymerase (5 Units/µL). The mixture was placed in a thermocycler (Mastercycler personal Eppendorf VWR, Belgium) with the following temperature profile: heating at 94°C over 6 min for the initial denaturation step, followed by 18 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 20 s, followed by a final elongation step of 72°C over 1 min 30 s. The *Taq* polymerase was added to the PCR reaction mixture after 4 min of the initial denaturation step. A gel electrophoresis of the PCR product was performed after each PCR. A 2% agarose gel was prepared in 1× Tris-acetate-EDTA (TAE) buffer with 10 µL of ethidium bromide for 100 mL of buffer. In each well of the agarose gel, 20 µL of PCR solution were placed after staining with a loading dye. A 50 base pair-DNA ladder was used to determine the length of the PCR product.

5.1.3. Preparation of ssDNA from biotinylated PCR product

A part of the double-stranded DNA obtained from the PCR was amplified with a reverse biotinylated primer (Eurogentec, Belgium) in order to separate the strands of the PCR product by using streptavidin magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen, Belgium), with a ratio of 0.1 mg of magnetic beads for 1 µg of dsDNA. The streptavidin magnetic beads were washed with 1× binding and washing (B & W) buffer (5 mM tris-HCl pH 7.5, 0.5 mM EDTA, 1 M NaCl) and resuspended in 2× B & W buffer to a concentration of 5 µg/µL. An equal amount of water solution of dsDNA was added to the solution of the streptavidin magnetic beads and the mixture was gently stirred for 25 min at RT. By applying a magnet, the magnetic beads were gathered onto the tube wall and the supernatant was discarded. The beads were washed twice with 1× B & W buffer and re-suspended in 0.15 M NaOH for 5 min at 37°C in order to release the non-biotinylated DNA strand. After applying a magnet, the solution was placed in a clean vial and immediately neutralized with an equal amount of 0.15 M acetic acid. The DNA was precipitated by adding 10% volume of 3 M sodium acetate and ice-cold ethanol to give a ratio of 70% in the final solution. This solution was incubated at −80°C for one hour and then centrifuged at 13,000 rpm at 4°C for 20 min. The solution was discarded; leaving approximately 50 µL at the bottom of the tube and 70% ethanol was added to the DNA pellet. The solution was centrifuged again with the same conditions and the step was repeated. The supernatant was discarded, leaving 20 µL of solution, which was evaporated in a speed vacuum device (Eppendorf Concentrator 5301, VWR, Belgium). The ssDNA was re-suspended in water and the concentration was determined by UV-Vis spectroscopy (Nanodrop, Thermo Scientific, Belgium).

5.1.4. Cloning and sequencing of aptamers

After each selection cycle, a part of the ssDNA selected was amplified by PCR with non-modified primers and cloned into the vector pCR2.1-TOPO (TOPO TA cloning kit, Invitrogen, Belgium) and transformed in *Escherichia coli* TOP 10 competent cells. Blue/white cloning was preferred to a normal cloning, as it allows the easy identification of colonies having inserted the aptamer fragments. A multiple cloning site (MCS) is present within the lac-Z sequence in the plasmid vector. When a plasmid vector containing foreign DNA is taken up by the host *E. coli*, the functional β-galactosidase enzyme is not produced. For screening the clones containing recombinant DNA, a chromogenic substrate known as X-gal is added to the agar plate. If β-galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by non-recombinant cells, therefore appear blue in color while the recombinant ones appear white. The desired recombinant colonies can be easily picked and cultured [63].

a) Preparation of solutions for cloning

*Ampicillin solution 100**

A concentrated solution of ampicillin was prepared by adding 200 mg of ampicillin salt (371.39 g/mol) in 40 mL of MQ water. The solution was filter-sterilized. The falcon of ampicillin solution was flamed before and after each use.

X-galactose solution

An X-galactose solution was prepared by dissolving 400 mg of X-galactose (Merck, Belgium) into 10 mL of DMF (40 mg/mL). The tube containing the solution was wrapped with foil paper and placed in the freezer at -20°C.

Luria-Broth liquid medium

A Luria-Broth medium (LB) was prepared by placing 15.5 g/L of Luria-Broth (Difco, Miller, Belgium) and 9.5 g/L of NaCl (Merck, Belgium) in MQ water. This solution was autoclaved.

LB agar for plates

In order to prepare agar plates for cloning, a solid medium was prepared by adding 15.5 g/L of LB medium (Difco Miller, Belgium), 9.5 g/L of NaCl (Merck, Belgium) and 12 g/L of select agar (Invitrogen, Belgium) in MQ water. This solution was autoclaved. After cooling to 50°C, 10 mL of ampicillin solution were added per liter of LB agar medium.

b) Preparation of plates for the cloning

After disinfection of the preparation table, the flame was placed on. The warm LB agar medium was poured over plates (~20 mL per plate), and the plates were cooled down at RT in order that the LB agar medium solidifies in the plates. Then, the plates were placed in an incubator at 37°C until use. Just before their use, 40 µL of X-galactose (40 mg/mL) was placed and spread over each plate.

c) Cloning of DNA aptamers

Ligation step

As the DNA aptamers were extracted from an agarose gel, a ligation step was applied in order to ensure that the A overhangs are present. For that, 30 µL of the PCR products were placed in an Eppendorf vial with 0.5 µL of dNTPs and 0.5 µL of *Taq* polymerase. The mixtures were kept for 15 min at 72°C, and then put on ice. The final products were checked by using Nanodrop.

Transformation in Escherichia coli competent cells

The cloning was performed with this final PCR product after ligation. For the cloning, 2 µL to 4 µL of each fresh PCR product were placed in a vial, with: 1 µL of salt solution, 1 µL of TOPO vector, and water to a total volume of 6 µL. The mixture was mixed gently and kept for 15 min at RT (22°C) and then put on ice. One vial of TOP10 competent cells was thawed on ice for each PCR product to be cloned. Then, 2 µL of the TOPO cloning kit were added and the mixtures were mixed gently. The mixtures were incubated on ice for 1 to 20 min. The cells were then heat-shocked at 42°C for 1 min without shaking. The tubes were immediately transferred to ice. Then, 250 µL of liquid LB medium are added to each cloning vial. The solutions were stirred at 37°C for 1 h. From 20 µL to 100 µL of the solutions were spread on agar plates. The plates were incubated at 37°C for 24 h.

Inoculation

The flame was kept on for the inoculation step, and all the tubes were flamed before and after use. In a falcon, 120 µL of 5 mg/mL ampicillin was added to 12 mL of liquid LB medium. Each white colony on LB plate was picked up with a tip, which was added to a tube of 2 mL of the liquid LB medium with ampicillin. The tubes containing the colonies were shacked between 12 and 16 h at 37°C in an incubator.

Plasmid DNA purification

The contents of the tubes containing the bacterial culture were centrifuged in Eppendorf tubes at 13,000 rpm at 4°C for 20 min. The supernatants were removed by inverting the Eppendorf tubes upside down and by pipetting the rest with a pipette. A plasmid DNA purification kit was used in order to extract and purify the cloned vectors (QIAprep Spin Miniprep kit, QIAgen, The Netherlands); and the DNA quantity recovered was determined using Nanodrop.

PCR and gel electrophoresis of the part of the vectors containing aptamer sequences

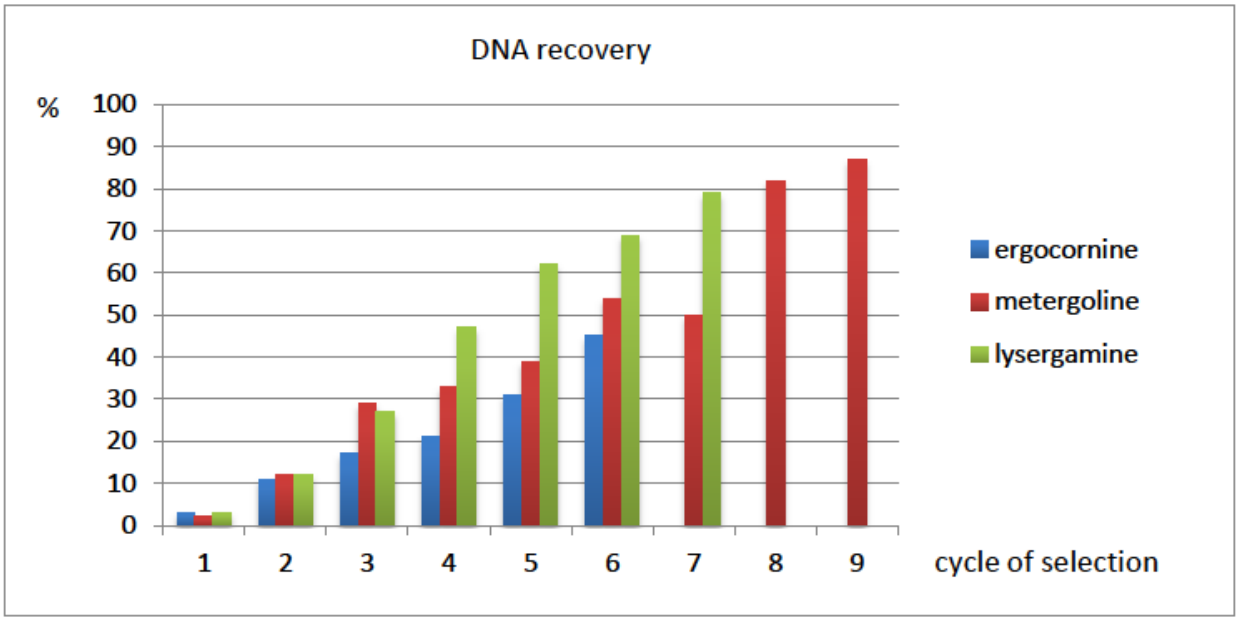
PCRs were performed with the vectors containing the aptamer sequences. The PCR products of the plasmid DNAs were analyzed by gel electrophoresis by preparing a 2% agarose gel with ethidium bromide the same way as described in the previous paragraph. The plasmids having inserted the selected aptamers were extracted, purified and resuspended in water to a concentration of 50 ng/µL; and were sent for sequencing (VIB Genetic Service Facility, Antwerp, Belgium).

5.2. Results and Discussion

5.2.1. Analysis of the recovery rate of aptamers after each cycle of selection by UV-Vis spectroscopy

A total of nine cycles of selection were performed with metergoline coated beads, seven cycles with lysergamine coated magnetic beads and six cycles with ergocornine coated magnetic beads. Negative-selections with ethanolamine coated magnetic beads were applied starting from the second selection cycle, in order to remove the non-specifically bound aptamers. After each selection cycle, the recovered ssDNAs were amplified and used as input for the following selection cycle. The amount of DNA recovered after elution from the ergot-coated magnetic beads was determined by UV-Vis spectroscopy and compared to the initial amount used for each selection cycle. The amount of ssDNA recovered after each selection cycle kept on increasing, for all the selections with ergocornine, metergoline and lysergamine (**Figure 31**). The ssDNA recovery increased from 2% to 87% for metergoline; from 3% to 79% for lysergamine; and from 3% to 45% for ergocornine. The lower recovery obtained with ergocornine can be explained by the instability of ergocornine coated magnetic beads. The selections for ergocornine were not carried out after the 6th cycle, as the amount of ergocornine on magnetic beads was too low to perform another selection cycle. However, for metergoline and lysergamine, the selection cycles were carried out until the obtention of optimal recovery rates.

Figure 31. *Recovery of ssDNA measured by UV-Vis spectroscopy after each cycle of selection with the different ergot alkaloid- coated magnetic beads.*



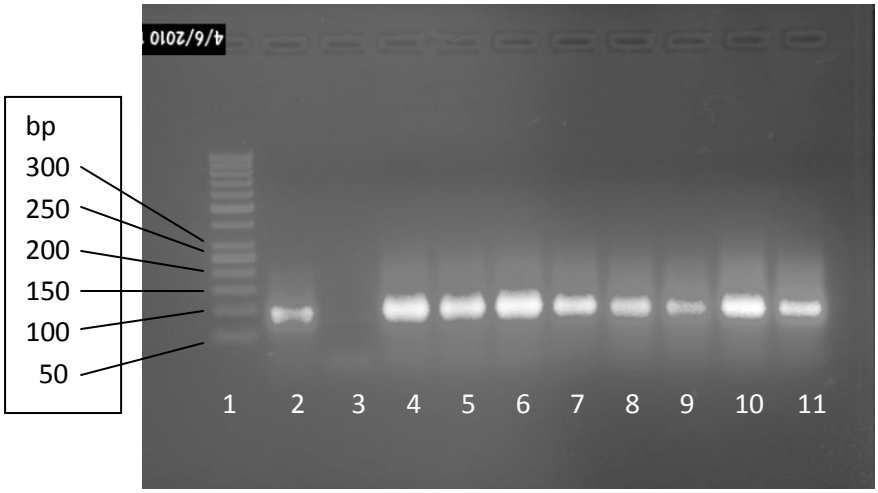
5.2.2. PCR of DNA aptamers and gel electrophoresis of PCR products

After each cycle of selection, the ssDNA aptamers selected for ergot alkaloids were amplified by PCR and analyzed using gel electrophoresis. The PCR parameters had to be optimized, as the ones found in the literature did not give a correct amplification, and only shorter byproducts were obtained with the general PCR parameters. The PCR parameters had to be adjusted in order to obtain PCR products of 80 bp. The comparison between the former PCR parameters and the optimized PCR parameters is given in **Table 2**. The optimization of the PCR parameters consisted in: a longer denaturation time in order to allow the DNA aptamers to unfold completely before amplification, a higher primer annealing temperature, an extra denaturation and primer annealing step before the final elongation time, and a reduced final elongation time, in order to prevent the DNA aptamers to refold during the process. All the PCR parameters were changed one by one, as the DNA aptamer selection is blind and the DNA composition is unknown at this stage of the selection. The PCR products were each time analyzed by gel electrophoresis. For that, the double-stranded DNA products were stained using ethidium bromide and placed in the wells of an agarose gel placed in an electrophoresis bath. A DNA ladder starting at 50 bp was used to determine the length of the PCR products. After the electrophoresis, the gel was placed on a UV lamp in order to visualize the PCR product (**Figure 32**). The DNA fragments having the correct length of 80 bp were extracted from the agarose gel. It is also possible to purify the PCR product by using a purification column, allowing the removal of small byproducts and primers. However, the purification column does not allow the removal of all the byproducts which can be present in the PCR product. Therefore, it was chosen to extract the DNA having the correct length by cutting the agarose gel and performing a gel extraction. This resulted in the obtention of cleaner and more consistent PCR products over the selection cycles.

Table 2. *Comparison between the general PCR parameters and optimized PCR parameters.*

General PCR parameters (*30)		**Optimized PCR parameters (*20)**	
Temperature	**Time**	**Temperature**	**Time**
94°C	5 min	94°C	6 min 30 s
94°C	30 s	94°C	30 s
53°C	20 s	55°C	20 s
72°C	20 s	72°C	20 s
72°C	5 min	94°C	3 min
4°C	pause	55°C	20 s
		72°C	2 min
		4°C	pause

Figure 32. *Photograph of agarose gel after DNA electrophoresis. Samples 1 to 10:* ***(1)*** *50 bp DNA ladder* ***(2)*** *Positive control (original ssDNA library)* ***(3)*** *Negative control (no DNA template)* ***(4-6)*** *DNA selected for metergoline* ***(7-11)*** *DNA selected for lysergamine.*

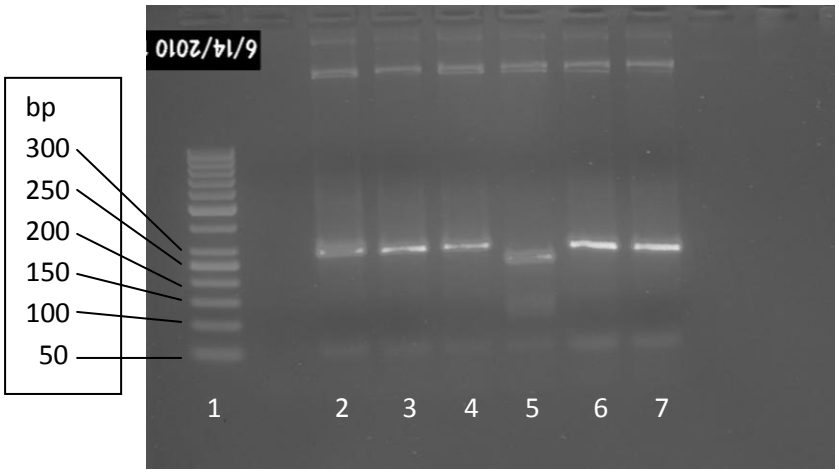


5.2.3. Cloning and sequencing of the selected aptamers

In order to perform a molecular cloning, some PCR reactions were performed with non-biotinylated primers and. A gel electrophoresis of the PCR product was carried out, and the DNA was extracted from the gel, allowing a better purification of the PCR product, in accordance with the results obtained by Zhou and Gomez-Sanchez [54]. After each cloning of aptamers, a PCR of the plasmid DNA and a gel electrophoresis were performed, in order to confirm if the cloning was successful or not. The length of the PCR product can be measured by using a DNA ladder.

After each selection cycle, the selected aptamers were cloned. After each cloning, a PCR and gel electrophoresis of the plasmids were performed. The plasmid PCR fragment used for the cloning had a length of 202 bp, so the total PCR product containing the plasmid PCR fragment and the DNA insert (aptamer) was expected to be 282 bp; as a 80 base-DNA library was used in the SELEX procedure. For the gel electrophoresis, DNA ladder of 50 bp was used, giving a bright spot every 50 bp. By comparing the length of the PCR product to the DNA ladder, it is possible to determine if the selected aptamers were successfully inserted into the plasmid DNA. A photograph of a typical gel after electrophoresis is shown in **Figure 33**, in which the samples are visible as white bands just below 300 bp. The plasmid DNA fragments giving a correct PCR product were purified and sent for sequencing (VIB, University of Antwerp, Belgium).

Figure 33. *Photograph of an agarose gel containing plasmid DNA after electrophoresis. Samples 1 to 7:* ***(1)*** *50 bp DNA ladder* ***(2)*** *plasmid with aptamer M3.1* ***(3)*** *plasmid with aptamer M3.2* ***(4)*** *plasmid with aptamer M3.3* ***(5)*** *plasmid with aptamer M3.5* ***(6)*** *plasmid with aptamer L2.1* ***(7)*** *plasmid with aptamer L2.2. The plasmid PCR fragment length itself is 202 bp, so the plasmid PCR fragments having inserted the selected aptamers are expected to have a length of 282 bp, which was the case for the samples 2, 3, 4, 6 and 7 (bright spot below 300 bp), which were sequenced. Sample 5 inserted a shorter DNA fragment, and was discarded.*



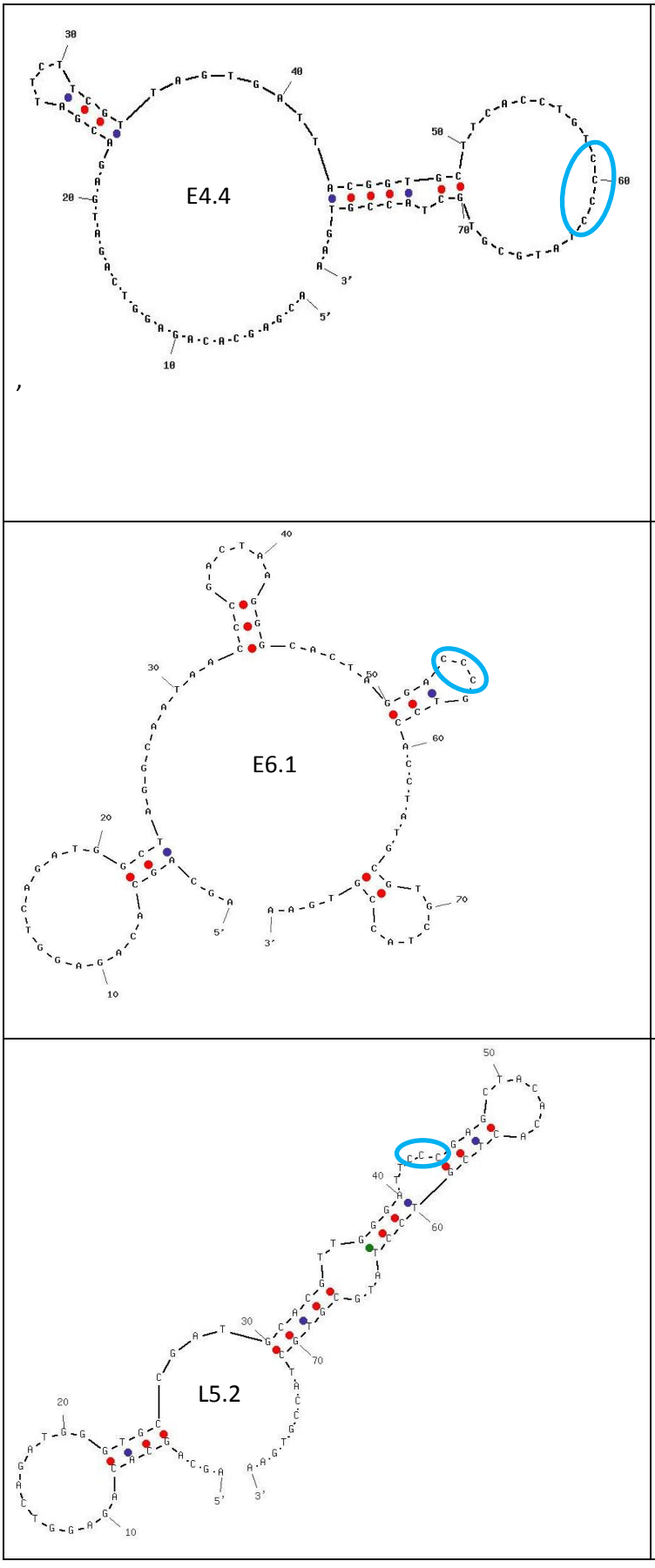
5.2.4. Prediction of the secondary and tertiary structures of the selected aptamers

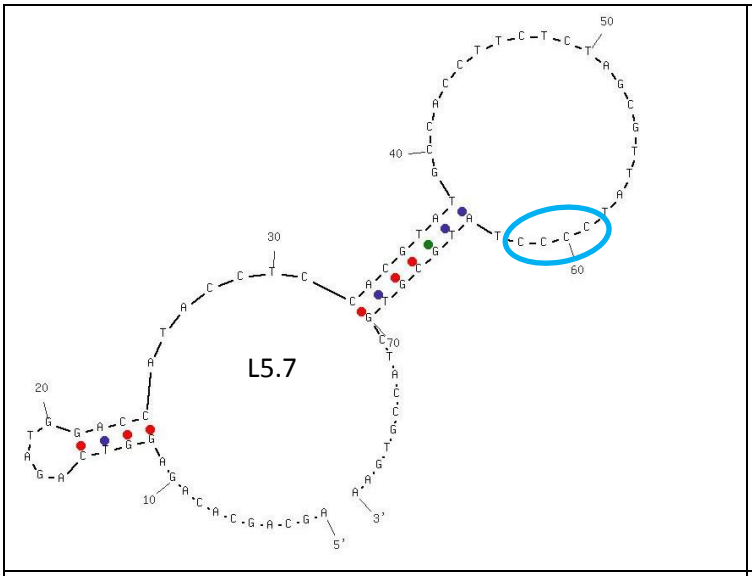
Some of the sequences obtained for the selected aptamers are shown in **Table 3**. The secondary structures of the aptamers were determined using IDT OligoAnalyzer software [56], in order to calculate the lowest energy levels of the secondary structures formed by the aptamers. The secondary structures corresponding to the selected aptamers shown in **Table 2**, are represented in **Figure 34**. It was observed that more than 80% of the selected aptamers had a consensus sequence of C bases (from 3 to 6 consecutive C bases), which are highlighted in the **Table 3** and circled in **Figure 34**. As it was observed that the pools were enriched in C bases after the selection procedure; therefore, it was suggested that motifs of consecutive C bases play a role as binding sites in the recognition of lysergamine and metergoline. In the literature, several aptamers were found to be enriched in one of the bases. For instance, G rich aptamers were reported for various target molecules [64-68]. The fact that one of the bases is significantly increased can be linked with the interaction of the aptamer with the target molecule. These regions are usually identified as binding sites or parts of them. For instance, a conformational change of the G-quartet of the thrombin aptamer was observed upon binding [67]. Some other studies on different target molecules have reported slight increments of one of the other nucleic acid bases, such as T or C [69, 70].

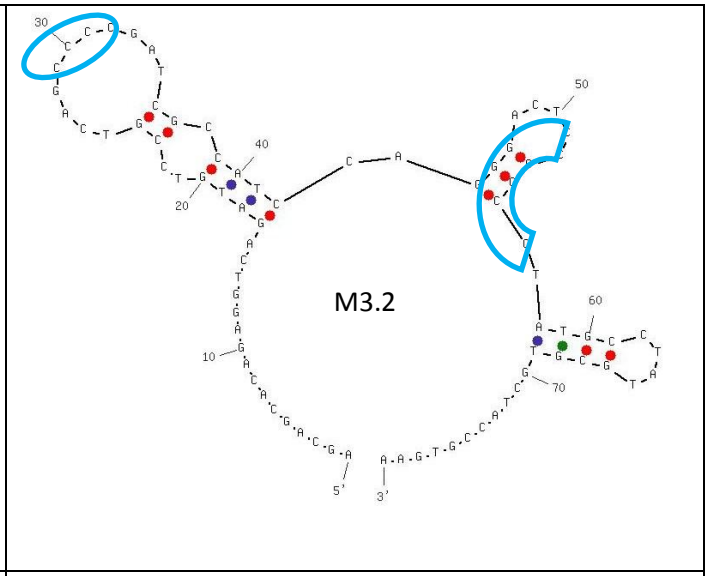
Table 3. *Examples of aptamer sequences and their GC content (with redundancy of C bases highlighted in blue).*

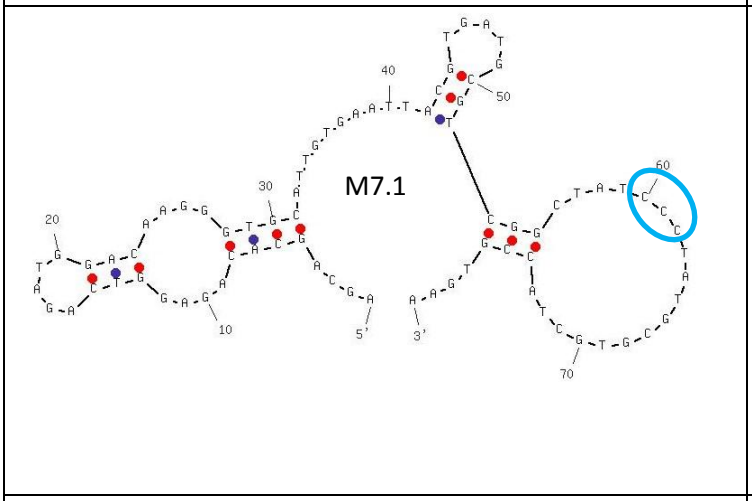
Aptamer	Sequence	GC content
E4.4	5'- ACG AGC ACA GAG GTC AGA TGA GAC GAT TCT TCG TTA GTG ATT ACG GTG CTT CAC CTG TCC CCT ATG CGT GCT ACC GTG AA -3'	51.2%
E5.4	5'- AGC AGC ACA GAG GTC AGA TGT GCG CCG CAG CCG CTG TTA CTC CAA CAT CCA CAT CGG GTA CCT ATG CGT GCT ACC GTG AA -3'	57.5%
E6.1	5'- AGC AGC ACA GAG GTC AGA TGG CTA GGC AAT AAC CCG ACT AAG GGC ACT AGG ACC CGT CCA CCT ATG CGT GCT ACC GTG AA -3'	56.2%
L5.1	5'- AGC AGC ACA GAG GTC AGA TGG CAC ACA CAG ATG TAC TCA GCC TCC CCC CGT CGT TGC CGC CCT ATG CGT GCT ACC GTG AA -3'	60.0%
L5.2	5'- AGC AGC ACA GAG GTC AGA TGG GTG CCG ATG CAC GTT GGG ATT CCC GAG CTA CAC ACT CGT CCT ATG CGT GCT ACC GTG AA -3'	57.5%
L5.5	5'- AGC AGC ACA GAG GTC AGA TGG GCA GGA TAC AAC ACG TTA CTA TCC ACT CTG CAC CGG CGG CCT ATG CGT GCT ACC GTG AA -3'	56.3%
L5.7	5'- AGC AGC ACA GAG GTC AGA TGG ACC ATA CCT CCA CGT ATG CCA CCT TCT CTA GCG TTA TCC CCT ATG CGT GCT ACC GTG AA -3'	53.8%
M3.2	5'- AGC AGC ACA GAG GTC AGA TGT CCG TCA GCC CCG ATC GCC ATC CAG GGA CTC CCC CCT ATG CCT ATG CGT GCT ACC GTG AA -3'	61.2%
M7.1	5'- AGC AGC ACA GAG GTC AGA TGG ACA AGG GTG CAT TGT GAA TTA CGT GAT GCG TCG GCT ATC CCT ATG CGT GCT ACC GTG AA -3'	52.5%
M7.2	5'- AGC AGC ACA GAG GTC AGA TGA TGA AAC AGC AAG TCA CGA CCC CAG ATG GGC ATA ACT CCC CCT ATG CGT GCT ACC GTG AA -3'	53.8%
M7.7	5'- AGC AGC ACA GAG GTC AGA TGG GAC AAA GCT GCA CTA CAG GAT GAT GCA CAA CTT CTC CTC CCT ATG CGT GCT ACC GTG AA -3'	52.5%
M8.2	5'- AGC AGC ACA GAG GTC AGA TGG CCA CCC CTC GAT GGC GCA GCG TCA CAC ACA GTC CCT ATC CCT ATG CGT GCT ACC GTG AA -3'	60.0%
M9.4	5'- AGC AGC ACA GAG GTC AGA TGC CGC TGC AAC TGG ACG CGG TTA CGT GAC GGT ATA CCC ATC CCT ATG CGT GCT ACC GTG AA -3'	57.5%
M9.17	5'- AGC AGC ACA GAG GTC AGA TGG CAG GTC CCT GAC GCC AAT CCC CTA TGG GGC TCC CGC TTC CCT ATG CGT GCT ACC GTG AA -3'	61.3%

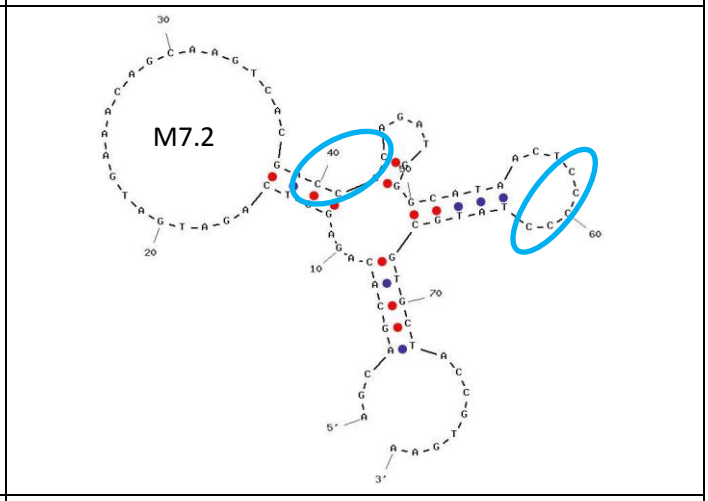
Figure 34. *Secondary structures of the selected DNA aptamers*

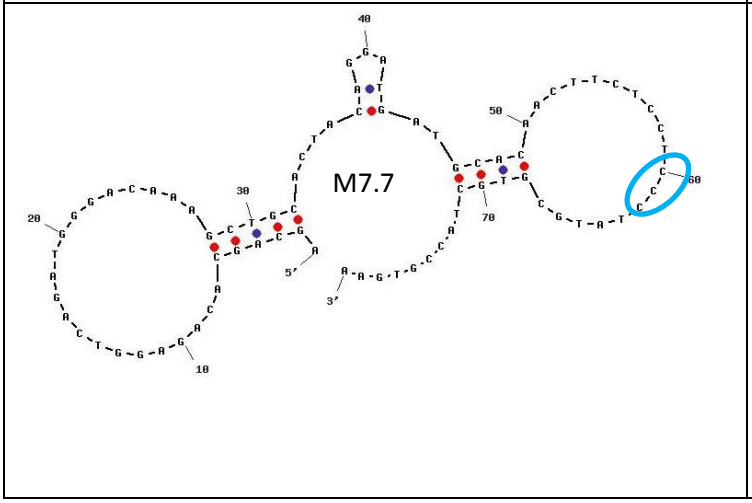

E4.4
E5.4
E6.1
L5.1
L5.2
L5.5

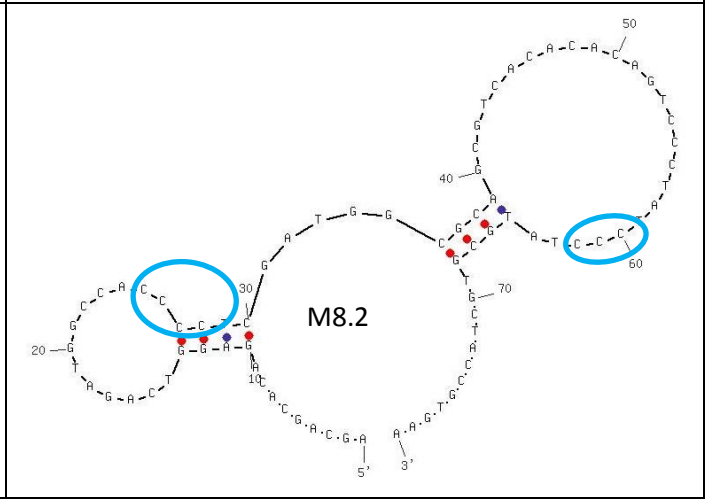

L5.7

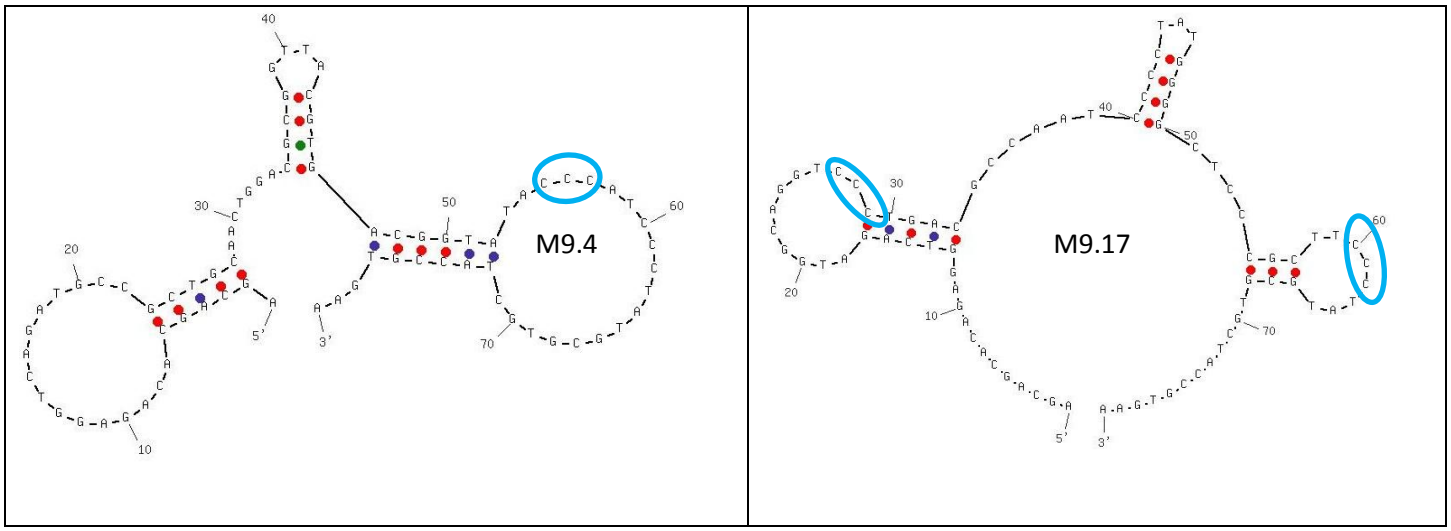

M3.2


M7.1


M7.2

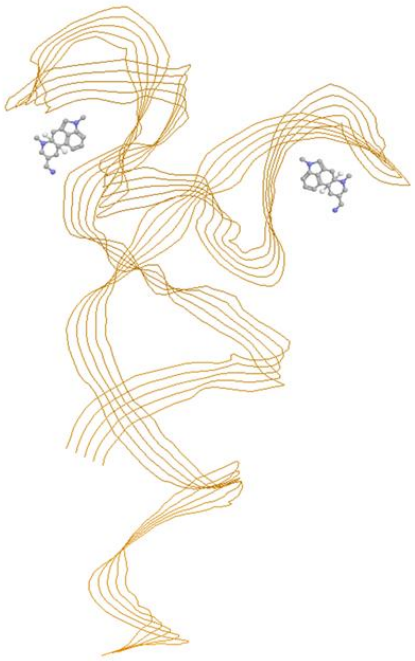

M7.7


M8.2



As a further analysis of aptamers, it is also interesting to determine their tertiary structure, in order to visualize the predicted binding sites implied in the recognition of the target molecule [57]. The tertiary structure of nucleic acids can be determined by using softwares such as RNA 1,2,3; in which experimental structures are automatically analyzed to deduce all of the hydrogen-bonded pairs in the structure and are classified according to the Leontis-Westhof rules [71]. Also, DNA aptamers and their target compounds can be assembled in softwares, such as RasMol [72]. The representation using RNA1,2,3 software of the best binding aptamer selected for ergot alkaloids in this study, aptamer M3.2, is shown in **Figure 35**.

Figure 35. *Representation of aptamer M3.2 complexing two molecules of lysergamine. The tertiary structure of the aptamer, determined by using RNA 1,2,3 software, and molecules of lysergamine (Chemdraw) were assembled in RasMol software.*



In this representation, it was observed that the two predicted binding sites were corresponding to cavities in the folded aptamer structure, situated at the opposite part of the stem of the aptamer.

6. Conclusion

The SELEX procedure with magnetic beads allowed the isolation of ssDNA aptamers from a library of 80 base ssDNA. The recovery of ssDNA after each selection cycle kept on increasing and reached a maximum (80%) for lysergamine and metergoline, meaning that the best binders were extracted for these two ergot alkaloids from the chosen library. For ergocornine, the selection procedure was stopped after the 6^{th} round of selection, as the ergocornine-coated magnetic beads were less stable than the other ergot alkaloid coated magnetic beads. It was observed that the majority of selected aptamers had a common sequence of C bases, which were suggested to play a role in the binding to ergot alkaloids. As the GC content of the selected aptamers was very high, it was necessary to adjust the PCR parameters in order to obtain a correct amplification with PCR. As GC bonds require more energy to separate, a more important denaturation time was necessary for instance to allow the aptamers to unfold completely before amplification. Also, it was necessary to shorten the final elongation time, as the aptamers could fold again at temperatures lower than 95°C. The secondary structures of the aptamers were observed to contain the C rich areas mainly in the secondary loops of the selected aptamers, predicted to act as binding sites for ergot alkaloids.

Partly based on:

Rouah-Martin, E.; Mehta, J.; Van Dorst, B.; De Saeger, S.; Dubruel, P.; Maes, B. U. W.; Lemiere, F.; Goormaghtigh, E.; Daems, D.; Herrebout, W.; Van Hove, F.; Blust, R. and Robbens, J. Aptamer-Based Molecular Recognition of Lysergamine, Metergoline and Small Ergot Alkaloids. *International Journal of Molecular Sciences* **2012**, *13*, 17138-17159.

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Chapter IV: Study of the complexes formed by ergot alkaloids and DNA aptamers

1. Introduction

Once aptamers are generated out of a library, they are compared in order to select the ones having the best affinity towards the target molecules. Several parameters can be evaluated about complexes such as: the chemical affinity or degree of binding, the kinetics of the reaction, the location of the binding sites, the thermodynamic parameters of the binding reaction, and the cooperativity in binding in case that the ligand possesses several binding sites. Complexes can be characterized by several manners and depending on their intrinsic properties, several techniques can be employed. The determination of the binding parameters of the complexes allows to compare the strength of the binding and the minimal amount of target molecules that can be measured. Several techniques allow the determination of chemical binding of the aptamer to the target molecule; such as quartz crystal microbalance, surface plasmon resonance, and fluorescence assays [1-3]. The structural analysis of complexes allows the determination of the binding sites, which can have some importance in target identification and biological interaction with the target molecule. Techniques such as ^{1}H-NMR and X-ray crystallography are generally used for the structural characterization of complexes [4, 5]. In the first part of this chapter, the general binding parameter of complexes will be explained. Then the different techniques mainly used for the characterization of complexes will be reviewed. Finally, the characterization of complexes of ergot alkaloids and DNA aptamers will be presented in the experimental part, and the results will be discussed.

2. Binding parameters of a complex

In the case of a simple complex made of one ligand and one target molecule, represented as compounds A and B; and the complex formed by the two compounds, represented as AB; the equilibrium can then be expressed by the following equation:

$$A + B \underset{K_{off}}{\overset{K_{on}}{\rightleftharpoons}} AB \qquad (1)$$

with K_{on} and K_{off} the kinetics of association and dissociation of the complex.

The equilibrium of the reaction is reached when the concentrations of the different components of the equilibrium equation are stable; which can be expressed as such:

$$\frac{d\,[AB]}{dt} = K_{on}\,[A]\,[B] - K_{off}\,[AB] = 0 \qquad (2)$$

The kinetics rate can be expressed as association or dissociation constant of the complex.

$$\frac{K_{off}}{K_{on}} = \frac{[A]\,[B]}{[AB]} = K_D \qquad (3)$$

with K_D the dissociation constant, invertly proportional to the association constant K_A, $K_D = 1/K_A$. Two strategies are possible to evaluate the binding of a complex: equilibrium experiment and kinetic experiment [1]. In an equilibrium experiment, the extent of the reaction is determined as a function of one of the reactant. Analysis of these data gives the association or dissociation constants of the complex. The dissociation constant, K_D, is often used to express the affinity of the ligand towards the target molecule, because the K_D is also giving the concentration of target molecule required to yield to a significant interaction with the ligand. In a kinetic experiment, the rates of the forward and reverse reactions are determined as a function of the concentration of one of the reactant. Analysis of these data gives the rate constants for the forward and the reverse reactions. The ratio of these rate constants gives the association or dissociation constants. The kinetic approach is more revealing because it gives the dynamics of the system and it allows the determination of equilibrium constant as well. However, kinetic experiments often require more sophisticated assays than equilibrium experiments [1].

3. Techniques for the determination of the binding parameters of a complex

Several techniques can be used for the analysis of complexes, depending on the chemical structure of the complex to be analyzed, the equipment available in the laboratory or the level of sensitivity to be reached. It is often important to determine the K_D of a complex because if the concentration of both the elements of the complex, it is possible to predict the complex is bound or not. The most common strategy for measuring K_D is to titrate a constant concentration of one ligand with an increasing concentration of the other. In case that the target molecule has an important molecular weight, such as certain proteins, separation-based techniques can be applied; such as dialysis, ultracentrifugation, gel and capillary electrophoresis and HPLC [6]. Otherwise, in case that the target molecule has a low molecular weight, mixture-based techniques can be used; such as fluorescence intensity and anisotropy, UV-Vis absorption and circular dichroism, surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) [6]. The most commonly used techniques are presented in this section.

3.1. Quartz Crystal Microbalance (QCM)

A quartz crystal microbalance (QCM) measures a mass per unit area, due to a change in frequency of a quartz crystal resonator. QCM technique relies on the piezoelectric properties of certain materials, such as quartz crystals. Piezoelectricity is defined as the electric charge that accumulates in certain solid materials, such as crystals, ceramics and even organic matter like bone, DNA and some proteins; in response to mechanical stress [7]. Applying an alternating current to the quartz crystal induces oscillations. The frequency of the oscillation of the quartz crystal is partially dependent on the thickness of the crystal. As mass is deposited on the surface of the crystal, the thickness increases; consequently the frequency of oscillation decreases from the initial value. This way, it is possible to immobilize one of the interaction partners, the ligand or the target molecule, on the surface of a quartz crystal, and to monitor the formation of the complex by passing the other interaction partner on the surface of the QCM chip. If a rigid layer is evenly deposited on one or both of the electrodes of the QCM chip, the resonant frequency will decrease proportionally to the mass of the adsorbed layer according to the Sauerbrey equation:

$$\Delta f = -[2 * f_0^2 * \Delta m] / [A * (\rho_q \mu_q)½] \quad (4)$$

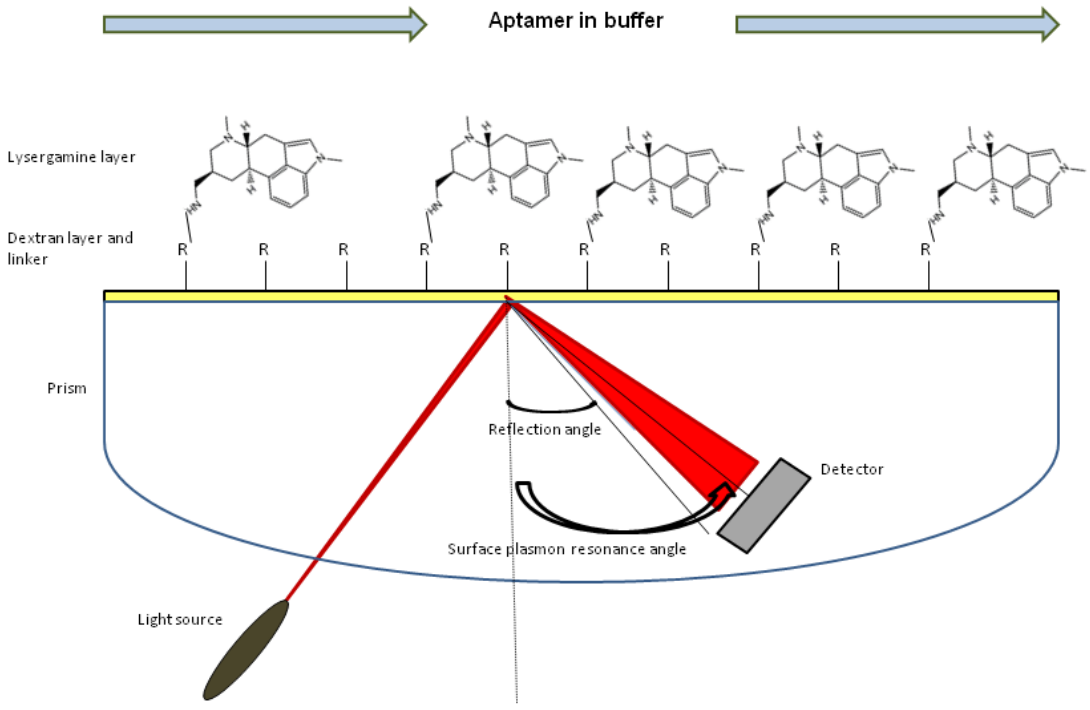
with,
Δf = measured frequency shift,
f_0 = resonant frequency of the fundamental mode of the crystal,
Δm = mass change per unit area (g/cm^2),
A = piezo-electrically active area,
ρ_q = density of quartz, 2.648 g/cm^3,
μ_q = shear modulus of quartz, 2.947´1011 $g/cm*s^2$.

The shift in resonance frequency caused by the binding of a soluble analyte to the immobilized ligand can be recorded by a frequency counter with high resolution. The curve resulting from the measured frequency versus time is called a sensorgram. As real-time measurements are performed, it is possible to deduce the equilibrium binding constants and affinity rate constants with QCM [8]. QCM technique usually has a limit of detection of 1 ng/cm^2. The QCM electrode surface can be easily chemically modified, allowing the analysis of a broad range of compounds, from small organic compounds to entire cells [9-11]. The electrode surface can be modified to create functional groups by different techniques: polymer coating, Langmuir films and chemical modifications with self-assembled monolayers. The preparation of self-assembled monolayers is usually performed by using organosulfur compounds on gold chips. The analyte can be immobilized by covalent linkage to the self-assembled monolayer or by non-covalent binding using a streptavidin modified surface and a biotinylated analyte.

3.2. Surface Plasmon Resonance (SPR)

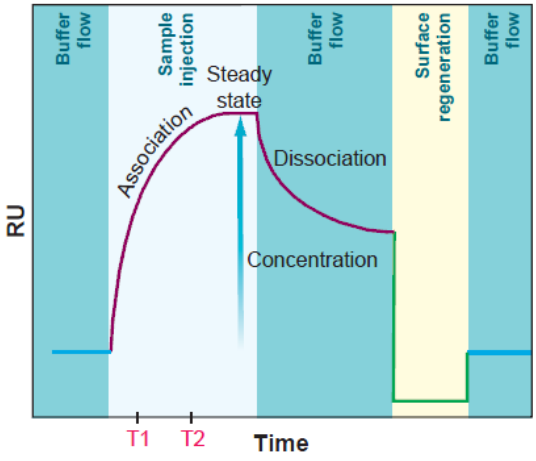
Surface plasmon resonance (SPR) is an optical technique which also allows the real-time monitoring of the adsorption of material on a chip and the formation of complexes with a high degree of sensitivity [3]. With SPR technique, it is possible to determine the equilibrium constants and kinetics of a molecular binding event. SPR technique requires the generation of a collective oscillation of electrons in a metallic gold chip, stimulated by an incident light. When the light strikes a metal, the light is usually reflected. But at a certain incidence angle, the energy of the light is converted to make the electrons of the metallic plaque oscillate (plasmon). The resonance condition is established when the frequency of the photons of the light beam matches the natural frequency of surface electrons oscillating against the restoring force of the positive nuclei [12]. The oscillating wave (plasmon wave) propagates through the outer surface of the gold chip, and any molecule added or removed from the surface induces a change of the resonance angle. Therefore, it is possible to monitor adsorption and binding events on a SPR chip by measuring the difference between the reference angle and the sample angle. **Figure 36** shows the principles of SPR technique, based on the scheme made by Sabban [13].

Figure 36. *Scheme of SPR configuration. In order to study binding events, one of the interaction partners is immobilized onto a functionalized dextran layer onto the SPR gold chip; the other interaction partner is passed through the surface. A light beam is sent at a certain fixed angle onto the chip in order to generate a plasmon wave, leading to the extinction of light in the reflected beam. This SPR angle varies with changes of refractive index and on the vicinity of the surface. The surface plasmon resonance angle is recorded by a detector and the shift is interpreted into addition or removal of material onto the chip.*



For a certain complex to be studied, one of the interaction partners is immobilized onto the surface of the SPR chip, and the other interaction partner is passed through the chip. Binding of the analyte in solution to the immobilized target leads to a change in refractive index (i. e. electron density) around the gold surface, which is detected as a change in the absorbance angle of totally reflected light by the SPR detector [14]. It has been shown that this change, arbitrary quantified as "resonance units" can be correlated with a mass increase on the chip surface, with 1 RU = 1 pg/mm^2 [15]. The adsorption/desorption of the analyte is recorded in real-time in a continuous flow of buffer. The binding and dissociation of the analyte to the immobilized ligand (or vice versa) are presented in a sensorgram of response versus time [16, 17]. The basic experimental protocol for the study of binding interaction can be summarized into the following process: (a) immobilization of one of the interaction partner, (b) injection of the second interaction partner using a series of concentrations and recording of the real-time interaction curves, (c) choice of an appropriate kinetics model and fitting of raw data to extract rate constants estimates. A typical sensorgram, corresponding to the process mentioned above for one chosen concentration of the second interaction partner is represented in **Figure 37**.

Figure 37. *SPR sensorgram [17]. The response measured in resonance units (RU) is recorded versus time, and is proportional to the mass of adsorbed material on the SPR chip. While the injection of the sample, the mass bound on the SPR chip keeps on increasing between the time-points T1 and T2 and finally reach a saturation (association phase). The sample is replaced by a flow of the buffer onto the SPR surface and the interaction partner is detached from the SPR chip (dissociation phase). The amount of interaction partner left on the chip after the dissociation phase is representative of the chemical affinity of the interaction partners.*



In a typical experiment, at the beginning of the injection of the interacting molecule, the sensorgram represents a changing ratio of the associating and dissociating molecules until a steady state is reached. After the end of injection, the pure dissociation can be observed. The kinetic association and dissociation rate constants (k_{on} and k_{off}) are calculated by globally

fitting the sensorgrams at various analyte concentrations to mathematical equations describing different binding models [18]. In addition, the affinity (equilibrium dissociation constant K_D) of the interaction can either be calculated from the rate constants ($K_D = k_{off}/k_{on}$) or by fitting a concentration-dependent plot of the steady state signals [18]. Different strategies can be used to immobilize either the target molecule, or the ligand, depending on the type of analysis to perform or the sensitivity to be reached [19]. For instance, in order to compare the affinity of the different aptamers selected for a target molecule, the target molecule is immobilized onto the SPR surface and several concentrations of the different aptamers are passed though the chip one by one after regeneration of the surface.

3.3. Isothermal Titration Calorimetry (ITC)

Isothermal titration calorimetry (ITC) is also a technique of choice for the characterization of complexes with aptamers, due to its accuracy and simultaneous determination of K_D, stoichiometry and thermodynamic parameters of the reaction [6, 20]. ITC is the only technique that can measure the enthalpy of the complex formation [21]. ITC relies on the fact that the formation of a complex is an exothermic or endothermic process and that the heat released or taken to the system can be monitored. A typical ITC experiment is carried out by the stepwise addition of one of the reactant into the reaction cell containing the other reactant [22]. Signal is measured as the amount of electric power needed to keep the temperature in the sample cell the same as the temperature in a control cell containing the same amount of buffer. The binding reaction created by the addition of the second reactant releases or absorbs a certain amount of heat (q_i) proportional to the amount of ligand binding $v*\Delta L_i$, as written in the equation below:

$$q_i = v * \Delta H * \Delta L_i \quad (5)$$

where v is the volume of the cell, ΔLi the increase in the concentration of bound ligand and ΔH the enthalpy of the binding reaction [22]. From these measurements, Gibbs energy change (ΔG) and entropy changes (ΔS) can be determined using the relationship:

$$\Delta G = \Delta H - T\Delta S = RT \ln K_D \quad (6)$$

where R is the gas constant and T the absolute temperature. In fitting data with the single set of identical sites model, initial guesses are made for the values of Ka, ΔH and stoichiometry. ΔQ(i) is calculated for each injection and compared to the measured values. The values of K_D, ΔH and the stoichiometry are varied until the best fit of the data is obtained using standard Marquardt methods [23]. A negative Gibbs energy ΔG is representative of a spontaneous process. ΔH is a measure of the energy content of the bonds broken and created, with a dominant contribution of hydrogen bonds. A negative ΔH value indicates an enthalpy change favoring the binding. ΔS is positive for entropically driven reactions and is an indicator of conformational changes and hydrophobic interactions. To

conclude, ITC is an interesting technique due to the fact that it gives some information about the thermodynamic data of the binding. Moreover, this technique does not require immobilization or labeling of the reactants. The main disadvantage of this technique is the fact that the determination of the binding constant is limited to 10^{-8} to 10^{-9} M^{-1} [22, 24]. Also, the enthalpy observed is the global enthalpy of the system; including the binding, ionization and conformation and takes into account any non-specific effects, such as buffer mismatch, pH mismatch and heat of dilution. Therefore, it is important to have appropriate controls when using this technique, and to be very rigourous with the experimental conditions in order to obtain reliable data.

3.4. Fluorescence techniques

Fluorescence techniques are also often used for the determination of equilibrium constants K_Ds of aptamers [6]. There are several techniques using fluorescence and several manners to conduct an experiment for the determination of equilibrium constants. It is not uncommon for the fluorescence of an aptamer to change upon binding to its target molecule. In the case that the binding induces a change in aptamer fluorescence, this signal can be used directly to estimate binding affinity by measuring the fluorescence intensity [6]. In case that the binding does not induce any change in aptamer fluorescence; it is possible to immobilize the target molecule onto a support and place different concentrations of fluorescent aptamer. By using a magnet, the solution can be discarded and the beads can be resuspended afterwards in a buffer, and it is possible to measure the fluorescence intensity of the aptamers bound to the target molecule. Fluorescence can determine equilibrium constant with a good accuracy. Many fluorometers, even the most basic models, can collect intensity data in a kinetic mode, allowing continuous collection of data points. Scores or hundreds of time points can be collected over the course of a binding reaction, yielding well-defined binding curves. In contrast, other methods can be adapted to collect kinetic data, but are not nearly as amenable to collecting large data sets [25]. An alternative, fluorescence correlation spectroscopy allows a correlation analysis of fluctuation of fluorescence intensity, and can be used for even more sensitive results [26]. Fluorescence anisotropy is another technique using fluorescence with polarized light, which can be used to measure the binding constants and kinetics of reactions that cause a change in the rotational time of the molecules. If the fluorophore is bound to a small molecule, the rate at which it tumbles can decrease significantly when it is bound tightly to a large molecule such as a protein. If the fluorophore is attached to the largest partner in a binding pair, the difference in polarization between bound and unbound states will be smaller, as the unbound protein will already be fairly stable and tumble slowly, and the measurement will be less accurate. The degree of binding is calculated by using the difference in anisotropy of the partially bound, free and fully bound (large excess of protein) states measured by titrating the two binding partners. Fluorescence techniques are efficient in general and relatively rapid to conduct, once the appropriate setup is determined. One disadvantage of these techniques requiring a label is

that the fluorophores can be large enough to potentially interfere with or otherwise influence binding [25].

3.5. Other techniques

In case that the target compound is voluminous, the free aptamers and aptamers bound to the target compounds can be separated by many techniques, such as dialysis, ultracentrifugation or ultrafiltration. In a dialysis experiment, two compartments are separated by a semi-permeable membrane. At the beginning of the experiment, one compartment contains the mixture of aptamer and target, while the other only contains buffer. If the aptamer is smaller than the target and the corresponding complex, only the aptamer can cross the membrane to the other compartment. Note that this method is only applicable in cases where the target is significantly larger than the aptamer. After equilibrium is achieved, free aptamer concentrations at different target concentrations are determined in the second compartment [6]. Ultrafiltration based techniques are similar to dialysis in that only free aptamer is able to pass through a filter membrane. In ultrafiltration the solution containing the aptamer and protein target is driven through the membrane using pressure, vacuum or centrifugal force. Aptamer bound to the protein target is retained by the membrane allowing the equilibrium distribution to be assessed across a range of protein target concentrations. The most widely used membrane material is nitrocellulose, which possesses long pores that are nucleic acid permeable but retain proteins as small as 2 kDa by hydrophobic adsorption [6]. Also affinity capillary electrophoresis can be applied for the separation of bound/unbound aptamer fractions and to determine equilibrium constants [27].

Some complementary techniques can be used for the structural characterization of aptamers such as X-ray crystallography and NMR. Crystal structures of aptamer-target complexes provide very detailed information on the interactions; they are therefore important for a thorough understanding of the aptamer-target binding [4]. This way, it is possible to confirm the location of binding sites for instance. Several crystal structures of aptamers in complex with their target molecules have been determined. In the case of small molecule targets, it was shown that aptamers often form a cage that surrounds the ligand. Also, it was observed that in structures of aptamers bound to nucleic acid binding proteins, the aptamers usually bind to the nucleic acid binding site and often mimic naturally occurring interactions [28]. Also, NMR approaches have been successfully applied to solve the solution structures of a range of ligand-RNA aptamer complexes [29].

4. Experimental Part: Analysis of the complexes of aptamers and ergot alkaloids

4.1 Materials and Methods

The complexes of aptamers and ergot alkaloids were analyzed by SPR, in order to determine the binding parameters of the different selected aptamers and to define the best binding aptamers which could be used in a biosensor. There are several methods available for the immobilization of the target molecule onto a SPR chip. The biotin/streptavidin system was used as coupling system [30]. For that, a biotinylated derivative of lysergamine was prepared in order to coat streptavidin SPR chips. A biotinylated derivative of ethanolamine was used as a negative reference. The different aptamers obtained from the SELEX procedure were runned through the lysergamine coated SPR chip in order to study the complexes formed.

4.1.1. Preparation of biotinylated lysergamine linker and reference

In order to prepare the biotinylated derivative of lysergamine to be used with streptavidin SPR chip, 6 mg (2.22×10^{-5} mol) of lysergamine were dissolved in 0.25 mL of dry DMF and a solution of 2 mg (3.4×10^{-6} mol) of NHS-PEG4-biotin linker (EZ-Link, Thermo Scientific, Belgium) in 0.25 mL of dry DMF was added. The mixture was stirred for 24 h at RT.

As a reference material, a NHS-PEG4-linker quenched with ethanolamine was prepared by dissolving 3 mg (4.97×10^{-5} mol) of ethanolamine (Sigma Aldrich, Belgium) in 0.25 µL of dry DMF and adding 2 mg (3.4×10^{-6} mol) of NHS-PEG4-biotin linker dissolved in 0.25 mL of dry DMF. The reaction mixture was stirred for 5 h at RT.

4.1.2. Characterization of biotinylated products by mass spectrometry (MS)

The products obtained from the reaction between the biotinylated linker and lysergamine or ethanolamine were characterized using mass spectrometry by placing 2 µL of the reaction mixtures in 198 µL of acetonitrile. For each sample, 5 µL were injected into a 0.2 mL/min flow of acetonitrile with 0.1% formic acid on an Acquity UPLC system (Waters, Elstree, UK). The mass spectra were recorded using electrospray ionization in positive ion mode on a Xevo TQ MS system (Waters, Elstree, UK).

4.1.3. Coating of streptavidin functionalized SPR chip with biotinylated linkers

As streptavidin supports restrict the use of organic solvents, 75 µL from each of the reaction mixtures were diluted in 300 µL of HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid (EDTA), 0.005% surfactant P20), in order to obtain a ratio of 25% of DMF in the final solution, which was then injected into the SPR chip (Biacore GE Healthcare, Belgium). In the SPR device (Biacore, GE Healthcare, Belgium), a streptavidin chip was used and 120 µL of the two different coating solutions were injected with a flow of 5 µL/min. After the coating reaction, the HBS-EP buffer was replaced by a continuous flow of binding buffer and the solutions of the different aptamers in binding buffer were loaded onto the coated SPR chip. The lysergamine coated surfaces were regenerated by applying pulses of 5 M urea. The analysis of the binding of ssDNA aptamers to the lysergamine coated

surface was compared with the binding to the ethanolamine coated surface, which was the most relevant reference for this analysis. However, several other references were tested such as a biotin coated streptavidin surface and a non-modified streptavidin surface.

4.2. Results and Discussion

4.2.1. Mass spectrometry analysis of the biotinylated linkers

As the focus of the study was to determine the binding between the selected aptamers towards the methylated ergoline part, lysergamine was used in the SPR analysis. The aptamers obtained from the selections for lysergamine and metergoline were tested. This way, it was possible to discard the aptamers binding to the phenyl part of metergoline. In order to immobilize the target molecule onto a SPR chip, the (strept)avidin/biotin interaction has often been used thanks to its efficiency and convenience of use for the immobilization of the target molecule. Several coupling reactions can be used to obtain a biotinylated derivative of the target molecule [30]. For this purpose, a biotinylated derivative of lysergamine with a NHS-polyethylene glycol-biotin (NHS-PEG4-biotin) linker was prepared (**Figure 38**) in order to coat a streptavidin coated SPR chip. An ethanolamine quenched linker was also prepared with a NHS-PEG4-biotin linker and was injected onto a streptavidin SPR chip as a reference (**Figure 39**).

Figure 38. *Scheme of the reaction between NHS-PEG4-biotin linker and lysergamine.*

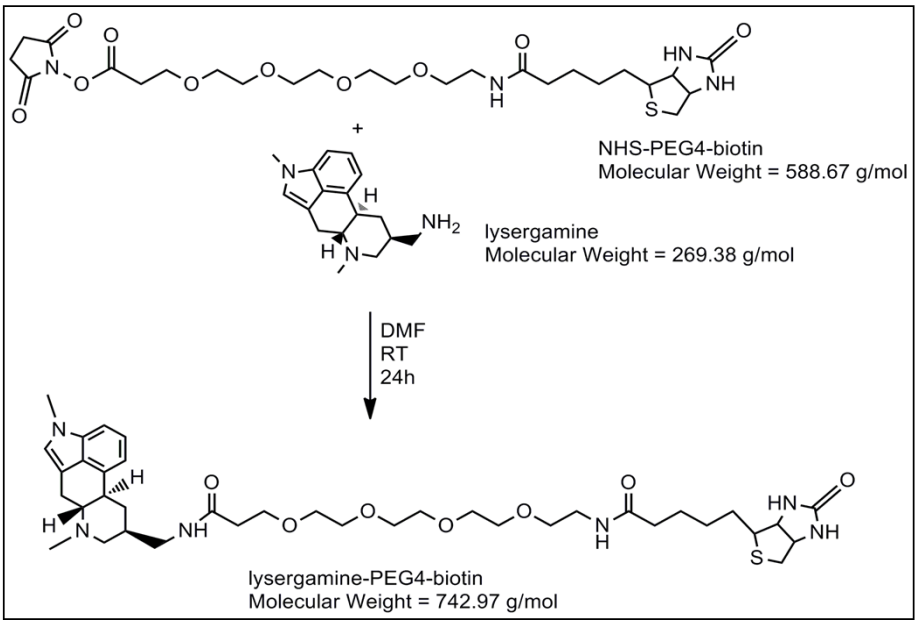
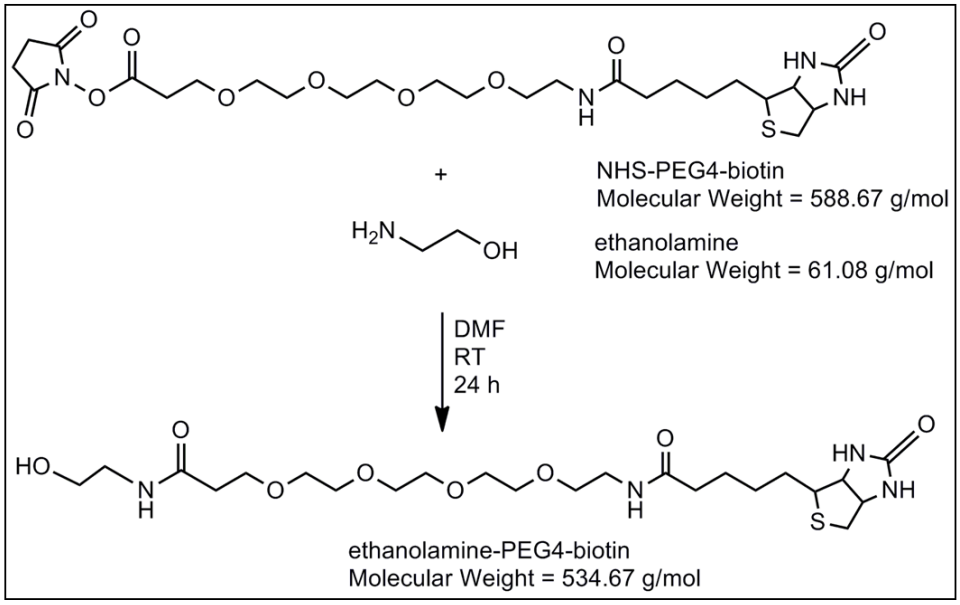


Figure 39. *Scheme of the reaction between NHS-PEG4-biotin linker and ethanolamine.*



The reaction products, biotinylated linker with lysergamine or ethanolamine, were analyzed using mass spectrometry in positive mode (**Figures 40 and 41**), in which the protonated reaction product lysergamine-PEG4-biotin was found at *m/z* 743.41 (*m/z* calculated 743.41) and the protonated reaction product ethanolamine-PEG4-biotin was found at *m/z* 535.29 (*m/z* calculated 535.28).

Figure 40. *Mass spectrum in positive mode of the reaction between NHS-PEG4-biotin linker and lysergamine.*

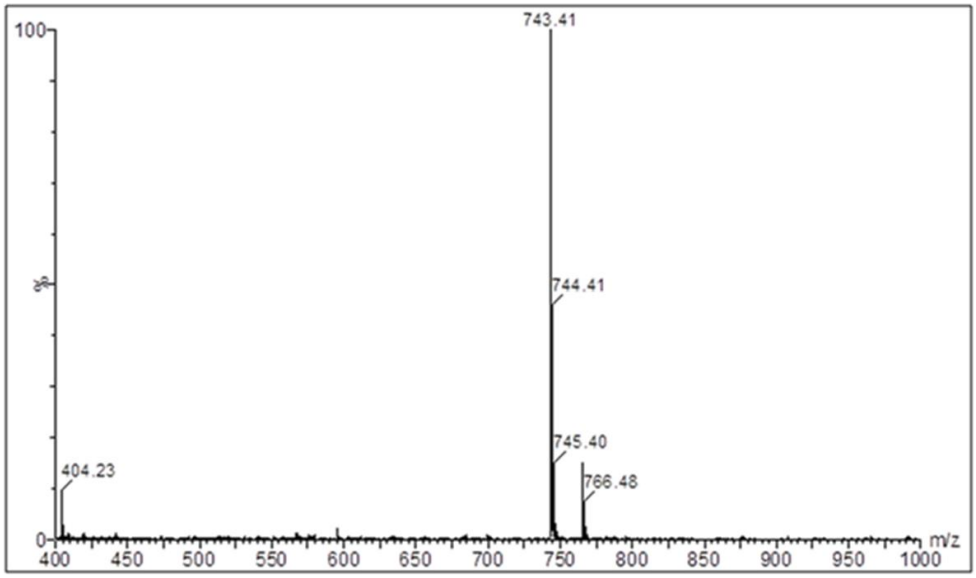
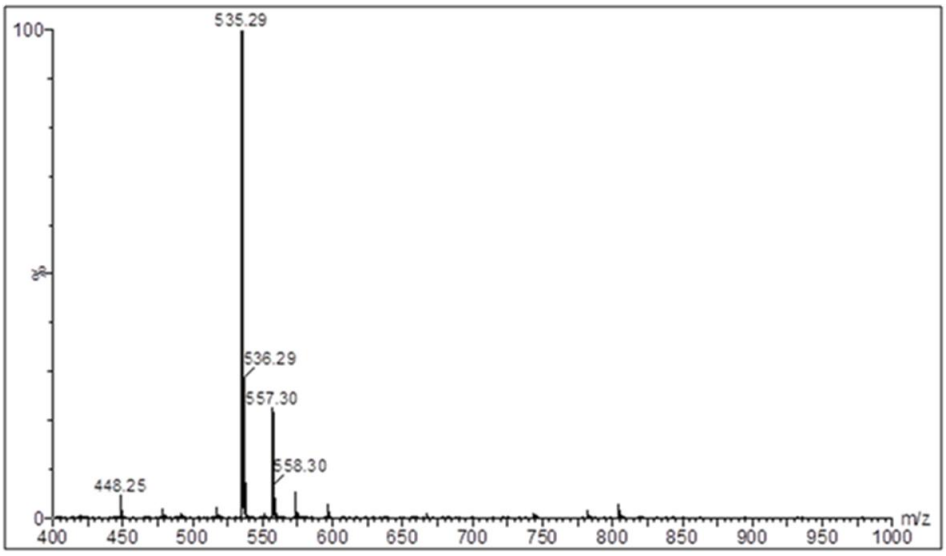


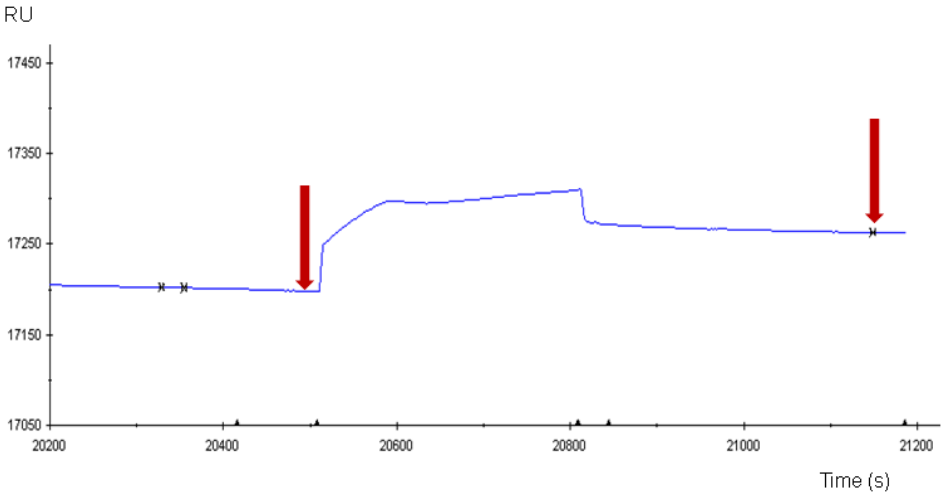
Figure 41. *Mass spectrum in positive mode of the reaction between NHS-PEG4-biotin linker and ethanolamine.*



4.2.2. SPR Measurements

The two biotinylated products presented in the previous section were injected into different channels of a streptavidin SPR chip with the same parameters (concentration, flow, injection time). The different solutions of aptamers were passed through the coated surfaces and the responses were calculated by subtracting the reference measurement from the sample measurement. The SPR responses obtained for the coating with biotinylated lysergamine was in the range of 500 resonance units (RU), and in the range of 350 RU for the biotinylated ethanolamine, which is a smaller molecule and therefore gave a lower response. Other references were tested, such as a non-modified streptavidin surface and a streptavidin surface coated with biotin. However, these two latter references gave lower responses (<5 RU) than the ones obtained for ethanolamine (~10 RU). Therefore, only the ethanolamine coated streptavidin surface was considered for the determination of K_Ds in the binding assay. Different concentrations of each selected aptamer were passed through the SPR chip coated with lysergamine. The responses given by each aptamer were recorded, as shown on **Figure 42**. The responses given by the same aptamers with the ethanolamine-coated SPR chip were subtracted, in order to determine the final responses. Triplicates of the measurements were performed with each different aptamer. In total, 70 selected aptamers were analyzed by SPR.

Figure 42. *Example of sensorgram obtained for the injection of one concentration of a given aptamer. The responses (RU) are measured before the injection of the aptamer (baseline) and after stabilization following the end of injection; as shown on the sensorgram with arrows.*



The dissociation constants of the complexes of lysergamine and aptamers were determined by least-squares fitting of the values of the responses given by the SPR analysis with a non-linear regression of the following equation:

$$y = \frac{B_{Max} * x}{K_D + x} \qquad (7)$$

with *y*, the degree of saturation; B_{Max}, the number of maximum binding sites; K_D, the dissociation constant and *x*, the concentration of ssDNA aptamer.

As shown in **Figures 43 and 44** and **Table 4**, aptamer M3.2 had a B_{Max} of 205.2 RU, representing approximately from half to one third of the values obtained for L5.2 and L5.7, respectively 575.8 and 531 RU. The three selected aptamers had dissociation constants (K_Ds) ranging from 44 nM^2 for aptamer M3.2 to 499 nM for aptamers L5.2 and L5.7 (Table 1). These three aptamers represent sensitive ligands for lysergamine, as the dissociation constants (K_Ds) obtained for the complexes are situated in the nanomolar range. These results are in accordance with those reported in the literature, usually ranging from millimolar to picomolar range for the most sensitive aptamers [31, 32]. The best fit for the values obtained for aptamer M3.2 corresponded to a model with two binding sites, instead of a model with one binding site for aptamers L5.2 and L5.7. The fact the aptamer M3.2 has the lowest dissociation constant (K_D) is in accordance with the primary structures of the sequences obtained from the selected aptamers, in which two common motifs of consecutive C bases were found in this aptamer, and the same common motif was present only once in the two other aptamers selected. Whether binding is cooperative or not can be determined from the shape of the binding curve [1]. It is difficult to distinguish non-

cooperative from cooperative binding from a plot of response versus the ligand (aptamer) concentration, but differences are easier to recognize when the response is plotted versus log ligand concentration [1]. From the shape of the plot of the response of M3.2 versus the logarithm of the concentration of M3.2, a negative cooperative binding was observed; thus the binding of one site to the target molecule prevents slightly the second binding site to access the target molecule. However, the affinity of aptamer M3.2 was overall better than the one of other aptamers selected and aptamer M3.2 was found to be the most sensitive to lysergamine.

Figure 43. *SPR responses (in resonance units) obtained for three selected aptamers: aptamer L5.2, aptamer L5.7 and aptamer M3.2.*

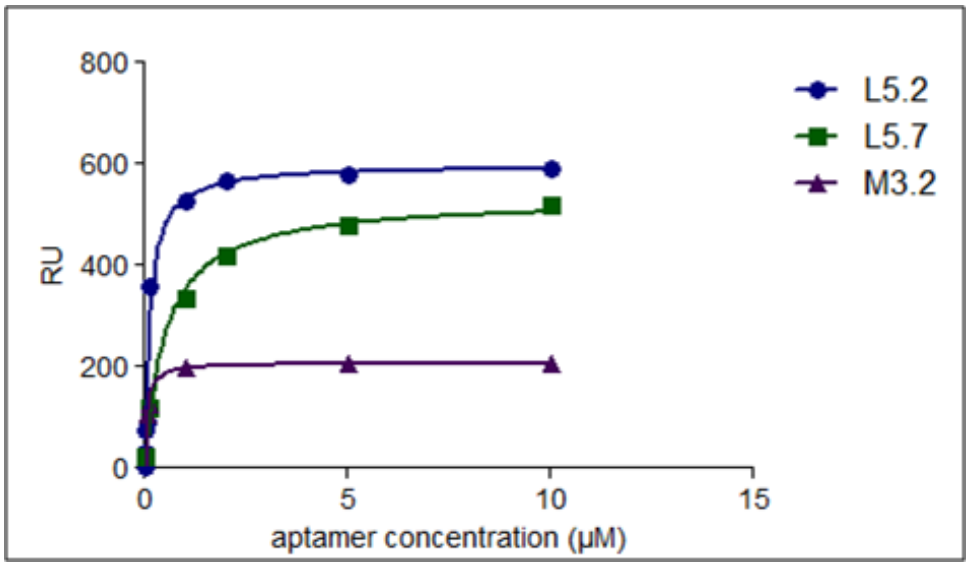


Figure 44. *SPR responses (log scale) obtained for aptamers L5.2, L5.7 and M3.2.*

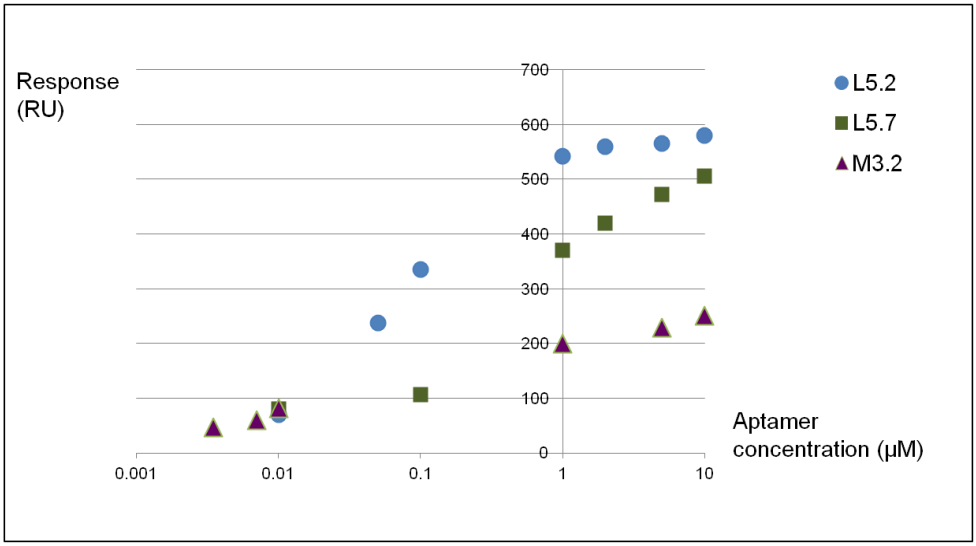


Table 4. *Calculations by GraphPad Prism of the dissociations constants (K_Ds) of complexes of lysergamine and the selected aptamers.*

Fitting Model	**Two-site specific binding**	**One-site specific binding**	
Best fit values for	**Aptamer M3.2**	**Aptamer L5.2**	**Aptamer L5.7**
B_{Max} (RU)	205.2	585.8	531.0
K_D	44 $nmol^2/L^2$	73 nmol/L	499 nmol/L
R^2	0.997	0.993	0.991
Number of points analyzed	6	7	6
Degree of freedom	2	5	4

5. Conclusion

A SPR analysis of the selected aptamers was conducted by using streptavidin chips coated with a biotinylated lysergamine, and reference streptavidin chips prepared with biotinylated ethanolamine. Other coupling reactions were tried, but the streptavidin/biotin system was generally preferred, as it is generally necessary to perform the coating inside the SPR machine, which sets some limitations in the chemical reactions that can be performed. The SPR analysis allowed the determination of the dissociation constants of the complexes of aptamers and lysergamine with a good sensitivity. The three best binding aptamers, which dissociations constants were determined to range from 44 nM to 499 nM, were kept for further work in the construction of an aptasensor. It was shown that aptamer M3.2 corresponded to a model with two binding sites, which was in accordance with the structure of this aptamer, having two motifs of consensus C bases.

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Chapter V: Development of an optical aptasensor for ergot alkaloids

1. Introduction

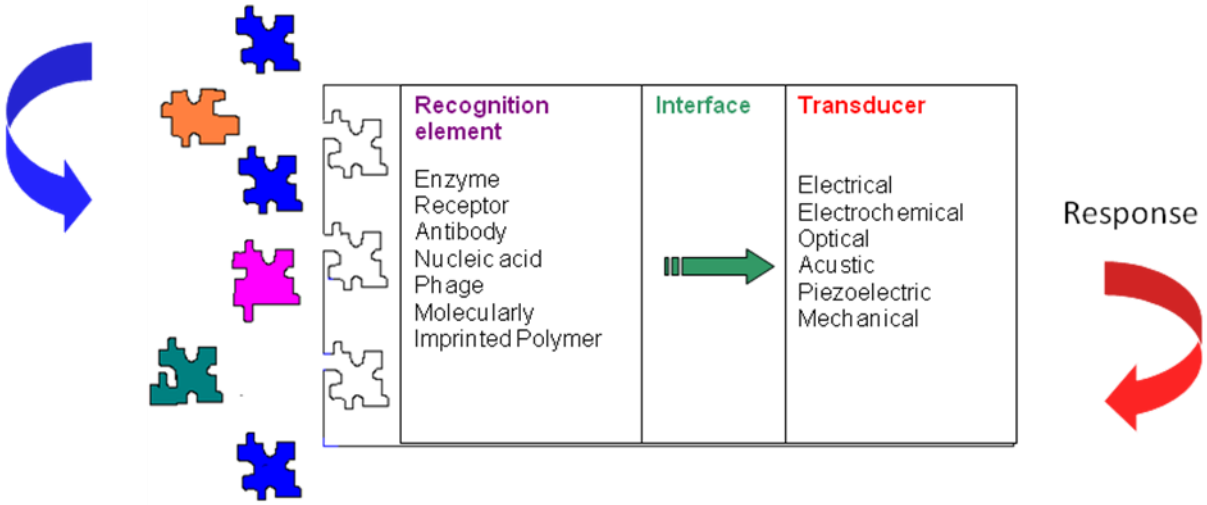
The food industry is the main party concerned with the presence of pathogenic microorganisms, toxins and pollutants; and failure to detect these contaminants may lead to dreadful effects. Although the safety of food has improved overall, food contamination remains a general issue worldwide [1, 2]. Also, the increasing number of potentially harmful pollutants in the environment calls for fast and cost-effective analytical techniques that can be used in extensive monitoring programs. The requirements, both in terms of time and costs, of most traditional analytical methods (e.g. chromatographic methods) often constitute an important limitation for their application on regular basis. Moreover, skilled personnel are required for the use of analytical equipment and data interpretation. In this context, biosensors appear to be suitable alternative or complementary tools to analytical techniques [3]. The main advantages of biosensors over traditional analytical techniques for the detection of food and environmental contaminants are their cost-effectiveness, rapidity and portability, which make *in situ* and real time monitoring possible, without extensive sample preparation [4]. The main reason why biosensors are still rarely used is their impracticability for real samples, as a biosensor developed for standards is not automatically applicable for real samples. Hence, the challenge for scientists is to develop or improve some good existing concepts for constructing biosensors applicable on real samples and usable in commercial sphere [5]. Lately, DNA aptamers have proven suitable for analytic and diagnostic applications [6-8]. Indeed, aptamers can bind with high affinity and specificity to a broad range of target molecules. They are synthetically evolved in the SELEX process, therefore their potential range of use is virtually unlimited [9]. The specificity of aptamers can be modulated, in order to restrict or broaden the field of application. It was shown that it was possible to obtain aptamers distinguishing between isomers or closely related compounds. For instance aptamers selected for theophylline and L-arginine could discriminate closely related chemical structures by factors as high as 4 orders of magnitude [10-12]. Also, the robustness and good resistance of aptamers can be exploited to functionalize or modify them with different types of labels and to immobilize them onto various types of surfaces [13]. In this chapter, an overview of the different types of biosensors will be presented with some examples of biosensors based on aptamers, or aptasensors. The production of an optical biosensor is one of the possible applications with the selected aptamers. Optical detection with gold nanoparticles is interesting because it is one of the most user-friendly and straightforward sensing system, providing an immediate yes/no answer associated with the color resulting from the test. The construction of an

optical aptamer-based biosensor for the detection of ergot alkaloids will be presented in the experimental part of this chapter.

2. The different types of biosensors

A biosensor is defined as a self-contained integrated device, consisting of a biological recognition element in direct contact with a transduction element, which converts the biological recognition event into a useable output signal [14]. The IUPAC has defined the terminology of a biosensor as an integrated receptor-transducer device, which is able to provide selective quantitative or semi-quantitative analytical information using a biological recognition element [15]. Biosensors should thus be distinguished from bioassays where the transducer is not an integral part of the analytical system [3]. As shown in **Figure 45**, different recognition elements, such as antibodies, nucleic acids or enzymes; and various transduction techniques can be used for the construction of biosensors [7]. Biosensors were classified depending on their transduction system: electrical, electrochemical, optical, piezoelectric and thermometric [5].

Figure 45. *Scheme of the general configuration of a biosensor including recognition element, interface and transducer.*



A transducer is defined as an analytical tool which provides an output quantity having a given relationship to the input quantity [5, 16]. Aptasensor based analysis is continuously evolving with various detection schemes ranging from label-free methods such as surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) measurements to label dependant methods such as electrochemistry, fluorescence, chemiluminescence, field effect transistors etc. Currently, optical, electrochemical and mass aptasensors constitute the predominant types under development [7, 13, 17] and therefore this chapter will focus on these techniques only.

2.1. Optical biosensors

Optical biosensors are based on the measurement of changes in the optical characteristics induced by the formation of complexes of the biological ligand and target compound. Generally, there are two detection protocols that can be implemented in optical biosensing: detection requiring labels such as fluorescent dyes or chromophores like gold nanoparticles, and label-free detection. In fluorescence detection, either target molecules or biorecognition molecules are labelled with fluorescent tags, such as dyes; the intensity of the fluorescence indicates the presence of the target molecules and the interaction strength between target and biorecognition molecules. Fluorescence-based detection can be extremely sensitive, with a detection limit down to a single molecule; it suffers from laborious labelling processes that may also interfere with the function of a biomolecule [18]. Also, quantitative analysis can be challenging due to the fluorescence signal bias. Colorimetric aptasensors using unmodified gold nanoparticles (AuNPs) have attracted much attention because of their practicality and they have been developed for various targets in the past several years [19]. Colorimetric aptasensors meet important criteria for sensors: ease of use, fast response, cost-effectiveness and absence of analytical instrumentation [19-22]. In label-free detection, target molecules are not labelled or altered, and are detected in their natural forms [18]. Surface plasmon resonance is an example of optical transduction. In this category, SPR biosensors are optical sensors using special electromagnetic waves surface plasmon - polaritons - to monitor interactions between an analyte in solution and a bioelement immobilized on the SPR sensor surface. Beside the fact that SPR biosensors can be very sensitive; they also allow real-time monitoring and they do not require labelled molecules [13].

- Fluorescence-based aptasensors

Fluorescence-based biosensors involve a fluorescent label, usually organic dyes; such as rhodamine, coumarin, cyanin or fluorescin [23]. A fluorometer is required for the measurement of the fluorescence intensity. It is possible to produce a fluorescence-based aptasensor either with a single fluorophore transduction signal, or with a fluorophore-quencher pair [24]. To produce an aptamer biosensor with a single fluorophore signal transducer, a fluorophore may be covalently linked to the aptamer or a fluorescent nucleotide analogue may be incorporated into the sequence at a position that is expected to undergo significant structural reorganization following ligand (L) binding. In this system, target-aptamer interaction is reported by either an increase or a decrease in fluorescence intensity resulting from the change in the electronic environment surrounding the fluorophore upon ligand binding [24]. The other category, fluorophore-quencher pair aptasensor are often referred as molecular beacons. In the traditional format, molecular beacons act like nucleic acid switches that are normally closed - or "off". Binding induces a conformational change that opens the hairpin structure of the nucleic acid, and as a result

the fluorescence is turned "on" [25]. The stem structure holds the fluorophore and the quencher in close proximity to one another, preventing the fluorophore from emitting a signal as a result of resonance energy transfer (RET) [25, 26]. Once the single-stranded loop of the molecular beacon binds to its target molecule, the conformational change results in a spatial separation of the fluorophore from the quencher; giving a fluorescence signal. An example of fluorescence energy transfer-based molecular beacon aptasensor is the one developed for thrombin [27]. A highly sensitive and specific fluorescence resonance energy transfer (FRET) aptasensor for thrombin detection was developed based on dye labelled aptamer assembled graphene. Due to the non covalent assembly between aptamer and graphene, fluorescence quenching of the dye takes place because of FRET. The addition of thrombin leads to the fluorescence recovery due to the formation of quadruplex–thrombin complexes which have weak affinity to graphene and keep the dyes away from graphene surface. Because of the high fluorescence quenching efficiency, unique structure, and electronic properties of graphene, the graphene aptasensor was shown to exhibit extraordinarily high sensitivity and excellent specificity in both buffer and blood serum. A detection limit as low as 31.3 pM was obtained based on the graphene FRET aptasensor. In another example, a fluorescence-based aptasensor was constructed using graphene oxide to quench a fluorescin-labelled aptamer (which binds to ochratoxin A) in its free form but not the folded form after OTA binding. This aptasensor was shown to be suitable for measurements in real food samples such as the 1% red wine with various concentrations of ochratoxin A [28].

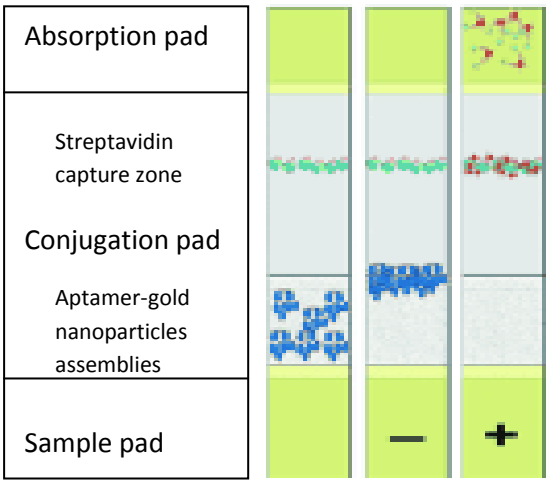
Alternatively, another type of nanoparticles, quantum dots (QD) can replace organic fluorophores. QDs are semiconducting inorganic fluorophores, nanocrystals usually comprised of atoms from elemental groups II and VI (e.g. cadmium sulfide and cadmium selenide) or groups III and V (e.g. indium phosphide and indium arsenide) [13]. QDs exhibit an energy band gap that determines the required wavelength of radiation absorption and emission spectra. QDs are extremely efficient at absorbing light and converting it into a highly stable fluorescent emission, making them up to 50 times brighter than conventional organic fluorophores. The small radii (in the nanometer scale) of these structures lead to electron confinement, giving rise to their unique optical and electronic properties. While the absorption spectrum of QDs is broad, their emission spectrum is narrow and is tunable by adjusting their size. In order to enhance their quantum yield and produce brighter signals, a surface capping layer, such as a zinc sulfide shell, may be deposited on their surfaces. Moreover, the use of QDs as signal transducers in place of fluorophores has an additional advantage owing to their resistance toward photobleaching [24]. Recent work has demonstrated that QDs can be used in the construction of biosensors that signal by fluorescence resonance energy transfer (FRET) [29]. For example, a quantum-dot based molecular beacon has been described in which the non fluorescent dye DABCYL was used to quench the fluorescence of the quantum dot. In the presence of a target DNA, the opening

of the molecular beacon resulted in an approximately fivefold increase in fluorescence of the quantum dot [30].

- Gold nanoparticles-based aptasensors

The use of gold nanoparticles is very interesting because no extra apparatus is required to see the color change. This methodology relies on the strongly distance-, shape and size-dependent optical properties of gold nanoparticles [20-22]. The conformational change of the aptamer occurring upon binding to its target molecule can induce a change of the electric charges surrounding the gold nanoparticles, inducing a color change; and also changes in the physical and chemical properties of the aggregates. The development of oligonucleotide-modified gold nanoparticles as probes for the selective and sensitive detection of complementary sequences by Mirkin and his coworkers back in 1997 found the possibility of chemically conjugating these particles with nucleic acids and exploiting their unique distance-dependent optical properties for signalling [31]. Oligonucleotide-modified gold nanoparticles can be used as such in solution or incorporated to paper tests or dipsticks. Dipsticks are cheap, mobile, easy to use and provide a fast detection of the target compound. They mainly give qualitative information (yes/no answer) and can be used for a first estimation. As no apparatus is required, they can be used in the field; giving them a great advantage compared to other techniques. Making use of oligonucleotide and gold nanoparticles, Liu and Lu designed a dipstick test for ATP and cocaine that use oligonucleotide-modified gold nanoparticles as signal transducers [32]. In this dipstick test using lateral flow diffusion, some successive layers of porous papers are assembled in a user-friendly test kit. The system, which is shown in **Figure 46**, can be described as such: a sample pad acts as a sponge and holds an excess of sample fluid. Once soaked, the fluid migrates to a second element, the conjugation pad, in which are stored the aptamers linked to gold nanoparticles. The differences of colors and migration properties of the complexes with the target molecule compared to free aptamer-gold nanoparticles structures can be exploited to detect the presence of the target molecule. In this work, the free aptamers-gold nanoparticles (aptamers tagged with biotin) structures, which can migrate further, are stopped by a streptavidin line; while the complexed aptamers will move less far and have a different color. An absorption pad is placed at the end of the test to absorb the fluid sample and to make it migrate along the paper test. In their experiment, the aptamers linked to gold nanopaticles formed aggregates, which were dissociated in presence of cocaine. A color change from blue to red occurred with the dissociation of the aggregates, which was exploited to migrate further on the conjugation pad, as indicated in **figure 46**.

Figure 46. *Scheme of a lateral flow diffusion test using aptamers and gold nanoparticles. The sample is loaded on the sample pad. The sample migrates by lateral flow diffusion on the conjugation pad, which contains gold nanoparticles linked to aptamers. In case that the sample contains the molecule of interest, the color change and difference in migration can be exploited for the specific detection of the target molecule; which will result in a colored band on the dipstick test*.



- Surface plasmon resonance aptasensors

SPR biosensors provide advantage of label-free real-time analytical technology [33]. In a typical SPR biosensing experiment, the aptamer (i.e., a ligand or biomolecule) is immobilized on an SPR-active gold-coated glass slide which forms one wall of a thin flow-cell, and the other interactant in an aqueous buffer solution is induced to flow across this surface, by injecting it through this flow-cell. The signal transducer used in SPR-based aptamer biosensors is generally gold covered disks coated with streptavidin. The presence of streptavidin, a biotin binding protein, enables the immobilization of biotinylated aptamers. When these chips are exposed to their targets, the interaction between the aptamers and their ligands would alter the local refractive index, and in turn change the surface plasmon resonance in response to excitation by the incident light. This fluctuation may be detected by monitoring the change in SPR angle [24]. This system has been applied by Tombelli and colleagues for the detection of the HIV-1 Tat (trans-activator of transcription) protein with high selectivity [34]. Also, Tang and colleagues developed a sensor with anti-thrombin DNA aptamers [35]. These groups further demonstrated that the sensor chips may be rinsed with 2 M NaCl solutions to prompt ligand dissociation following target detection, rendering them reusable. SPR aptasensors also offer the advantage of a high sensitivity. For instance, the SPR aptasensor developed by Ashley and Li for the detection of catalase had a limit of detection (LOD) in the nanomolar range (20.5 nM) and a dynamic range of 15-1000 nM, and showed a good specificity toward catalase [36]. SPR aptasensors are therefore very interesting; however this technique requires skilled personnel and is rather expensive.

2.2. Electrochemical biosensors

Besides optical detection techniques, electrochemical techniques offer great possibilities for sensing applications, as they allow a good quantification of analytes [37, 38]. The construction of electrochemical aptasensors is made possible if the binding of the aptamer to its target molecule can either generate a measurable current (amperometric), a measurable potential or charge accumulation (potentiometric); measurably alter the conductive properties of a medium (conductimetric); changes of both resistance and reactance (impedimetric), or measurable potentiometric effect at the gates of the electrodes (field-effect) [18]. Amperometric biosensors are the most widespread electrochemical biosensors, and they utilize for their biochemical reaction mediators, i.e. molecules which are able to transfer electrons. They can participate in the redox reaction with the biological component and help in the faster electron transfer [5]. In potentiometric biosensors, the difference in potential that is generated across an ion-selective membrane separating two solutions at virtually zero current flow is measured. Electrochemical transduction usually offers a high sensitivity, which can be enhanced by attaching biocatalytic labels or redox groups to the aptamer-target complex. It is possible to covalently attach the aptamers onto metallic surfaces, such as electrodes, for the realization of aptamer-based electrochemical biosensors; either by directly fixing thiol-modified DNA onto gold electrodes, or by using a coating previously placed on the electrodes with functionalized DNA [39, 40]. Some very sensitive aptasensors were developed using electrochemical transduction. For instance, Wei and Ho have reported an amperometric aptasensor for the detection of botulinum neurotoxin having a LOD of $40*10^{-9}$ g/L or 400 fM [41]. Another electrochemical aptasensor developed by Min and coworkers, using impedance spectroscopy, could successfully detect its target molecule, interferon-g, to a level of 100 fM [42]. In 2012, Ng and coworkers developed an electrochemical aptasensor for the detection of several microcystin congeners, including microcystin LR (MC-LR), MC-LA, and MC-YR [43]. In the proposed technique, microcystin-specific DNA aptamers were immobilized onto the electrode surface by self-assembly via thiol-chemistry and exposed to a solution containing the redox cations $[Ru(NH3)6]^{3+}$. In the absence of the target, the redox cations bound to the phosphate backbone of the immobilized aptamers gave a large reduction peak measured via square wave voltammetry. Introduction of microcystins caused a decrease in the voltammetric current within the dynamic range of 10 pM to 10 nM microcystin, with an LOD of 7.5–12.8 pM.

2.3. Mass-sensitive biosensors

Last but not least, numerous mass-based sensors have been elaborated using aptamers. These aptasensors detecting mass-change include micro cantilever, gravimetric and surface-acoustic wave-based sensors. These sensors are well adapted to large molecular weight target molecules such as proteins for instance. Micro cantilevers are thin, silicon-based, gold

coated surfaces hanging from a solid support. A surface adsorption results in a bending of the cantilever, which is detected either electrically by a metal oxide semi-conductor field effects transistor or optically by changes in angle reflection. In 2004, Savran and colleagues synthesized a cantilever-based aptamer biosensor, which makes use of two cantilevers connected by a solid support. One of the cantilevers, serving as the sensor, was functionalized with thiol-modified aptamers specific for *Taq* polymerase. The second cantilever, functionalized with single-stranded nonspecific DNA sequences, serves as the reference. The binding of the *Taq* polymerase to the aptamers causes the sensor cantilever to bend. The extent of bending depends on the ligand concentration and can be measured using the optical lever technique [44]. In piezoelectric aptasensors, such as quartz crystals, a mass loading resulting from target binding to the aptamers decreases the velocity of surface acoustic waves propagating along the signal transducer, consequently decreasing the resonance frequency of these waves or changing the phase shift between input and output waves. Thus, monitoring the change in one of these two properties can provide an indication of the absence or presence of the analyte of interest [24]. QCM aptasensors offer the advantages of high specificity, reusability, low detection limit, no label, and no sample pre-treatment. Moreover, quantification is possible with QCM technique. For instance, a QCM aptasensor developed by Yao and coworkers for the detection of IgE could successfully quantify IgE concentrations in buffer and in human serum as low as 2.5 µg/L [45]. Also, the team Minunni and coworkers fabricated an aptasensor to detect HIV-1 Tat protein by immobilizing an RNA aptamer on a piezoelectric quartz crystal, which was shown to have a detection limit of 0.25 ppm [46]. Another example is the QCM aptasensor developed by Dong and Zhao for the detection of mercury in water, having a detection limit of 0.24 nM, which resulted to be better by three orders of magnitude than the previous methods reported for the quantification of mercury [47].

3. Experimental Part: Development of an aptamer-based colorimetric test for ergot alkaloids

3.1. Materials and Methods

In this study, a colorimetric aptasensor was developed for ergot alkaloids, based on one of the selected aptamers and gold nanoparticles. For that, the selected aptamer is hybridized to two small DNA fragments linked to colloidal gold by a thiol bond. Upon binding to the target molecule, a conformational change of the aptamer can occur. This conformational change can lead to a modification of the chemical environment surrounding the gold nanoparticles, inducing a color change of the solution. The procedure developed by Liu and Lu [22] was followed with the aptamer selected for ergot alkaloids. The following labelled DNA sequences (Eurogentec, Belgium) were used in the colorimetric test: 5'-ACTCATCTGTGAAGAGAAGCAGCACAGAGGTCAGATGTCCGTCAGCCCCGATCGCCATCCAGGGACT

CCCCCCTATGCCTATGCGTGCTACCGTGAA-3' (modified aptamer M3.2), 5'-TCACAGATGAGT-C3-SH-3' (3'-SH oligo) and 5'-SH-C6-TGCTGCTTCTCT-3' (5'-SH oligo). In a microcentrifuge tube were placed: 1.6 µL of 1 mM 3'-SH oligo and 7.4 µL of water. In a second microcentrifuge tube, the same quantity was applied with 5'-SH oligo. Then, 1 µL of 500 mM acetate buffer (pH 5.2) and 1.5 µL of tris(2-carboxyethylphosphine) TCEP-HCl were added to each tube to activate the thiol moiety. After 1 h of activation, 3 mL of 15 nm colloidal gold solution were added to each tube containing TCEP-activated oligos and the mixtures were gently stirred. The tubes were stored in a drawer for 24 h. Then, 30 µL of 500 mM Tris acetate buffer (pH 8.2) was added dropwise to each tube and 300 µL of 1 M NaCl was added. The tubes were stored for another day. The functionalized particles were centrifuged at 13,000 rpm for 15 min at RT. The supernatant was discarded and the particles were dispersed in 200 µL of 100 mM NaCl buffer. The two solutions of functionalized gold nanoparticles were mixed together. Then, 10 µL of 10 µM modified aptamer M3.2 were added and the mixtures were incubated at 4°C overnight. The aggregates were centrifuged at 2,000 rpm for 1 min at RT. The supernatant was discarded and the aggregates were dispersed in 500 µL of 300 mM NaCl buffer. For the optimization of salt concentration, 50 µL of the aggregates solution were taken for each analysis. The phase transition of the aptamer-gold nanoparticles aggregates was studied by recording the UV absorption at 260 nm as a function of the temperature with different NaCl concentrations (from 150 mM to 300 mM), this in order to find the appropriate NaCl concentration for which the UV-Vis absorption increases sharply at RT. For the final test, two series of samples with different concentrations of gold nanoparticles solutions were prepared: a concentrated solution (Series II), in which 30 µL of aggregates solution were taken and the NaCl concentration was adjusted to 250 mM by adding 100 mM NaCl buffer; and a more diluted series of 40 µL of solution of gold nanoparticles diluted twice (Series I), with a final NaCl concentration of 250 mM. For the specificity test, each compound was prepared in a solution of DMF with a concentration of 50 mM and 1 µL of each of the solutions was added to the test tubes containing the aptamer-gold nanoparticles system. A sample of ergot contaminated flour provided by the European Food Safety Authority (sample EFSA/82/RE), having a known concentration of ergot alkaloids [48, 49], was tested. The study made by the team of Diana Di Mavungu et al. has shown that the distribution of ergot was homogeneous. The sample of ergot contaminated flour was prepared by adding 300 mg of contaminated flour sample to 500 µL of DMF and vortexing for 1 min. This way, the ergot alkaloids were extracted from the flour sample. Then, 1 µL of this solution was used for the test. A preliminary test with 1 µL of DMF was also performed and did not show any effect on the solution of DNA-functionalized gold nanoparticles. The UV-Vis spectra of the samples from Series I were measured by a Synergy Mx UV-Vis spectrophotometer (Biotek, Germany), and were analyzed using Gen5 software.

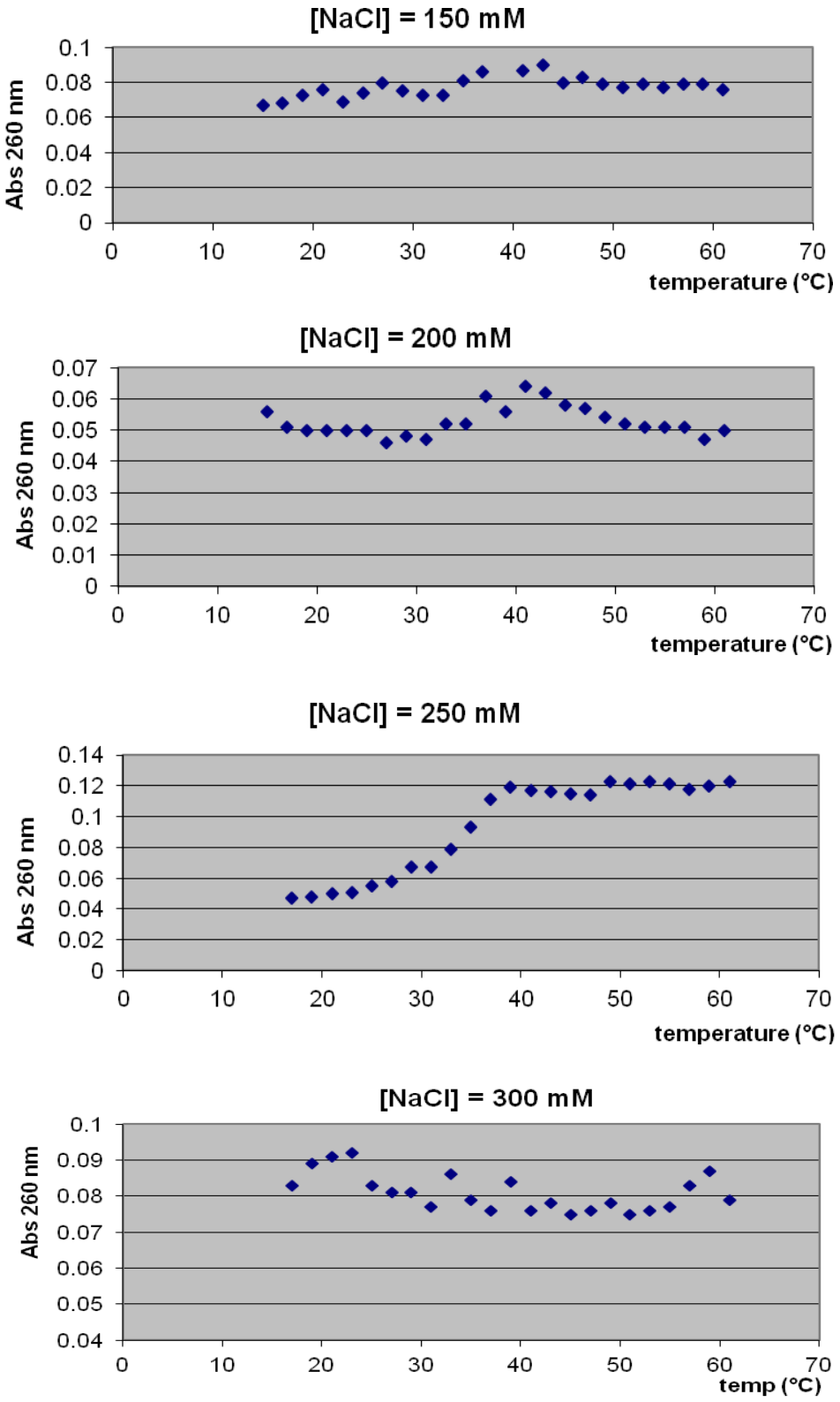
The construction of a dipstick test based on the colorimetric reaction was also studied. The mobility of the complexes was studied by placing 2 µL of the solutions of complexes on a

conjugation pad, and testing different solutions of DMF in water, with ratios going from 1:9 to 9:1.

3.2. Results and Discussion

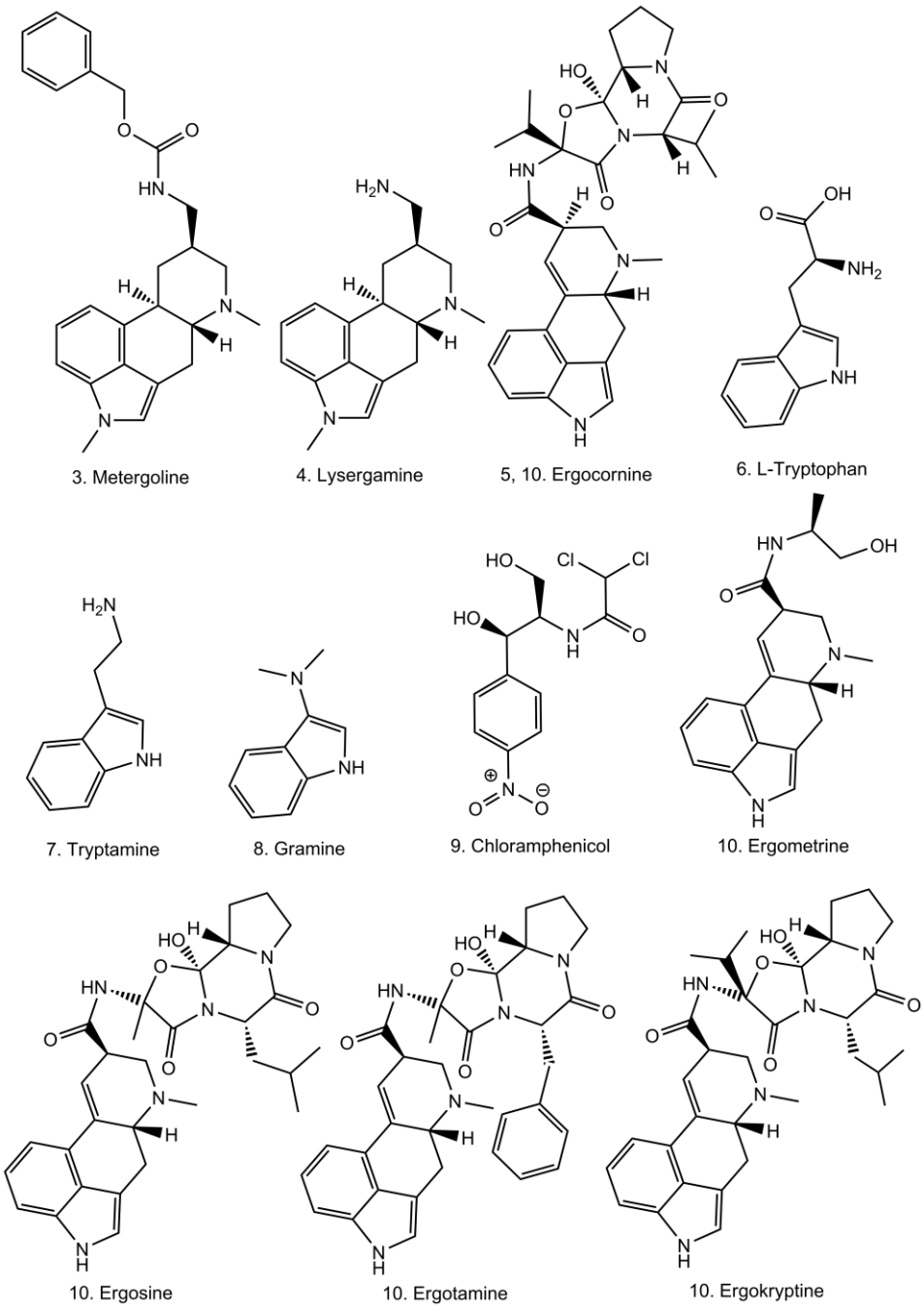
In the study of phase transition of the aptamer-gold nanoparticles solutions shown in **Figure 47**, the absorbance of the solutions having different concentrations of NaCl were recorded at 260 nm as a function of temperature. Initially, the extinction may be constant or decrease slightly with increasing temperature. Initially, the extinction at 260 nm may be constant or decrease slightly with increasing temperature. At a given NaCl concentration, the extinction begins to increase sharply after reaching a certain temperature. The optimal temperature for detection is usually situated around ∼2–3 °C below this. The sample for which the sudden increase of extinction occurs around room temperature is the best suited for colorimetric reaction. In this study, several concentrations of NaCl were tested for our solutions of aptamers linked to gold nanoparticles: 150 mM, 200 mM, 250 mM and 300 mM. The sample having the concentration of 250 mM NaCl showed a sharp increase of the extinction coefficient around 25°C (**Figure 47**). Therefore, an optimal concentration of 250 mM NaCl was chosen for the rest of the study, as the melting temperature obtained with this parameter was the closest to RT (~25°C).

Figure 47. *Absorbance intensities versus temperature for different aptamer-functionalized gold nanoparticles solutions containing varying concentrations of NaCl.*



Once the modified version of aptamer M3.2 was fixed onto gold nanoparticles, a homogenous red solution was obtained. The solution was partitioned in several tubes, in order to test the different samples containing ergot alkaloids, ergot-alkaloid free samples and possible interfering compounds. For that, solutions of different ergot alkaloids in dimethylformamide (DMF) were tested: lysergamine, metergoline, ergocornine and an extract of ergot contaminated flour containing several ergot alkaloids such as ergometrine, ergosine, ergotamine, ergocornine, ergokryptine, ergocristine and their epimers in various proportions (**Figure 48**).

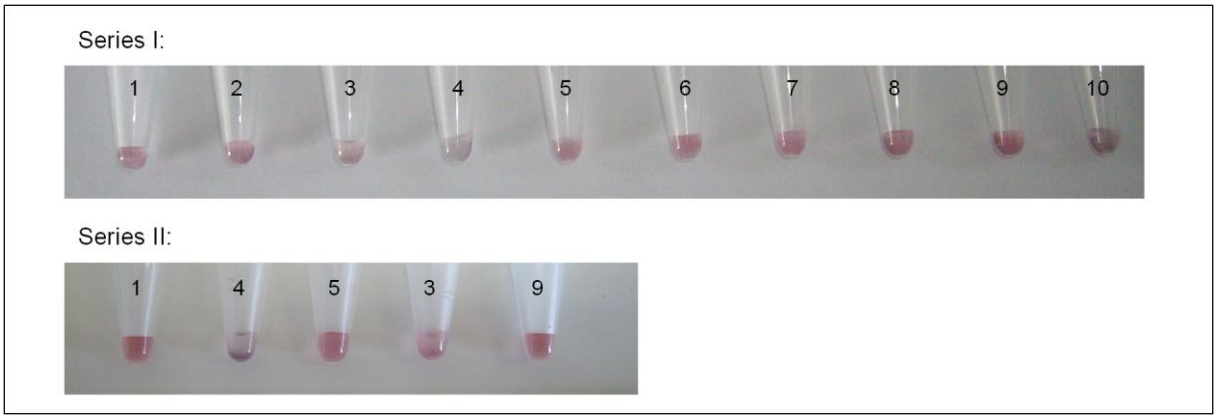
Figure 48. *Chemical structures of the different compounds tested in the colorimetric assay.*



As the ergoline skeleton of ergot alkaloids contains a two-member ring structure named indole, several compounds having an indole group which could be possibly found in food samples, such as L-tryptophan, gramine and tryptamine were also tested (**Figure 48**). In addition, another molecule differing from ergot alkaloids and containing a phenyl group, chloramphenicol, was also tested (**Figure 48**). The solutions of the different compounds were prepared in DMF with a concentration of 50 mM and for each sample, 1 µL was taken and placed in the tubes containing the solution of aptamers linked to gold nanoparticles. A rather high concentration of compounds was chosen in order to provide a qualitative assay clear enough to be seen by the naked eye. The colorimetric test was performed with a diluted solution of gold nanoparticles (Series I) and was measured by UV-Vis spectroscopy. A second set of samples was prepared with a more concentrated solution of gold nanoparticles and some of the compounds from Series I. In **Figure 49**, are shown the results of the colorimetric assay with aptamer M3.2 linked to gold nanoparticles, in which a precipitate was obtained with lysergamine (sample 4), metergoline (sample 3) and the ergot contaminated flour extract (sample 10) and a color change occurred in the presence of lysergamine and the ergot contaminated flour extract. The color change was much less pronounced in the case of metergoline (sample 3). The other molecules tested: the solvent DMF (sample 2), ergocornine (sample 5), L-tryptophan (sample 6), tryptamine (sample 7), gramine (sample 8), and chloramphenicol (sample 9), did not generate any change.

Figure 49. *Colorimetric reaction based on gold nanoparticles linked to aptamer M3.2.*

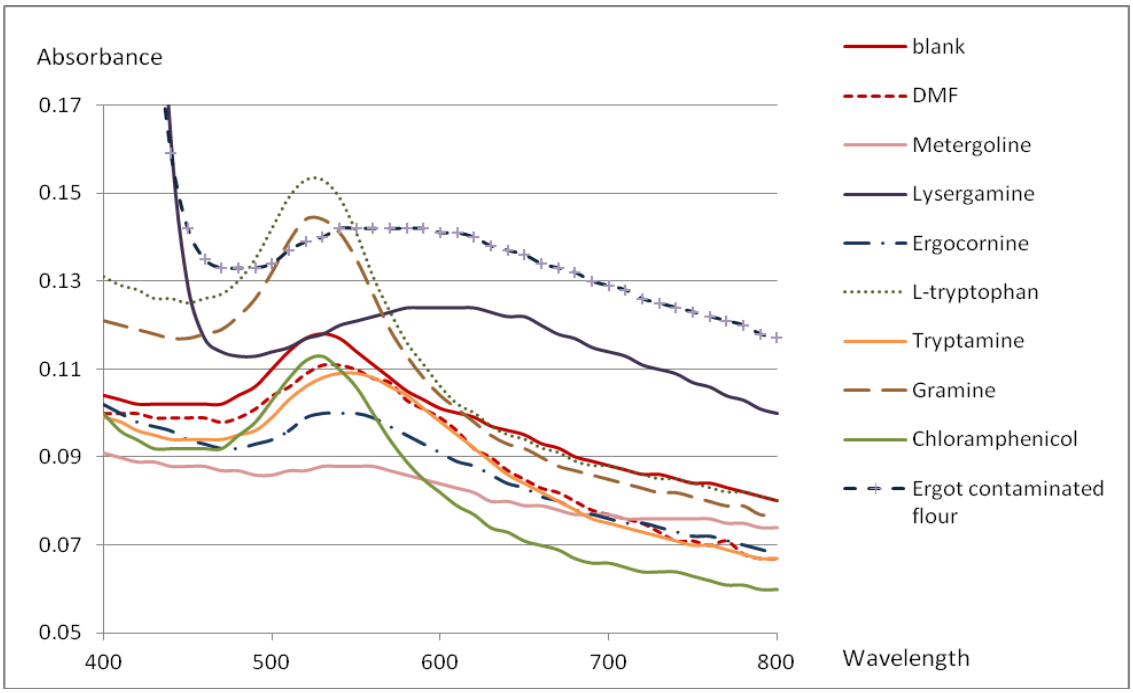
(1) Reference, no compound added **(2)** DMF **(3)** Metergoline **(4)** Lysergamine
(5) Ergocornine **(6)** L-Tryptophan **(7)** Tryptamine **(8)** Gramine **(9)** Chloramphenicol
(10) Ergot contaminated flour sample.



In **Figure 50** is presented the UV-Vis spectra of the different solutions of the samples 1 to 10. In this analysis, it was observed that the solution of gold nanoparticles linked to aptamer M3.2 (sample 1) had a maximum of absorption at 525 nm. The same absorption profile was also obtained for the samples 2, 5, 6, 7, 8 and 9; containing respectively: the solvent DMF, ergocornine, L-tryptophan, tryptamine, gramine and chloramphenicol. In the case of lysergamine (sample 4) and the extract of flour contaminated sample (sample 10), the

maximum of absorption was shifted to 600 nm. In the case of metergoline (sample 3), a fading of the absorption peak between 530 nm and 600 nm was observed. From this colorimetric assay, it was possible to conclude that aptamer M3.2 could generate a specific binding reaction in the presence of ergot alkaloids and that it was able to distinguish between the different ergot alkaloids tested. First, in the case of lysergamine, it was suggested that two molecules of lysergamine could saturate the two binding sites of aptamers M3.2, resulting in the formation of a purple precipitate and a shift of the UV-Vis absorption spectrum. In the case of metergoline, which is more voluminous than lysergamine, it was concluded that only one binding site of aptamer M3.2 was occupied, resulting in the formation of a precipitate with a much reduced color change. The difference of behaviour towards lysergamine and metergoline is highlighted in the Series II (**Figure 49**), in which a more concentrated solution of aptamers linked to gold nanoparticles was used for the colorimetric assay, enabling to evaluate with the naked eye the difference of color of the precipitates obtained with these two different molecules.

Figure 50. *UV-Vis absorbance spectra of samples 1 to 10 from Series I, ranging from 400 nm to 800 nm.*



As aptamer M3.2 was originally selected for metergoline, and not lysergamine, it is supposed that one of the two binding sites of aptamer M3.2 was disabled by the linkage to gold nanoparticles in the colorimetric assay and that only smaller molecules than metergoline could fit the second binding site. The sample of ergot contaminated flour extract (sample 10) gave a strong color change and shift in the absorption spectrum from 525 nm to 600 nm as well as lysergamine, even though this sample did not contain any molecule of lysergamine,

but a mixture of different natural ergot alkaloids (**Table 5**). As the sample of ergocornine (sample 5), did not lead to any change, it is supposed that the aptamer did not interact with such a compound in the real ergot sample, nor the other voluminous ergot alkaloids such as ergosine, ergotamine or ergokryptine (**Figure 48**). It was then concluded that aptamer M3.2 could interact with small natural ergot alkaloids such as ergometrine (**Figure 48**), having a structure and size close to the ones of lysergamine. The interfering compounds having an indole group; such as gramine, L-tryptophan or gramine; did not generate any change. It was then possible to deduce that aptamer M3.2 was specific to the ergoline structure, containing or not a methylation on position N1. Regarding the specificity, the team Rankin et al. [50] has shown that the specificity that aptamers could reach a very high level and reported an aptamer distinguishing molecules only differing in a methyl group. In our study, the methylation of the ergoline skeleton was not the discriminating factor, but rather the size of the ergot alkaloid tested. The specificity of this system of gold nanoparticles linked to aptamer M3.2 could be expressed as restricted to the molecules possessing an ergoline ring and smaller in size than metergoline.

Table 5. *Ergot composition of ergot contaminated flour sample (sample 10), determined by LC-MS [48, 49].*

Ergot alkaloid	Concentration (µg/kg)	Total epimers	% of ergot alkaloid content
Ergometrine	106	132	4.3
Ergometrinine	26		
Ergosine	1567	1567	51
Ergosinine	< LOQ		
Ergotamine	22	24	0.8
Ergotaminine	2		
Ergocornine	65	84	2.7
Ergocorninine	19		
Ergokryptine	1251	1259	41
Ergokryptinine	8		
Ergocristine	<LOQ	3	0.1
Ergocristinine	3		
Total	3068	3068	100

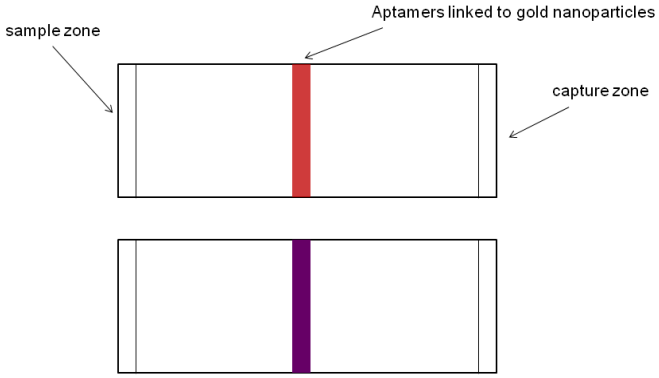
The solutions containing the complexes of gold nanoparticles linked to aptamers and ergot alkaloids were placed on a conjugation pad, and their mobility was tested with solutions containing different ratios of DMF in water (from 1:9 to 9:1). It was observed that the complexes were immobile in all the conditions tested.

4. Conclusion and future perspectives

Aptamer M3.2, having the lowest dissociation constant, was linked to gold nanoparticles in order to generate a specific biosensing technique for the detection of ergot alkaloids. This selected aptamer was tested with lysergamine, metergoline, ergocornine and an extract of ergot contaminated flour that contained six major ergot alkaloids and their epimers. The aptamer allowed the detection of the smaller alkaloid molecules (lysergamine and ergometrine) without false positives from similar indole compounds (tryptophan, gramine and tryptamine) [51]. It was also shown that the sensing system of aptamer and gold nanoparticles could produce a specific color change and precipitation with small ergot alkaloids in both buffers and flour sample extract. Aptamers are advantageous because they are very specific and also because of the easy and fast sample preparation. Aptamer-based test kits are not available on the market yet, but represent a viable option in a near future for the specific detection of ergot alkaloids. This aptamer-based sensing solution can be used as such for the detection of ergot alkaloids in real samples; although a more extensive study of a certain number of ergot contaminated flour samples is necessary in order to determine the applicability of this sensing solution and also to set the limits of detection and quantification of this system. Also, this system can be further developed into a dipstick test. Some preliminary tests were realized, but it was observed that the complexes did not migrate on a paper test. Various buffers were tested but the clusters of complexes were not mobile at all. So, it would be necessary to change the format of the dipstick; and maybe place the aptamers further on the conjugation pad for instance, in order to visualize clearly the band obtained in case of positive result. A scheme of the suggested format of the dipstick test is given in **Figure 51**.

The limitation in time of the laboratory work for this project and the fact that the elaboration of aptamer-modified gold nanoparticles is laborious and time-consuming did not allow the reformatting of the dipstick test. However, the aptamer-based sensing solution seems to be a very interesting option for the fast and simple detection of ergot alkaloids in flour samples. The immediate structural modification in presence of small ergot alkaloids and the absence of interference by other compounds shows the good applicability of the selected aptamers for ergot alkaloids for the construction of optical aptasensors.

Figure 51. *Suggested format of the dipstick test for ergot alkaloids based on DNA aptamers. On top: result obtained in the case of a negative result (red band); down: result obtained in the case of a positive result (purple band).*



Partly based on:

Rouah-Martin, E.; Mehta, J.; Van Dorst, B.; De Saeger, S.; Dubruel, P.; Maes, B.U.W.; Lemiere, F.; Goormaghtigh, E.; Daems, D.; Herrebout, W.; Van Hove, F.; Blust, R. and Robbens, J. Aptamer-Based Molecular Recognition of Lysergamine, Metergoline and Small Ergot Alkaloids. *International Journal of Molecular Sciences* **2012**, *13*, 17138-17159.

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Chapter VI: Extraction of ergot alkaloids from ergot contaminated rye feed with DNA aptamers

1. Introduction

Based on the specific recognition that can be obtained with aptamers, it was suggested that aptamers could also have a use for the extraction of ergot alkaloids from ergot contaminated cereal crop samples. The extraction of natural compounds is currently performed by using chemical techniques, such as liquid-liquid or solid-liquid extraction, pressurized-liquid extraction, sub- and supercritical extractions, and microwave- and ultrasound-assisted extractions [1]. However, the extraction of natural compounds is usually laborious despite the general improvement of these techniques and remains a challenging task [1]. As secondary metabolites usually only represent about 0.0001% or 1 ppm of the total biomass and are dispersed in the biological material, several purification steps are usually necessary to remove the unwanted matrix, which often implies important amounts of solvents [1-3]. Moreover, the separation of compounds having similar chemical structures can be problematic and often require elaborate and expensive analytical equipments [2]. Industrial extraction still remains a very polluting activity with a low cost-efficiency, generating important amounts of waste. The extracted compounds moreover have to be purified several times after extraction, due to the difficulty to avoid impurities in the final product with an extraction procedure only. Regarding ergot alkaloids, several chemical techniques, mainly using liquid-liquid extraction, were developed and are currently in use [4-7]. Also, a solid-phase extraction based on silicates allowing a better recovery of ergot alkaloids was proposed by Votruba and Flieger [8]. These techniques usually provide a good recovery of ergot alkaloids, but they are laborious and destructive for the sample; therefore they cannot be applied for food decontamination. Other techniques were recently explored. For instance, the team of Lenain and coworkers have elaborated a solid-phase extraction system for the removal of ergot alkaloids from food and feed based on molecularly imprinted polymers (MIPs) [9].

In this study, the use of aptamers as recognition elements of a solid phase extraction system for the extraction of ergot alkaloids was evaluated. This study had two main objectives: to define if ergot alkaloids from a real sample would bind to a given DNA aptamer and to determine if interfering compounds would bind to the DNA aptamers. This way, it was possible to investigate if aptamers were suited for the decontamination of food contaminants or in a more general way, for the specific extraction of compounds from complex matrices. As aptamers were mainly developed for sensing purposes, there are only scarce examples in the literature about the application of aptamers for extraction purposes, as this type of system was only developed for two compounds: ochratoxin A [10] and

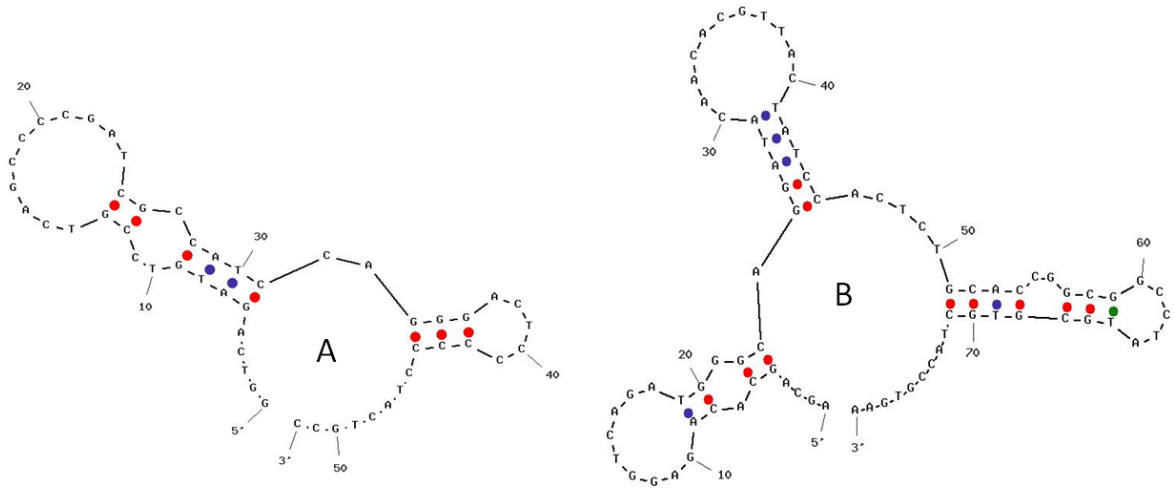
cocaine [11]. However, nucleic acids are widely used in the life sciences and diagnostics and the chemistry of nucleic acids is well-known, as nucleic acids are often used in solid phases for hybridizations assays. An important number of attachment methods for nucleic acids have been published, varying widely in chemical mechanism, ease of use and attachment stability [12-18]. For the immobilization of nucleic acids, different types of solid supports can be used, such as organic polymers or inorganic oxides for instance. Silica gel presents a high thermal, mechanical and chemical stability and enables chemical modifications through the silanol groups dispersed on its surface [19]. Moreover, the chemistry of silica is rather well-known. Finally, silica gel represents an ecological material with a low impact on the environment. The covalent coupling of organic molecules and biomolecules can be done by chemical pre-treatment of silica particles with organosilanes, such as dichlorodimethylsilane (DDS) [20, 21], 3-chloropropyltrimethoxysilane (CTS) [22, 23], or 3 aminopropyltriethoxysilane (APTS) [24]. In this study, it was chosen to immobilize DNA aptamers onto a silica gel and to use this system for the specific extraction of ergot alkaloids from a rye feed sample. An acidic water buffer was used for the extraction of ergot alkaloids and as eluent for the aptamer-based solid extraction system. In the previous application, the colorimetric reaction, DMF was used for the extraction of ergot alkaloids, which was then diluted in water. Here, it was chosen to work directly with a water buffer, as ergot alkaloids are soluble in acidic solutions. The content of the fractions eluted from DNA aptamers was analyzed by mass spectrometry, in order to identify the potential ergot alkaloids that could be eluted from the DNA aptamers, and to seek for the presence of interfering compounds, or contamination in the sample after elution.

2. Experimental Part: Extraction of ergot alkaloids with an aptamer-based solid phase extraction system

2.1. Materials and Methods

An aptamer, named aptamer M3.2, having a dissociation constant of 44 $nmol^2/L^2$, was previously selected for the ergoline group, and reported in our previous research article [25]. The original aptamer M3.2 consists of 80 bases, but in this experiment, only the fragment containing the predicted binding sites (**Figure 52**) was used for the functionalization of the silica gel. The following 3'-aminated version of the shortened aptamer M3.2 was used 5'-GGTCAGATGTCCGTCAGCCCCGATCGCCATCCAGGGACTCCCCCCTACTGCC-3'-NH_2. The full-length aptamer M3.2 can be seen on **Figure 34**. Another aptamer selected for ergot alkaloids, aptamer L5.5 having a dissociation constant of 0.66 µM, was also tested in this study. The 3'-aminated aptamer L5.5 was used, having the following sequence 5'-AGCAGCACAGAGGTCAGATGGGCAGGATACAACACGTTACTATCCACTCTGCACCGGCGGCCTATGCGTGCTACCGTGAA-3'-NH_2. The secondary structures of aptamers M3.2 and L5.5 were determined by using IDT Oligoanalyzer software [26], and are shown in **Figure 52**.

Figure 52. *Secondary structures of aptamers M3.2* ***(A)*** *and L5.5* ***(B)*** *determined by IDT oligoanalyzer software.*



2.1.1. Silica functionalization with aminated DNA

A narrow particle range silica gel of 10-20 µm (Merck, Germany) was used to prepare the aptamer-functionalized silica gel. For each sample, 300 mg of silica were placed in Eppendorf tubes.

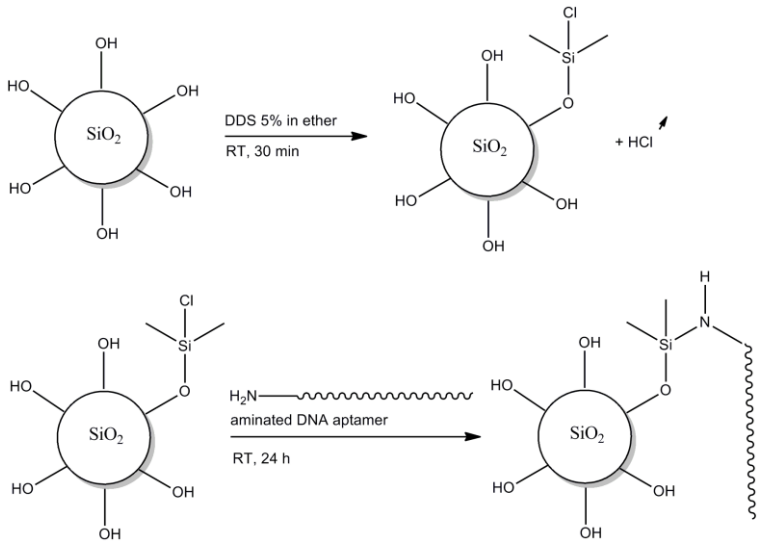
Sample 1 was kept as unmodified silica gel used as a reference. Sample 1 was washed two times with 500 µL of ultrapure MQ water and placed in 500 µL of MQ water.

The silica gels were activated by a treatment with DDS, in order to enable functionalization with aminated molecules [27]. Samples 2 and 3 were pre-treated with DDS, in order to be functionalized afterwards with the aminated version of two aptamers selected for ergot alkaloids (**Figure 53**). The silica gels of samples 2 and 3 were first washed with 200 µL of ether and placed in 500 µL of 5% ether solution of DDS (Sigma-Aldrich, Belgium) and the solutions were gently stirred for 30 min at room temperature (RT). Then, the solutions were poured in centrifugation columns and the solutions were centrifuged at 13,000 rpm during 1 min. The silica gels were washed two times with 300 µL of methanol and centrifuged in centrifugation columns as well. The silica was then washed three times with 300 µL of MQ water and placed in 300 µL of MQ water.

For the sample 2, 346 µg of modified 3'-aminated aptamer M3.2 (40 nmol) (Eurogentec, Belgium) was placed in 200 µL of MQ water. Sample 3 was prepared by placing 1.6 mg of 3'-aminated aptamer L5.5 (71 nmol) (Eurogentec, Belgium) in 200 µL of MQ water. The two solutions of 3'-NH_2 modified aptamers were mixed with the solutions of DDS treated silica, and the mixtures were gently stirred for 24 h at RT. The samples were then centrifuged at

13,000 rpm during 2 min. Then, the silica samples were washed three times with 500 µL of MQ water and finally placed in 500 µL of MQ water.

Figure 53. *Chemical representation of the functionalization of silica gel using dimethyldichlorosilane and aminated DNA aptamer.*



2.1.2. Sample preparation

A sample of ergot contaminated rye feed, having a known concentration of ergot alkaloids, was used for the extraction of ergot alkaloids. A sample of 0.5 g of the ergot contaminated flour sample was placed in 5 mL of 0.1 M HCl and gently stirred for 1 h at RT. Then, the solution was transferred to Eppendorf tubes, which were centrifuged at 13,000 rpm for 2 min at RT. The supernatant was taken and placed in clean vials.

2.1.3. Specific extraction of ergot alkaloids using the aptamer-functionalized silica gel

For each sample, 500 µL of rye feed extract were used. The reference sample was prepared by adding 500 µL of rye feed extract to the solution of unmodified silica gel. The sample 2 was prepared by mixing 500 µL of rye feed extract with the solution of aptamer M3.2 functionalized silica gel. Finally, sample 3 was prepared by mixing 500 µL of rye feed extract with the solution of aptamer L5.5 functionalized silica gel. The three solutions were gently mixed for 1 h at RT. Then, the supernatant was discarded after centrifugation in centrifugation tubes. For the elution step, the silica gels were recovered and placed in clean Eppendorf tubes with 500 µL 0.1 M HCl and were gently stirred at 70°C for 15 min. The silica gels were centrifuged in centrifugation tubes at 13,000 rpm for 1 min and the supernatants were kept for the mass spectrometry analysis.

2.1.4. LC-QTOF-MS analysis

Ergot alkaloids were separated and identified by liquid-chromatography quadrupole time-of-flight LC-QTOF-MS having a HPLC 1290 series coupled to a 6530 quadrupole-time-of-flight mass spectrometer (QTOF-MS) (Agilent Technologies). Chromatographic separation was achieved using a Zorbax Eclipse Plus C18 column (100 mm x 2.1 mm x 1.8 µm, Agilent Technologies) and following mobile phase composition: water 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Gradient elution was as follows: initial 5% B, linear change of B to 99%, from 0 to 5.0 min followed by isocratic elution at 99% B from 5.0 to 8.0 min, then linear change of B% to 5% until 8.1 min, then maintained at 5% B until 12.0 min in order to re-equilibrate the column. The flow rate was 0.3 mL/min and the injection volume 5 µL. Total run time was 12.0 min. The column was kept at a constant temperature of 45°C. The QTOF-MS was run in ESI positive mode scanning m/z from 50 to 1,000 amu at a scan rate of 3 spectra/s. An Agilent Jetstream source was used with the following parameters: gas temperature 275°C, gas flow 8 L/min, nebulizer pressure 40 psi, sheath gas temperature 325°C, sheath gas flow 11 L/min. Nozzle, capillary, fragmentor, and skimmer voltages were set to 0 V, 3500 V, 110 V, and 65 V, respectively. The instrument was calibrated during run times by monitoring positive ions with m/z 121.0508 and 922.0098. The MassHunter (Agilent Technologies) software was used for data acquisition and processing.

2.2. Results and Discussion

The ergot alkaloid content of the sample of ergot contaminated rye feed given by the LC-MS analysis performed by Diana Di Mavungu et al. [27] is shown in **Table 6**. The total ergot alkaloid concentration of this sample given by this analysis was 1081 µg/kg, representing a moderately high ergot contaminated sample. Ergotamine, ergosine, ergocornine and ergokryptine were the main ergot alkaloids of this sample according to this study.

Table 6. *LC-MS analysis of the ergot alkaloid content of the ergot contaminated rye sample [27].*

Ergot alkaloid	**Concentration (µg/kg)**
Ergometrine/inine	50
Ergosine/inine	416
Ergotamine/inine	337
Ergocornine/inine	126
Ergokryptine/inine	126
Ergocristine/inine	26
Total Ergot Content	1081

The ergot alkaloids eluted from the aptamer-functionalized silica gel were analyzed by LC-QTOF-MS analysis. LC-QTOF-MS was used because it provides high mass accuracy (typically 2 to 10 ppm error) and resolution (>20,000) which is necessary to generate a formula for an unknown compound with a given m/z value. Another advantage of the LC-QTOF-MS is its ability to fragment compounds providing high resolution mass spectra of the product ions to confirm the generated formula. The screening of the six main ergot alkaloids was based on a self-made csv (comma separated value) database. The accurate mass given with four digits was compared to the theoretical monoisotopic masses. The error was given in ppm.

In the LC-QTOF-MS analysis of the samples after extraction using the aptamer-functionalized silica gels, the following data were analyzed: the presence of the six main ergot alkaloids, the presence of the ergot alkaloid precursor fragment at m/z 223.1283, and finally the presence of other compounds than ergot alkaloids. The samples were analyzed in positive ion mode; and the hydrogen atom mass was subtracted in the final results. **Table 7** shows the compounds found in the three samples tested. In sample 1, resulting from the elution from non-functionalized silica gel, no ergot alkaloid could be found, neither the ergot alkaloid precursor fragment. No other compound was detected in this sample.

Table 7. *Analysis of the ergot alkaloid content after elution from silica gels by LC-QTOF-MS (positive mode).*

Sample	Ergot alkaloids found	Retention Time (min)	Theoretical monoisotopic mass	Determined monoisotopic mass	Score mass	Diff mass ppm	Fragment 223.1283 at RT
1 **Elution silica**	None						
2 **Elution aptamer M3.2**	Ergosine Ergokryptine	5.55 5.7	547.2794 575.3107	547.2874 575.3085	98.11 92.05	-1.88 3.88	Yes Yes
3 **Elution aptamer L5.5**	Ergosine Ergokryptine Ergocornine	5.59 5.8 5.669	547.2794 575.3107 561.2951	547.2797 575.3112 561.2916	99.88 99.71 81.13	-0.48 -0.72 6.2	Yes Yes Yes

In sample 2, resulting from the elution from aptamer M3.2 functionalized silica gel, two ergot alkaloids, were found: ergosine at m/z 547.2874 (theoretical m/z 547.2794) and

ergokryptine 575.3085 (theoretical m/z 575.3107). The fragment at m/z 223.1283 was found for both the ergot alkaloids. No other compound or impurity were found, or were at a very low level not detectable by this analysis. In sample 3, resulting from the elution from aptamer L5.5 functionalized silica gel, three ergot alkaloids were found: ergosine at m/z 547.2797 (theoretical m/z 547.2794), ergokryptine at m/z at 575.3112 (theoretical m/z 575.3107) and ergocornine at m/z 561.2916 (theoretical m/z 561.2951). The ergot alkaloid precursor fragment at m/z 223.1283 was found for the three ergot alkaloids found in the sample. No other compound or impurity was detected in this sample as well. These results show that the presence of aptamers in the silica gel allowed the specific extraction of ergot alkaloids from the sample. However, the other ergot alkaloids present in the sample such as ergotamine, ergometrine and ergocristine, were not retained by the aptamer-functionalized silica gels; or were present at a very low level not detectable by this analysis.

In comparison to the other recognition elements developed for ergot alkaloids, it was observed that aptamer are highly specific, restricted to a few related chemical compounds within the ergot family group. Similar specificity was obtained with antibodies [28, 29]. In comparison to the MIPs developed for ergot alkaloids, it was observed that the aptamer-based extraction was more specific [9, 30]. Depending on the use of the extraction system, it can be said that MIPs are suited for a general extraction of ergot alkaloids, while aptamers can be used for a more specific extraction of some ergot alkaloids. It is also possible to envisage a more complete extraction of ergot alkaloids from samples by coupling several different aptamers, having a different specificity, to silica gel. Aptamers offer the possibility to modulate the extraction process. As the original aptamer M3.2 fixed to gold nanoparticles could detect small ergot alkaloids, and that the same shortened aptamer fixed to silica gel could bind to voluminous ergot alkaloids, it can be envisaged to combine the original and the shortened aptamer in order to extract a broad range of ergot alkaloid structures. Concerning the extraction solution, only 0.1 M HCl was used; which can be easily quenched by 0.1 M basic solution. Other diluted acidic solutions could probably give the same result, however the limitation in time did not allow further investigation on the topic. Even though more samples would be needed for a more extensive study of this aptamer-based solid phase extraction, this study shows the applicability of aptamers for extraction purposes, offering a new range of possibilities for the specific extraction of toxins, natural compounds or pollutants from food or environmental matrices.

3. Conclusion

Aptamer-functionalized silica gels were realized with two of the aptamers selected for ergot alkaloids, in order to extract ergot alkaloids from rye feed. The elution contents from the aptamer-functionalized silica gels were measured by LC-QTOF-MS. It was shown that the one of the aptamer-functionalized silica gel could successfully extract three ergot alkaloids from a rye feed sample, namely ergosine, ergokryptine and ergocornine; while the system based

on the other aptamer could retain ergosine and ergokryptine. The other compounds present in the sample were not retained by such a system. The DNA aptamers were not denatured by the silica functionalization, nor by the acidic solution used for the extraction of ergot alkaloids. The specificity of aptamers for ergot alkaloids was therefore confirmed with this study. Then, a more quantitative analysis would be necessary in order to completely validate the applicability of aptamers for extraction purposes. Also, the reaction used for silica functionalization could be slightly changed to use less harsh conditions, as this reaction generate vaporous chlorhydric acid, which can be dangerous at a bigger scale. However, this study shows that it is possible to use aptamers for the specific extraction of compounds from turbid matrices; in addition to their use in sensing systems. Depending on the field of application, e.g. medical purposes or food decontamination, one or several aptamers having a different affinity towards ergot alkaloids can be grafted onto silica gel; enabling a total or a partial removal of ergot alkaloids from ergot contaminated samples. These results show that this type of system made of aptamer-grafted silica gel has a great potential for some applications in food clean-up, extraction of toxins or contaminants in various types of matrices, as well as the specific isolation of natural compounds from turbid matrices.

In the recent context of green and sustainable chemistry, the use of aptamers can solve issues encountered with chemical extractions. The overall environmental impact of an industrial extraction cycle is not easy to estimate; however it is known that it requires at least the 50% of the energy of the whole industrial process [31]. Then, in spite of the high energy consumption and the large amount of solvents required, the yield is most of the times indicated in decimals [31]. A general definition of green chemistry is the invention, design and application of chemical products and processes to reduce or to eliminate the use and generation of hazardous substances. In relation to green extraction of natural products, this definition can be modified as follows: “Green Extraction is based on the discovery and design of extraction processes which will reduce energy consumption, allows use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product” [31]. The listing of the six principles of green extraction gives an overview of the essential points to be observed by scientists and industries regarding the implementation of new extraction procedures [31]:

Principle 1: Innovation by selection of varieties and use of renewable plant resources.
Principle 2: Use of alternative solvents and principally water or agro-solvents.
Principle 3: Reduce energy consumption by energy recovery and using innovative technologies.
Principle 4: Production of co-products instead of waste to include the bio- and agro-refining industry.
Principle 5: Reduce unit operations and favour safe, robust and controlled processes.
Principle 6: Aim for a non denatured and biodegradable extract without contaminants.

Recent trends in extraction techniques have largely focused on finding solutions that minimize the use of solvents, or replacing them with water solutions or alternative solvents such as supercritical fluids and ionic liquids [32, 33]. The use of aptamers can help to drastically reduce the amounts of solvents required, as specific extraction of the compounds of interest can be obtained in a one-step procedure mainly involving water, instead of a series of extractions steps with solvents; which fulfills the Principle 2 and 5. Moreover, the high specificity of aptamers allows the obtention of a high quality extract, with no contamination by other compounds, even if they have a closely related chemical structure; in accordance with the Principles 3 and 6. In case that this process would be applied to food decontamination at the same time as ergot alkaloid extraction for pharmaceutical purposes, this process would also satisfy the Principle 4 of the green extraction chart, regarding the production of co-products instead of waste. Therefore, the use of DNA aptamers for extraction purposes appears to be a viable option, which entirely follows the principles of green and sustainable chemistry.

Partly based on:

Rouah-Martin, E.; Maho, W.; Mehta, J.; De Saeger, S.; Covaci, A.; Van Dorst, B.; Blust, R. and Robbens, J. Aptamer-based extraction of ergot alkaloids from ergot contaminated rye feed. *Advances in Bioscience and Biotechnology* **2014**, 5, 692-698.

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Conclusion

1. General conclusion about the ergot project

The aim of this study was to develop a fast and specific biosensor for ergot alkaloids. For that it was first necessary to choose which ergot alkaloid to work on, as the family of ergot alkaloids comprises more than 40 molecules. In the **Introduction**, the different species producing ergot alkaloids and the devastating effects of ergot contamination were presented, the sanitary risks for humans and animals were documented, as well as the safety measures that were implemented in several countries. It was shown in **Chapter II** that the ergot alkaloids group was very complex and could be separated in three subcategories: lysergic acid and its simple amides, clavines and peptide ergot alkaloids (ergopeptines and ergopeptames). The ergot alkaloids pattern in each case can vary enormously, depending on the type of fungus, type of plant parasited, climate and environmental conditions (temperature, humidity, light); and harvesting time. Thus, the choice of the target molecule was a critical step in this study. Moreover, some ergot alkaloids can be difficult to obtain, due to regulations, price and amounts available. Indeed, some ergopeptines are only available in minute amounts such as micrograms, for an expensive price. Some other ergot alkaloids are prohibited, such as lysergic acid and some of its simple amides, and thus totally unavailable in European countries even for research purposes. Finally, only a few ergot alkaloids, such as metergoline or ergocornine, could be provided in reasonable amounts. Ergocornine is a rather stable molecule, and lacks chemical reactivity; however, ergocornine was used to functionalize magnetic beads for the selection of aptamers. Then, metergoline was also studied. The chemical structure of metergoline differs slightly from the other ergot alkaloids, but it contains an ergoline structure; so it could still be used as an ergot alkaloid template. Metergoline was then used as such, and also it was also cleaved into a smaller molecule, lysergamine, which is very close in structure to lysergic acid; and has a reactive primary amine which could be used for chemical reactions. The selection of aptamers for lysergamine and metergoline was presented in **Chapter III**. Lysergamine and metergoline were grafted onto NHS-functionalized magnetic beads. The ssDNA library was incubated with the ergot alkaloid coated magnetic beads. The partition between the ssDNA sequences binding to the ergot alkaloid coated magnetic beads was performed by applying a magnet, attracting the magnetic beads and the ssDNA sequences bound, and pipetting off the supernatant containing the unbound fraction of the ssDNA library used. The magnetic beads were washed and the binding sequences were eluted, amplified by PCR and reused for another cycle of selection after strand separation. Starting from the second cycle of selection, negative selections were applied by using the ethanolamine coated magnetic beads. This way, it was possible to remove the ssDNA sequences that were not specific to lysergamine or metergoline. Starting from the second cycle of selection, the recovered ssDNA sequences were sequenced and analyzed. A PCR was performed after each round of

selection with non-biotinylated primers. The purified PCR products were inserted into vectors and transformed into *Escherichia coli*, in order to obtain sufficient amounts of copies of each selected aptamer. After, the sequencing of the selected aptamers, it was found that more than 80% of the sequences had a common motif of C bases, which was suggested to play a role in the binding to lysergamine and metergoline. The analysis of the complexes of the selected aptamers and lysergamine was done by using SPR technique (**Chapter IV**), in which a streptavidin SPR chip was coated with lysergamine, linked to a biotinylated linker, and the selected aptamers were passed through the surface. It was possible to monitor in real-time the formation and dissociation of complexes and the data were used to determine the binding parameters of the complexes, such as dissociation constants and cooperativity. The best binding aptamer, having a dissociation constant in the nanomolar range (44 nM) was kept for further work in this study. The SELEX procedure applied could lead to the obtention of aptamers with high affinity towards ergot alkaloids, which is comparable to the one of antibodies towards their targets. In **Chapter V** was shown one of the applications which can be based on this aptamer: the construction of an optical aptasensor. In order to develop a colorimetric sensing test for ergot alkaloids, one of the selected aptamers was linked to gold nanoparticles in order to produce a color reaction in presence of ergot alkaloids, due to a conformational change of the edifices made by aptamers and gold nanoparticles. It was observed that a color change from red to dark purple occurred in the presence of lysergamine, with a precipitation of the complexes. The addition of metergoline resulted in a precipitation of the complexes, without color change. The addition of ergocornine, a more voluminous ergot alkaloid, did not generate any change to the aptamer sensing solution. Compounds having an indole moiety were tested in order to determine if they could possibly interfere with ergot alkaloids, as the indole 2-member ring structure is included in the 4-member ergoline ring. Then, indole-containing molecules which could be possibly found in food matrices, such as gramine, L-tryptophan and tryptamine, were added to the solution of aptamers linked to gold nanoparticles; and no change occurred. This result shows the high specificity that can be obtained with aptamers, which can discriminate between similar chemical structures. A sample of ergot contaminated flour from EFSA was tested with this system, resulting in an immediate color change with precipitation of the complexes. It was suggested that only one small ergot alkaloid contained in the sample, ergometrine, could bind to the aptamer linked to gold nanoparticles. These results confirm the exceptional features of aptamers as recognition elements for the construction of sensors: high specificity and absence of reaction with the matrix or possible interfering compounds, and good thermal and chemical stability. Indeed, during their selection and amplification, aptamers were heated up to 94°C and regained their hairpin structure without degradation when cooling down. Also, aptamers could be used in acidic buffers and mixtures of small amounts of solvents such as DMF mixed with water for instance. The robustness of aptamers offers a great range of biological applications. Therefore, it was concluded that aptamers were interesting tools for the construction of sensors and assays. Then, in order to

further test the specificity of aptamers and to further extend the field of application of aptamers, solid phase extraction systems were constructed based on two of the selected aptamers; which was presented in the **Chapter VI**. It was shown that aptamer-based solid phase extraction systems could successfully extract ergot alkaloids very specifically. The system based on shortened aptamer M3.2 could specifically extract ergosine and ergokryptine; while the system based on aptamer L5.5 could specifically extract ergosine, ergokryptine and ergocornine, without contaminants. Although, the amount of ergot alkaloids recovered was not quantified with this analysis; these results showed that aptamers were also suited for the specific extraction of compounds from complex matrices, without any interference with other compounds from a complex matrix. The chemical stability of DNA aptamers and absence of degradation in chemical, environmental or biological media also render them a very good option for fast and specific extraction tools.

However, some improvements could be brought to the general selection procedure in order to increase its efficiency and to reduce the time necessary for its completion; as the manual SELEX procedure was laborious and time-consuming. Some techniques using sophisticated equipment for the separation of aptamer-target complexes were recently developed to generally improve the SELEX procedure. For instance, automated systems can be used for the selection procedure in order to reduce the time needed for the selection [1-4]. Automation also allows the reduction of variability in the selection process, leading to a better reproducibility of the protocols. Then, other modifications can be brought in order to improve the affinity of the selected aptamers. For instance, capillary electrophoresis can be applied in order to improve the separation process between sequences bound to the target from the rest of the library used [1, 5, 6]. CE-SELEX utilizes electrophoresis to separate binding sequences from inactive sequences by a mobility shift to allow separation. Nucleic acids that are bound to the target migrate with a different mobility from the unbound sequences, and then the selected nucleic acids are collected afterwards. Bound nucleic acids are then amplified and purified off-chip for multiple rounds of selection, similar to the protocol in a conventional SELEX. Capillary-electrophoresis SELEX (CE-SELEX) is capable of isolating high-affinity aptamers in as little as 2-4 rounds of selection, and some research groups have been able to identify aptamers with low nanomolar dissociation constants by using CE-SELEX [7]. However, CE-SELEX is limited in terms of its properties and target size. Especially, low-molecular weight targets such as small organic compounds are not suitable in CE-SELEX because the migration velocity of oligonucleotide-target complexes is not effectively discriminated with one of the unbound oligonucleotides [8]. So, the use of CE-SELEX for ergot alkaloids may have been critical for this reason. However, another technique could have been used, named atomic force microscopy SELEX (AFM-SELEX), in order to produce aptamers with a very high affinity, with dissociation constants in the picomolar range [9, 10]. Atomic force microscopy (AFM) detects the adhesion or affinity force between a sample surface and cantilever, dynamically. This feature is useful as a method for the selection of aptamers that bind to their targets with very high affinity. Also, this technique

allows the obtention of DNA aptamers within fewer rounds of selection, as compared with a conventional SELEX strategy. Considered together, it can be concluded that AFM-SELEX strategy could have been a viable option for the screening of DNA aptamers with high affinity for ergot alkaloids.

2. Future perspectives and other possible applications of aptamers selected for ergot alkaloids

The sensing solution based on DNA aptamers can be used in a dipstick test. A format slightly different from the existing formats would be necessary though, as the complexes of aptamers and ergot alkaloids were not mobile and did not migrate at all on the conjugation pad. So, the complexes of aptamers and ergot alkaloids were remaining at the place where the sample was loaded; making the result not visible enough, as the sample can be very colored itself. However, it is feasible to place the aptamer line further on the dipstick test, in order to obtain a purple line in the middle of the conjugation pad in case of positive result; or a red line at the end of the conjugation pad in case of negative result. The aptamer selected for lysergamine can be also used with several other transduction platforms for the construction of various aptasensors. The high specificity of the selected aptamer towards the ergoline skeleton and the conformational change occurring upon binding is well-adapted for optical sensors using labels or techniques such as surface plasmon resonance. The realization of mass-based sensors is also a possibility. Finally, the production of an electrochemical sensor using potentiometry is under study (team of Luc Nagels, University of Antwerp, Belgium).

Other types of applications can be done using the selected aptamers. For instance, it was shown that it was possible to produce a solid-phase extraction system based on aptamers, in order to specifically extract ergot alkaloids from rye. Further investigation is required to test the efficiency of this system, and to know the recovery of ergot alkaloids. However, it could be concluded that DNA aptamers could also be incorporated in extraction or purification systems in order to specifically remove pollutants, contaminants or other molecules of interest. For instance, natural compounds can be simply isolated from crude extracts in a one-step procedure, avoiding expensive and laborious chemical extraction processes.

3. Concluding remarks

For the detection of contaminants, analytical methods are currently used. They are sensitive and selective, but time-consuming; and days to weeks are usually needed to get a result [11]. The increasing public concern over environment safety has led to a search for technologies capable of rapidly identifying contamination problems at source. For such reasons, biosensors are becoming increasingly important in the environmental monitoring of toxins and pathogens [12]. Also, point-of-care-testing (POCT), or diagnostic testing that is

done on site, is a field in which biosensors could have a major impact in the future; allowing the patient and medical personnel to get results rapidly and easily [13]. POCT allows for faster diagnosis and has the potential to significantly reduce costs [13-15]. For the production of sensing devices and assays, biological ligands usually offer the advantage of specificity compared to chemical ligands. Among the different biological molecules; peptides, nucleic acid, sugars and fatty acids; mainly proteinic structures have a role of recognition in living organisms. For such reasons, the introduction of biological tools to sensors started with proteins, such as enzymes or antibodies. Immunosensors, allow a very specific identification of a particular compound; while enzyme-based biosensors usually allow the detection of broad families of pollutants. Thus, enzyme-based biosensors can offer a general toxicity "index" [16, 17]. However, one disadvantage of these two recognition elements is that proteins are not stable edifices and are prone to degradation; as they are permanently synthesized and degraded in living organisms. Also, the production of proteins is complex and can require the use of living organisms for their production, which poses ethical issues as well. Therefore, novel recognition elements such as DNA and peptide aptamers, and MIPs, are expected to complete, and sometimes replace the use of proteins in sensing systems in the future [18]. The increased stability of these recognition elements extends the lifetime of the biosensors and reduces the transportation cost by avoiding the need of cooled transport [18]. Among these novel recognition elements, MIPs are the most stable and robust structures. MIPs constitute powerful extraction tools, and also a promising alternative for sensing purposes. However, the affinity accomplished by these synthetic receptors is usually several orders of magnitude below that of the antibodies; and they can sometimes lack specificity as well. In comparison, peptide and nucleic acid aptamers were shown to be more specific than MIPs and seem to be very well-suited for the construction of sensing systems. It was observed that aptamers could demonstrate an affinity and specificity similar to those of monoclonal antibodies; and that sometimes even more sensitive assays could be constructed when aptamers were used instead of antibodies [19, 20]. Aptamers have some advantages over antibodies, such as easy chemical synthesis, easy modification, and robustness, thus, aptamers are considered as a valid alternative to antibodies in sensing systems [21, 22]. Peptide aptamers started to be developed recently and are less frequently used than nucleic acid aptamers, however they are also expected to gain popularity in a recent future. Peptide aptamers are not currently selected via the same process as nucleic acids, but they can be obtained by using techniques such as phage display or ribosomal display. The diversity of chemical structures of peptides is broad, as the 20 common peptides are usually found in living organisms, which have very different chemical structures. Moreover some organisms can produce their own peptides, enlarging the diversity of chemical structures. Due to their exceptional features, it is expected that nucleic acid and peptide aptamers will increasingly be used in sensors. The potential growth of the market is mainly attributed to future diagnostics, drug discovery and research applications. However, there are some few limitations and challenges to overcome for the complete

implementation of aptamers in sensing devices. The main obstacle to aptamer use in diagnostics is related to the lack of standardized protocols [19]; and only few systems already overcame the lab status [23]. In addition, the aptamer technology has to be extensively tested in a clinical sample matrix to establish reliability and accuracy [24]. Another challenge for the development of biosensors in POC diagnostics is to develop a fully integrated biochemical analysis system that is capable of performing all procedures on a single platform [25]. Finally, aptamers can be sensitive to nucleases; which sometimes presents a problem on diagnostic assays based on the assay of disease markers in biological fluids [26]. This problem can be partly overcome though; as reports have been published detailing approaches to make oligonucleotide sequences resistant to nucleases by modifying the oligonucleotide backbone using modified bases for nuclease resistance [26]. However, we did not encounter any problem regarding nucleases in our study with biological samples from cereals crops. An important research effort is now devoted to the improvement of aptamer technology and the obtention of marketable products [8, 27, 28]. Aptamers is an emerging market, which is growing rapidly in various application areas, including therapeutics and diagnostics. The global aptamers market was valued at $287 million in 2013 and is expected to reach $2.1 billion by 2018 [29]. Also, a new horizon is opening up towards the isolation of specific compounds with aptamers, which can be applied in extraction processes in the context of green and sustainable chemistry; an area of growing importance nowadays. So, beside the fact that aptamers are the most efficient detection tools available nowadays for diagnostic tests and biosensors; we believe that they can also bring major improvements in extraction purposes, and render possible processes that fully fulfill the conditions of green and sustainable chemistry.

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Summary of the project

Ergot alkaloids are toxins produced by fungi of the genus *Claviceps*. These toxins can be found in food items deriving from cereal crops, as the fungi of *Claviceps* genus mainly contaminate cereal crops and grasses. The contamination of food by ergot alkaloids can cause a severe disease called ergotism, which can be lethal. Therefore, the detection of ergot alkaloids in food and feed is crucial to food safety. Complementary to analytical techniques (mass spectrometry, HPLC), which are currently used for screening food samples, this project aimed to produce a biosensor, or fast sensing technique allowing a first estimation of the presence of ergot alkaloids in food samples. The main focus of the study was to select single-stranded DNA aptamers specific to template ergot alkaloids, and to incorporate them to a transducing technique in order to produce a signal in presence of ergot alkaloids. In a general manner, biological recognition elements represent interesting tools because of their complex structures, which can be very specific to a compound or a family of compounds. Among the different biological recognition elements (cells, proteins, sugars, nucleic acids), ssDNA was chosen because of its interesting features. First, it offers a wide diversity of shapes thanks to the unique tridimensional structure of each sequence, allowing a good specificity and sensitivity towards a given target. Moreover DNA is a relatively stable molecule, which can easily be functionalized. Finally, the synthetic production of DNA is animal-friendly, can be done in large scales and is relatively cheap. In this study, ssDNA aptamers were generated from a library by following an iterative procedure called SELEX (Systematic Evolution of Ligands by EXponential enrichment), consisting in incubating the ssDNA library with the ergot alkaloids and separating the aptamers bound to the toxins from the rest of the library. The resulting aptamers were eluted with a DNA chelating buffer and were amplified by polymerase chain reaction (PCR). After strand separation, the ssDNA aptamers were used as a new pool for another cycle of selection, resulting in a competition between the binding elements. After each cycle of selection, the aptamers were cloned in order to be sequenced and their affinity for ergot alkaloids was determined by using surface plasmon resonance (SPR). Aptamers having dissociation constants in nanomolar range were obtained. It was also observed that an important number of the aptamers selected for ergot alkaloids had a common sequence of several C bases, suggested to play a role in the binding to ergot alkaloids. The aptamer having the lowest dissociation constant of 44 nM was used in a colorimetric assay with gold nanoparticles. Using this system, a specific color reaction from red to purple, and the precipitation of complexes were observed in presence of small ergot alkaloids. It was shown that compounds which are different from ergot alkaloids, but which have some common structural parts, did not interfere with the aptamer-gold nanoparticles sensing system. A sample of ergot contaminated flour provided by the EFSA was tested using this system, and gave a positive result, comparable to the one obtained with lysergamine in buffer. These very promising results show the applicability of ssDNA aptamers for the construction of

biosensors. The aptamer-based sensing solution can be used as such or can be incorporated in a sensing paper-test, or dipstick, for the fast detection of ergot alkaloids in food and feed matrices for instance. Finally, this selected ssDNA aptamer can be used in different sensing formats, such as electrochemical devices for instance, in order to construct more quantitative sensing tools. A complementary analysis was done by implementing aptamers in a solid-phase extraction system. It was shown that aptamers could specifically extract ergot alkaloids from ergot contaminated rye, and that no other compound was retained by such a system. Moreover, the aptamers were not degraded by the acidic medium used for the solubilisation of ergot alkaloids. It could be concluded that aptamers were also suited for the removal or isolation of specific compounds from complex matrices.

Samenvatting van het project

Ergotalkaloïden zijn toxische stoffen geproduceerd door schimmels van het genus *Claviceps.* Deze toxines kunnen voorkomen in voedingsmiddelen afkomstig van granen, gezien de schimmels van het genus *Claviceps* voornamelijk granen en grassen contamineren. De besmetting van voedsel door ergotalkaloïden kan leiden tot een ernstige, mogelijks dodelijke ziekte, genaamd ergotisme. De detectie van ergotalkaloïden in levensmiddelen en diervoeders is daarom van cruciaal belang voor de voedselveiligheid. Complementair aan analytische technieken (massaspectrometrie, HPLC), die momenteel worden gebruikt voor het screenen van voedselstalen, is in dit project een biosensor of snel detectiesysteem ontwikkeld om een eerste schatting van de aanwezige ergotalkaloïden in levensmiddelen te kunnen maken.De belangrijkste focus van de studie was om enkelstreng DNA aptameren te selecteren specifiek om ergotalkaloïden te binden en deze aptameren te incorporeren in een transducerend platform dat een signaal produceert in aanwezigheid van ergotalkaloïden. In het algemeen zijn biologische herkenningselementen interessante molecules vanwege hun complexe structuur, die heel specifiek een verbinding of groep van verbindingen kunnen herkennen. Van de verschillende biologische herkenningselementen (cellen, eiwitten, suikers, nucleïnezuren), werd ssDNA gekozen vanwege zijn interessante eigenschappen. Ten eerste biedt het een grote diversiteit aan conformaties dankzij de unieke driedimensionale structuur van elke sequentie wat een goede specificiteit en gevoeligheid heeft voor een bepaalde doelmolecule.Bovendien is DNA een relatief stabiele molecule, die gemakkelijk gefunctionaliseerd kan worden. Tot slot is de synthetische productie van DNA aptameren diervriendelijk, mogelijk op grote schaal en relatief goedkoop.In deze studie werden ssDNA aptameren uit een bibliotheek geselecteerd door het volgen van een iteratieve procedure, genaamd SELEX (Systematic Evolution of Ligands by EXponential enrichment), bestaande uit het incuberen van een ssDNA bibliotheek met de ergotalkaloïden en het scheiden van de aptameren gebonden aan het toxine van de rest van de bibliotheek. De aptameren werden geëlueerd met een DNA bindende buffer en geamplificeerd via PCR (Polymerase Chain Reaction). Na het scheiden van de DNA strengen werden de ssDNA aptameren gebruikt als nieuwe pool voor een verdere selectiecyclus, resulterend in een competitie tussen de aptameerbindingselementen. Na elke selectiecyclus werden de aptameren gekloneerd voor DNA sequentie en werd hun affiniteit voor ergotalkaloïden bepaald door SPR (Surface Plasmon Resonance). Er werden aptameren verkregen met een dissociatieconstante in de micro- en nanomolaire grootteorde. Er werd opgemerkt dat bijna alle voor ergotalkaloïden geselecteerde aptameren een gemeenschappelijke sequentie hadden van verschillende C-basen, waaruit kan verondersteld worden dat deze een rol speelt in de binding aan ergotalkaloïden. Het aptameer met de beste dissociatieconstante (44 nM) werd gebruikt in een colorimetrische test met goud nanopartikels. Met dit systeem werd een specifieke kleurreactie van rood naar paars en de vorming van precipitaten van complexen waargenomen in aanwezigheid van kleine ergotalkaloïden. Er werd aangetoond dat

molecules die verschillend zijn van ergotalkaloïden, maar toch een gelijkaardige structuur hebben, niet interfereren met het goud-aptameer nanopartikel detectiesysteem. Een staal van ergotalkaloïde verontreinigde bloem - ter beschikking gesteld door EFSA (European Food Safety Authority) - werd getest met behulp van dit systeem, en gaf een positief resultaat, vergelijkbaar met de resultaten verkregen met ergotalkaloïden in buffer. Deze veelbelovende resultaten illustreren de toepasbaarheid van ssDNA aptameren voor de bouw van biosensoren. De colorimetrische reactie kan worden gebruikt in een dipstick, voor de snelle detectie van ergotalkaloïden in levensmiddelen en diervoeders. Tenslotte kan het geselecteerde ssDNA aptameer in verschillende sensorformats worden gebruikt, zoals bijvoorbeeld elektrochemische sensoren, om een kwantitatief detectieinstrument te ontwikkelen.

Een staal van ergotalkaloïde verontreinigd meel - door het EFSA aangeleverd- werd getest met dit systeem, en leverde een positief resultaat op, vergelijkbaar met de data zoals verkregen voor lysergamine in buffer. Deze veelbelovende resultaten tonen de toepasbaarheid van ssDNA aptameren voor de constructie van biosensoren. De aptameergebaseerde sensingoplossing kan als zodanig worden gebruikt of in een dipstick worden geïntegreerd voor de snelle detectie van ergot alkaloïden bijvoorbeeld in matrices van levensmiddelen en diervoeders. Tenslotte kan het geselecteerde ssDNA aptameer worden gebruikt in verschillende sensing formats, zoals elektrochemische instrumenten om een meer kwantitatieve detectie te kunnen uitvoeren.

Een aanvullende analyse werd uitgevoerd door het implementeren van aptameren in een vaste-fase-extractie systeem. Er werd aangetoond dat aptameren specifiek ergotalkaloïden van gecontamineerde rogge kunnen extraheren op een specifieke manier, en dat geen andere verbindingen werden weerhouden in een dergelijk systeem. Bovendien waren de aptameren niet gedegradeerd in het zure medium dat nodig is voor het oplossen van ergotalkaloïden. Daarom kan geconcludeerd worden dat aptameren ook geschikt zijn voor de verwijdering of isolatie van specifieke verbindingen uit complexe matrices.

Curriculum vitae

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Addendum

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Int. J. Mol. Sci. **2012**, *13*, 17138-17159; doi:10.3390/ijms131217138

OPEN ACCESS
International Journal of
Molecular Sciences
ISSN 1422-0067
www.mdpi.com/journal/ijms

Article

Aptamer-Based Molecular Recognition of Lysergamine, Metergoline and Small Ergot Alkaloids

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Received: 18 September 2012; in revised form: 26 November 2012 / Accepted: 5 December 2012 / Published: 14 December 2012

Abstract: Ergot alkaloids are mycotoxins produced by fungi of the genus *Claviceps*, which infect cereal crops and grasses. The uptake of ergot alkaloid contaminated cereal products can be lethal to humans and animals. For food safety assessment, analytical techniques are

currently used to determine the presence of ergot alkaloids in food and feed samples. However, the number of samples which can be analyzed is limited, due to the cost of the equipment and the need for skilled personnel. In order to compensate for the lack of rapid tests for the detection of ergot alkaloids, the aim of this study was to develop a specific recognition element for ergot alkaloids, which could be further applied to produce a colorimetric reaction in the presence of these toxins. As recognition elements, single-stranded DNA ligands were selected by using an iterative selection procedure named SELEX, *i.e.*, Systematic Evolution of Ligands by EXponential enrichment. After several selection cycles, the resulting aptamers were cloned and sequenced. A surface plasmon resonance analysis enabled determination of the dissociation constants of the complexes of aptamers and lysergamine. Dissociation constants in the nanomolar range were obtained with three selected aptamers. One of the selected aptamers, having a dissociation constant of 44 nM, was linked to gold nanoparticles and it was possible to produce a colorimetric reaction in the presence of lysergamine. This system could also be applied to small ergot alkaloids in an ergot contaminated flour sample.

Keywords: single-stranded nucleic acid; ergot alkaloids; SELEX procedure; surface plasmon resonance

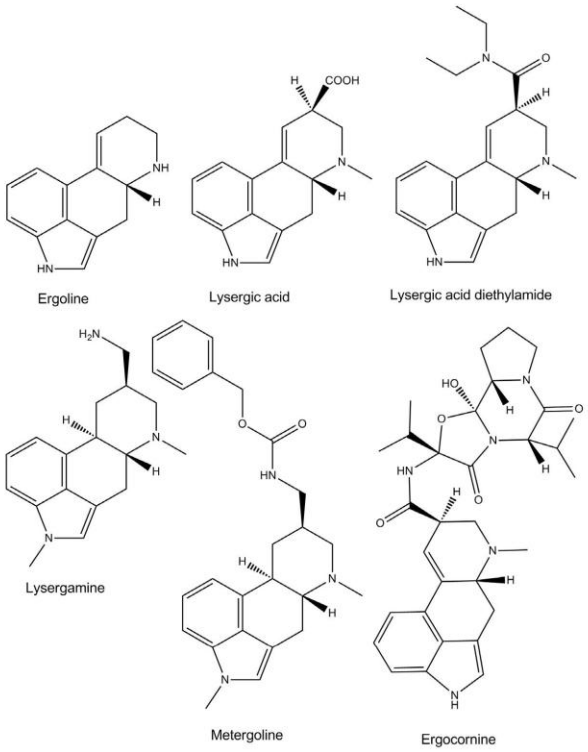
1. Introduction

Ergot alkaloids represent a family of more than 40 highly biologically active molecules which can have poisoning effects [1]. Although some ergot alkaloids and semi-synthetic derivatives have interesting medical properties and are used for medical purposes, most of them are toxic and their absorption can be lethal to humans and animals [2–4]. Ergot alkaloids have a common four-membered ring called ergoline, which interacts with adrenergic, serotinergic and dopaminergic receptors [5]. The ergoline skeleton is substituted on position C8, which supports another more or less elaborated chemical group, which is characteristic for each of the different molecules of the family (Figure 1). Ergot contaminated food samples contain a mixture of ergot alkaloids, in varying proportions. The presence of ergot alkaloids in food and feed samples is currently determined by liquid chromatography-mass spectrometry analysis [6]. However, the number of samples which can be analyzed is restricted due to the cost of the equipment and the need for skilled personnel. Therefore, the aim of this study was to offer a simple and reliable detection method for ergot alkaloids in food and environmental samples.

As recognition elements of sensors, aptamers have emerged in the last two decades and have shown an ability to provide reliable sensing tools, and have increasingly gained interest thanks to their advantageous features [7]. Aptamers are artificial ligands, made of nucleic acids or peptides, which can specifically bind to a target molecule or a family of structurally related compounds. The range of possible targets is extremely wide, going from small organic molecules to entire cells or bacteria [8–11]. The elaborate and unique tridimensional structure of aptamers allows them to specifically recognize their targets with a high sensitivity; they can sometimes distinguish between

molecules very close in structure to the target molecule. The synthetic production of aptamers then allows the production of billions of different nucleic acid sequences on a large scale and fulfills the research requirements for affinity molecules. Another important aspect of the synthetic production of aptamers is animal-friendliness, in comparison to antibody production, which usually requires extraction from animals. Therefore, aptamers can be considered as innovative recognition tools, offering an ethical and cost-effective alternative to antibodies. Finally, nucleic acid aptamers are stable molecules and can easily be functionalized, allowing their immobilization onto different supports in order to be included as recognition elements in sensors.

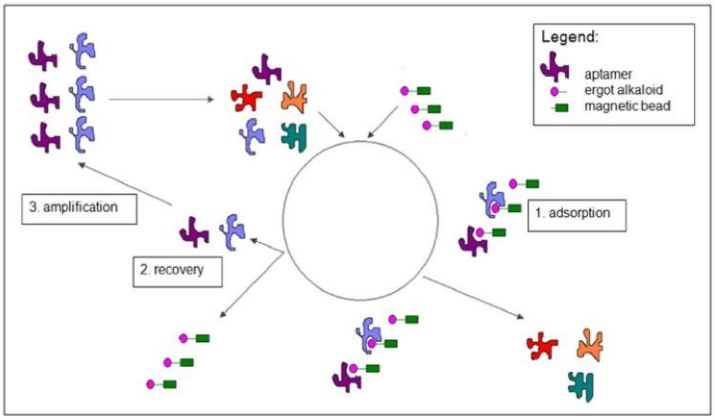
Figure 1. Chemical structures of ergoline and some natural and semi-synthetic ergot alkaloids.



Aptamers are obtained by following an iterative selection procedure called SELEX, *i.e.*, Systematic evolution of ligands by EXponential enrichment, which consists of incubation cycles of an aptamer library with the target molecule and recovery of the fraction of the library bound to the target molecule. In this iterative selection procedure, the pool is enriched in binding material, as the unbound fraction of the library used is discarded and the fraction bound to the target molecule is amplified by a polymerase chain reaction (PCR) and then used for other selection cycles [12–15]. The complexes of aptamers and target molecules can be characterized by surface plasmon resonance (SPR), enabling comparison of the binding of the selected aptamers [16]. Further development of the sensors can be carried out by optimizing the binding aptamers.

As ergot alkaloids are a wide group of molecules, it was firstly necessary to decide whether to select an aptamer recognizing specifically one ergot alkaloid, or a more general aptamer able to detect several ergot alkaloids. Considering that the composition of ergot alkaloids can vary enormously from one sample to another, it was decided to work on the chemical part which is common to all the ergot alkaloids, a four-membered ring named ergoline (Figure 1). For this reason, it would have been interesting to work on the natural precursor of ergot alkaloids, lysergic acid, which consists of an ergoline skeleton supporting a carboxylic acid moiety on atom C8 (Figure 1). Unfortunately, lysergic acid, which is also the precursor of the even more potent semi-synthetic lysergic acid diethylamide (LSD), has now been discontinued by most of the chemical firms who had produced it. Moreover, it was not possible to order such a compound in a European country. The focus of this study was therefore directed towards more readily available ergot alkaloids close in structure, metergoline and especially lysergamine (Figure 1). These two ergot alkaloids, metergoline [17] and lysergamine, are mainly composed of an ergoline skeleton, common to all ergot alkaloids, but which is methylated in contrast to the naturally occurring ones. These molecules also contain reactive groups such as a primary amine in the case of lysergamine and a non-fully substituted carbamate moiety in the case of metergoline. For the selection procedure, lysergamine and metergoline were immobilized onto magnetic beads in order to separate the DNA bound to the ergot alkaloids from the rest of the DNA library (Figure 2). Counter-selections were performed with quenched magnetic beads in order to eliminate the non-specifically bound aptamers. The aptamers selected for ergot alkaloids were amplified by PCR and used for the following selection cycles after strand separation of the PCR product. After each selection cycle, the aptamers were inserted into vectors grown in *Escherichia coli* and sequenced after a DNA extraction. The dissociation constants of the complexes of the selected aptamers and lysergamine were determined by SPR. In this analysis, only lysergamine was tested, because its structure is closer to the ergoline ring than metergoline. However, the selection of aptamers for metergoline is reported in this article, because it generated the most sensitive aptamer for the ergoline structure. A colorimetric test was developed by using this aptamer, which was fixed onto gold nanoparticles. The binding of lysergamine or metergoline to the aptamer system with gold nanoparticles resulted in precipitation of the complexes, and a color change occurred. The aptamer-gold nanoparticles system was applied to test a real sample of ergot contaminated flour, for which the same precipitation and color change were observed.

Figure 2. Scheme of SELEX procedure (Systematic Evolution of Ligands by EXponential enrichment) for ergot alkaloids. (**1**) Adsorption. A random 80-base ssDNA library is incubated with the ergot alkaloid coated magnetic beads. By applying a magnetic stand, the fraction of ssDNA which is not bound to the ergot alkaloid coated magnetic beads is discarded. (**2**) Recovery. After washing steps, the fraction of ssDNA bound to the ergot alkaloid coated magnetic beads is eluted from the ergot alkaloid coated magnetic beads. (**3**) Amplification. The selected ssDNA aptamers are amplified by polymerase chain reaction (PCR) in order to be used as input for the following selection cycle.



2. Results and Discussion

2.1. Coating of Magnetic Beads with Lysergamine and Metergoline

In order to select aptamers, it is usually necessary to immobilize the target molecule onto a support, in order to separate the single-stranded DNA (ssDNA) bound to the target molecule from the rest of the library. Several materials can be used, such as silica columns onto which the target molecule is grafted [18], or magnetic beads [19]. Magnetic beads are convenient supports as they can be used with rather small quantities of material and are easy to handle from a practical point of view. They form a suspension in liquids and they can be incubated in a buffer solution containing the ssDNA library. Then, by applying a magnetic stand, the magnetic beads are gathered onto the wall of the tube and the supernatant containing the unbound fraction of the library can be easily discarded. The weakly bound ssDNA fragments are removed by washing the magnetic beads. The ssDNA bound to the target molecule are eluted from the magnetic beads by applying a chelating elution buffer and heating the solution. In this work, two ergot alkaloids, lysergamine and metergoline, were fixed onto *N*-hydroxysuccinimide-activated (NHS-activated) magnetic beads. Lysergamine had been previously obtained by cleavage of metergoline (Figure 3) in order to obtain a structure closer to that of ergoline, moreover possessing a reactive primary amine moiety [20]. The product resulting from metergoline cleavage was characterized by mass spectrometry (Figure S1). The protonated molecule at *m/z* 270.24 (*m/z* calculated 270.19) was observed in the mass spectrum, confirming that lysergamine was obtained by hydrogenolysis of the benzyloxycarbonyl group of metergoline. The NHS ester groups of the

magnetic beads were used to react either with the primary amine of lysergamine or the carbamate moiety of metergoline (Figure 4). A solution of ethanolamine was used to quench the unreacted NHS esters of the magnetic beads. Ethanolamine quenched magnetic beads were also prepared for counter-selections (Figure 4), in order to eliminate the non-specific binding of the aptamers to ethanolamine or the rest of the magnetic beads. The coating of magnetic beads was analyzed by Fourier transform infrared attenuated total reflection (FTIR-ATR) by evaporating concentrated solutions of reagents and products. The advantage of the use of infrared spectroscopy for these types of reaction products is that it enables the analysis of surface coating of the magnetic beads and the determination of the chemical bonds formed or broken. In this way, it is even possible to find out the orientation of the molecules immobilized onto the magnetic beads. Figure 5 shows the infrared analysis of the coupling between the amine group of lysergamine and NHS-beads, which was characterized by the loss of the peaks of the primary amine (ν_{max} cm^{-1} 3298 and 3355) and the formation of an amide bond (ν_{max} cm^{-1} 1679). The stretching of the C–N peak at ν_{max} cm^{-1} 1089 increased in the spectrum of the lysergamine coated beads, due to the formation of the amide bond. The characteristic peaks of lysergamine were found in the spectrum of lysergamine coated beads at ν_{max} cm^{-1}: 2968, 2896, 1451, 1422, 1389, 1304, 1049, 880 and 679. The covalent binding of lysergamine occurs on the primary amine which is opposite to the methylated ergoline part. It was then possible to conclude that the methylated ergoline skeleton was situated on the outer part of the magnetic beads, consequently being accessible to the aptamer library in a further step of the selection procedure. Figure 6 shows the infrared spectra obtained from the coating of NHS-functionalized magnetic beads by metergoline, in which the loss of the N-H stretching peak of the carbamate group at ν_{max} cm^{-1} 3400 and an increase of C–N stretching peak at ν_{max} cm^{-1} 1090 due to the new bond formed between the magnetic beads and metergoline, were observed. The characteristic absorption peaks of metergoline were also found in the spectrum of metergoline coated magnetic beads. It was thus possible to determine that the molecules of metergoline were attached to the magnetic beads at the center of the molecules, and that both the ergoline skeleton and the other cyclic part were exposed to the ssDNA library for the selection procedure.

Figure 3. Synthesis of lysergamine by hydrogenolysis of the benzyloxycarbonyl group of metergoline.

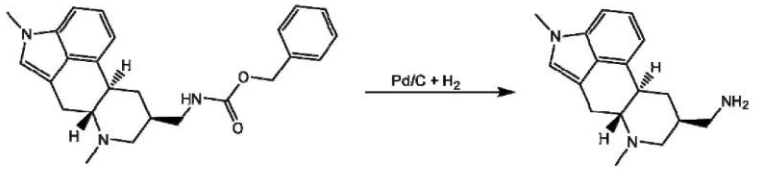


Figure 4. Scheme of the coating of *N*-hydroxysuccinimide-activated (NHS-activated) magnetic beads (**a**) reaction with metergoline (**b**) reaction with lysergamine (**c**) reaction with ethanolamine.

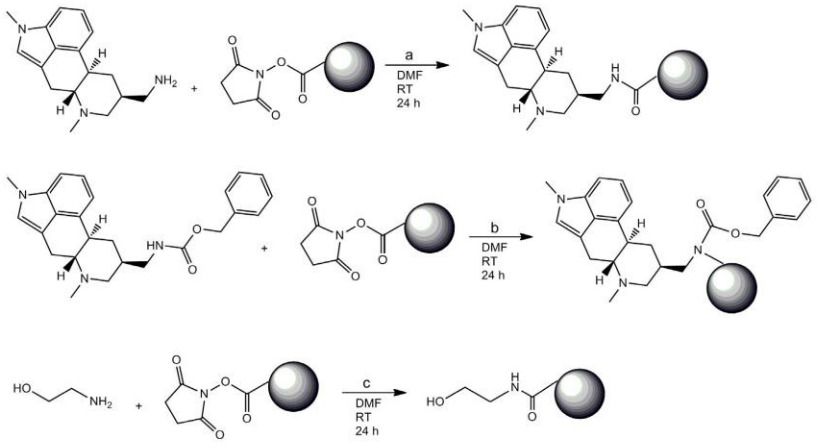


Figure 5. Infrared spectra of the coating of NHS-functionalized magnetic beads with lysergamine. (**A**) Range from 600 cm^{-1} to 1800 cm^{-1} (**B**) Range from 2600 cm^{-1} to 3800 cm^{-1}. (**a**) Lysergamine (**b**) Ethanolamine quenched NHS-activated magnetic beads (**c**) Lysergamine coated NHS-activated magnetic beads.

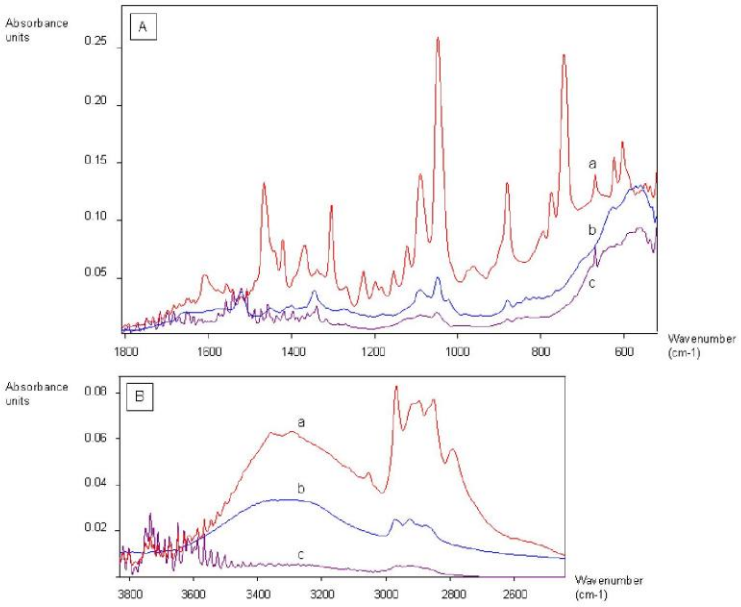
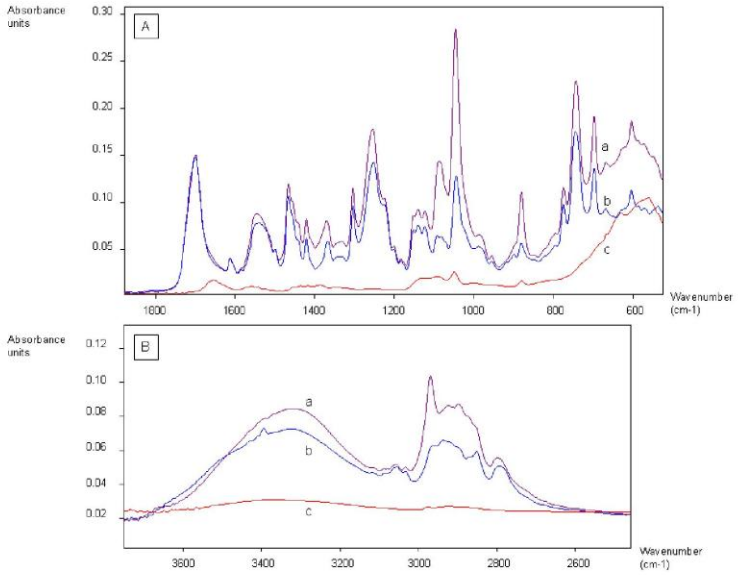


Figure 6. Infrared spectra of the coating of NHS-functionalized magnetic beads with metergoline. (**A**) Range from 600 cm^{-1} to 1800 cm^{-1} (**B**) Range from 2600 cm^{-1} to 3600 cm^{-1}. (**a**) Metergoline coated NHS magnetic beads (**b**) Metergoline (**c**) Ethanolamine quenched NHS magnetic beads.

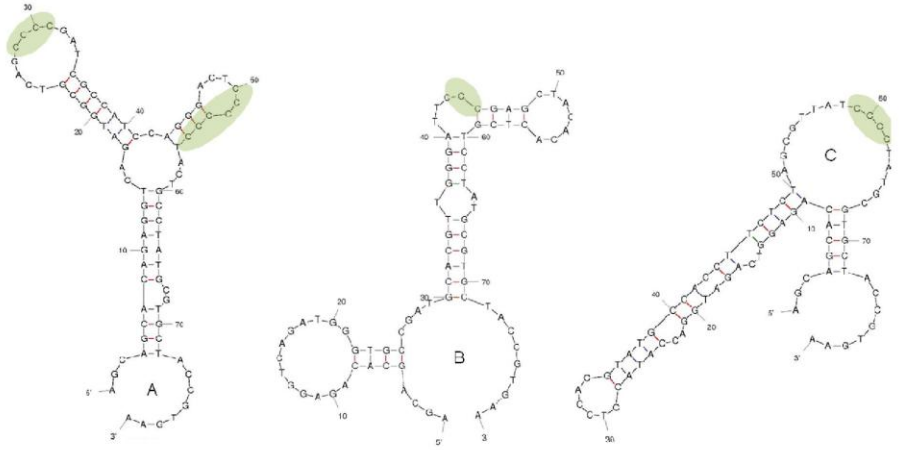


2.2. Selection of DNA Aptamers for Lysergamine and Metergoline

Ergot alkaloid coated magnetic beads were used for the selection of aptamers from an 80 base ssDNA library, consisting of 40 random nucleotides region flanked by two fixed primer regions of 20 bases. The selections for lysergamine and metergoline coated beads were carried out in parallel. A total of nine selection cycles were performed with metergoline coated beads and seven cycles with lysergamine coated beads. Counter-selections with ethanolamine coated magnetic beads were applied starting from the second selection cycle, in order to remove the non-specifically bound aptamers. After each selection cycle, the recovered ssDNA was amplified and used as input for the following selection cycle. The amount of ssDNA recovered after each selection cycle increased consistently; going from 2% recovery for the first cycle to 87% recovery for the ninth selection cycle for metergoline; and from 3% recovery to 79% recovery after the seventh selection cycle for lysergamine. The resulting aptamers were cloned and sequenced. The dissociation constants were then determined by SPR. The secondary structures of three selected aptamers (Figure 7) were determined by Mfold software [21]. After the last selection cycles with metergoline and lysergamine, it was observed that more than 80% of the selected aptamers had a common motif made of consecutive C bases (from 3 to 6). As a random ssDNA library was used, it was observed that the pools were enriched in C bases after the selection procedure. Therefore, it could be suggested that motifs of consecutive C bases play a role as binding sites in the recognition of lysergamine and metergoline. In the literature, several aptamers were found to be enriched in one of the bases. For instance, G rich aptamers were reported for various target

molecules [22–26]. The fact that one of the bases is significantly increased can be linked with the interaction of the aptamer with the target molecule. These regions are usually identified as binding sites or parts of them. For instance, a conformational change of the G-quartet of the thrombin aptamer was observed upon binding [25]. Some other studies on different target molecules have reported slight increments of one of the other nucleic acid bases, such as T or C [27,28].

Figure 7. Secondary structures of three aptamers selected for metergoline and lysergamine calculated by Mfold software with the predicted binding sites with common motifs of C bases in green areas. (**A**) Aptamer M3.2 (**B**) Aptamer L5.2 (**C**) Aptamer L5.7.



2.3. SPR Analysis of the Complexes of Aptamers and Lysergamine

2.3.1. Coating of SPR Streptavidin Chip with Biotinylated Derivatives of Lysergamine and Ethanolamine

As the focus of the study was to determine the binding between the selected aptamers towards the methylated ergoline part, only lysergamine was used in the SPR analysis. The aptamers obtained from the selections for lysergamine and metergoline were tested. This way, it was possible to discard the aptamers binding to the phenyl part of metergoline. In order to immobilize the target molecule onto a SPR chip, the (strept)avidin/biotin interaction has often been used due to its efficiency and convenience of use for the immobilization of the target molecule. Several coupling reactions can be used to obtain a biotinylated derivative of the target molecule [29]. For this purpose, a biotinylated derivative of lysergamine with a NHS-polyethylene glycol-biotin (NHS-PEG4-biotin) linker was prepared (Figure S2) in order to coat a streptavidin coated SPR chip. An ethanolamine quenched linker was also prepared with a NHS-PEG4-biotin linker and was injected onto a streptavidin SPR chip as a reference (Figure S3). The reaction products, biotinylated linker with lysergamine or ethanolamine, were analyzed using mass spectrometry in positive mode (Figures S4 and S5), in which the protonated reaction product lysergamine-PEG4-biotin was found at *m/z* 743.41 (*m/z* calculated 743.41) and the protonated reaction product ethanolamine-PEG4-biotin was found at *m/z* 535.29 (*m/z* calculated 535.28). These two reaction products were injected into different channels of the SPR chip with the

same parameters (concentration, flow, injection time). The different solutions of aptamers were passed through the coated surfaces and the responses were calculated by subtracting the reference measurement from the sample measurement. The SPR responses obtained for the coating with biotinylated lysergamine was in the range of 500 resonance units (RU), and in the range of 350 RU for the biotinylated ethanolamine, which is a smaller molecule and therefore gives a lower response.

2.3.2. SPR Measurements

The dissociation constants of the complexes of lysergamine and aptamers were determined by least-squares fitting of the values of the responses given by the SPR analysis with a non-linear regression of the following equation:

$$[y = B_{max} \times x/(K_d + x)] \quad (1)$$

with y, the degree of saturation; B_{max}, the number of maximum binding sites; K_d, the dissociation constant and x, the concentration of ssDNA aptamer.

For the SPR analysis, different concentrations of the selected aptamers were injected onto a streptavidin surface coated with lysergamine. The responses obtained for ethanolamine were subtracted from the responses obtained for lysergamine. Triplicates of the measurements were performed with each different aptamer. Other references were tested, such as a non-modified streptavidin surface and a streptavidin surface coated with biotin. However, these two latter references gave lower responses (<5 RU) than the ones obtained for ethanolamine (<10 RU). Therefore, only the ethanolamine coated streptavidin surface was considered for the determination of K_ds in the binding assay. As shown in Figure 8 and Table 1, aptamer M3.2 had a B_{max} of 205.2 RU, representing approximately one third of the values obtained for L5.2 and L5.7, respectively 575.8 and 531 RU. The three selected aptamers had dissociation constants (K_ds) ranging from 44 nM^2 for aptamer M3.2 to 499 nM for aptamers L5.2 and L5.7 (Table 1). These three aptamers represent sensitive ligands for lysergamine, as the dissociation constants (K_ds) obtained for the complexes are to be found in the nanomolar range. These results are in accordance with those reported in the literature, usually ranging from millimolar to picomolar range for the most sensitive aptamers [9,30]. The best fit for the values obtained for aptamer M3.2 corresponded to a model with two binding sites, instead of a model with one binding site for aptamers L5.2 and L5.7. The fact the aptamer M3.2 gave the lowest dissociation constant (K_d) and is in accordance with the primary structures of the sequences obtained from the selected aptamers, in which two common motifs of consecutive C bases were found in this aptamer, and the same common motif was present only once in the two other aptamers selected. The value of B_{max} obtained for aptamer M3.2, representing one third of the values obtained for the other aptamers and not a half as expected, can be explained by considering that the binding of this aptamer to two molecules of lysergamine can cover the area of three molecules of lysergamine on the SPR chip.

Figure 8. SPR responses of the aptamers binding to lysergamine.

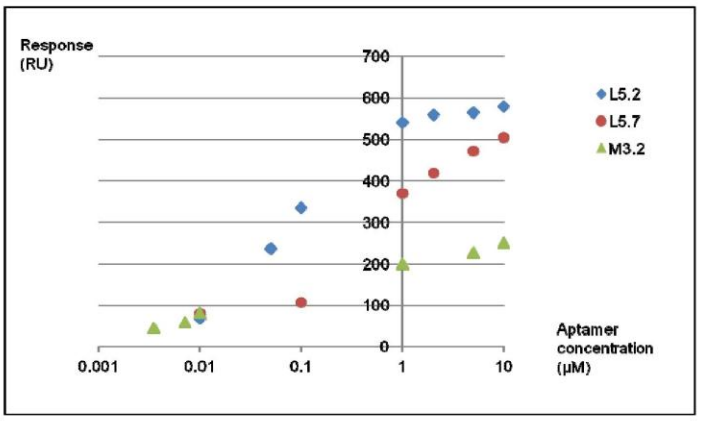


Table 1. Calculations by GraphPad Prism of the dissociations constants (K_ds) of complexes of lysergamine and the selected aptamers.

Fitting Model	Two-site specific binding	One-site specific binding	
Best fit values for	Aptamer M3.2	Aptamer L5.2	Aptamer L5.7
B_{max} (RU)	205.2	585.8	531.0
K_d	44 nmol^2/L^2	73 nmol/L	499 nmol/L
R^2	0.997	0.993	0.991
Number of points analyzed	6	7	6
Degree of freedom	2	5	4

2.4. Colorimetric Reaction with Gold Nanoparticles

Aptamers can be linked to gold nanoparticles by following the methodology elaborated by Liu and Lu [31,32] in order to produce a colorimetric reaction in the presence of the target of interest. For this, the selected aptamer is hybridized to two small DNA fragments linked to colloidal gold by a thiol bond. Upon binding to the target molecule, a conformational change in the aptamer can occur. This conformational change can lead to a modification of the chemical environment surrounding the gold nanoparticles, inducing a color change of the solution. Once the modified version of aptamer M3.2 was fixed onto gold nanoparticles, solutions of different ergot alkaloids in dimethylformamide (DMF) were tested: lysergamine, metergoline, ergocornine and an extract of ergot contaminated flour containing several ergot alkaloids such as ergometrine, ergosine, ergotamine, ergocornine, ergokryptine, ergocristine and their epimers in various proportions (Figure 9). As the ergoline skeleton of ergot alkaloids contains a two-membered ring structure named indole, several compounds having an indole group which could be possibly found in food samples, such as L-tryptophan, gramine and tryptamine were also tested (Figure 9). In addition, another molecule differing from ergot alkaloids and containing a phenyl group, chloramphenicol, was also tested (Figure 9). The solutions of the different compounds were prepared in DMF with a concentration of 50 mM and for each sample, 1 μL was taken and placed in the tubes containing the solution of aptamers linked to gold nanoparticles. A rather high

concentration of compounds was chosen in order to provide a qualitative assay clear enough to be seen by the naked eye. The colorimetric test was performed with a diluted solution of gold nanoparticles (Series I) and was measured by UV-Vis spectroscopy. A second set of samples was prepared with a more concentrated solution of gold nanoparticles and some of the compounds from Series I. Figure 10 shows the results of the colorimetric assay with aptamer M3.2 linked to gold nanoparticles, in which a precipitate was obtained with lysergamine (sample 3), metergoline (sample 4) and the ergot contaminated flour extract (sample 10); and a color change occurred in the presence of lysergamine and the ergot contaminated flour extract. The color change was much less pronounced in the case of metergoline (sample 3). The other molecules tested: the solvent DMF (sample 2), ergocornine (sample 5), L-tryptophan (sample 6), tryptamine (sample 7), gramine (sample 8), and chloramphenicol (sample 9), did not generate any change. In Figure 11, the UV-Vis spectra are presented of the different solutions of samples 1 to 10. In this analysis, it was observed that the solution of gold nanoparticles linked to aptamer M3.2 (sample 1) had a maximum absorption at 525 nm. The same absorption profile was also obtained for samples 2, 5, 6, 7, 8 and 9; containing respectively: the solvent DMF, ergocornine, L-tryptophan, tryptamine, gramine and chloramphenicol. In the case of lysergamine (sample 4) and the extract of a contaminated sample of flour (sample 10), the maximum absorption was shifted to 600 nm. In the case of metergoline (sample 3), a fading of the absorption peak between 530 nm and 600 nm was observed. From this colorimetric assay, it was possible to conclude that aptamer M3.2 could generate a specific binding reaction in the presence of ergot alkaloids and that it was able to distinguish between the different ergot alkaloids tested. First, in the case of lysergamine, it was suggested that two molecules of lysergamine could saturate the two binding sites of aptamers M3.2, resulting in the formation of a purple precipitate and a shift of the UV-Vis absorption spectrum. In the case of metergoline, which is more voluminous than lysergamine, it was concluded that only one binding site of aptamer M3.2 was occupied, resulting in the formation of a precipitate with a much reduced color change. The difference in behavior towards lysergamine and metergoline is highlighted in Series II (Figure 10), in which a more concentrated solution of aptamers linked to gold nanoparticles was used for the colorimetric assay, enabling the evaluation with the naked eye of the difference in color of the precipitates obtained with these two different molecules. As aptamer M3.2 was originally selected for metergoline, and not lysergamine, it was supposed that one of the two binding sites of aptamer M3.2 was disabled by the linkage to gold nanoparticles in the colorimetric assay and that only smaller molecules than metergoline could fit the second binding site. The sample of ergot contaminated flour extract (sample 10) gave a strong color change and a shift in the absorption spectrum from 525 nm to 600 nm as well as showing lysergamine, even though this sample did not contain any molecules of lysergamine, but a mixture of different natural ergot alkaloids (Table 2). As the sample of ergocornine (sample 5), did not lead to any change, it is supposed that the aptamer did not interact with such a compound in the real ergot sample, nor the other voluminous ergot alkaloids such as ergosine, ergotamine or ergokryptine (Figure 9). It was then concluded that the aptamer M3.2 could interact with small natural ergot alkaloids such as ergometrine (Figure 9), having a structure and size close to those of lysergamine. The interfering compounds having an indole group; such as gramine, L-tryptophan or gramine; did not generate any change. It was then possible to deduce that aptamer M3.2 was specific to the ergoline structure, with or without a methylation on position N1. Regarding specificity, the team of Rankin *et al.* [33] showed that aptamers could reach a very high

level of specificity and reported an aptamer distinguishing molecules only differing by a methyl group. In our study, the methylation of the ergoline skeleton was not the discriminating factor, but rather the size of the ergot alkaloid tested. The specificity could be expressed as being restricted to molecules possessing an ergoline ring and smaller in size than metergoline.

Figure 9. Chemical structures of the compounds tested in the colorimetric reaction assigned with the sample number of the assay.

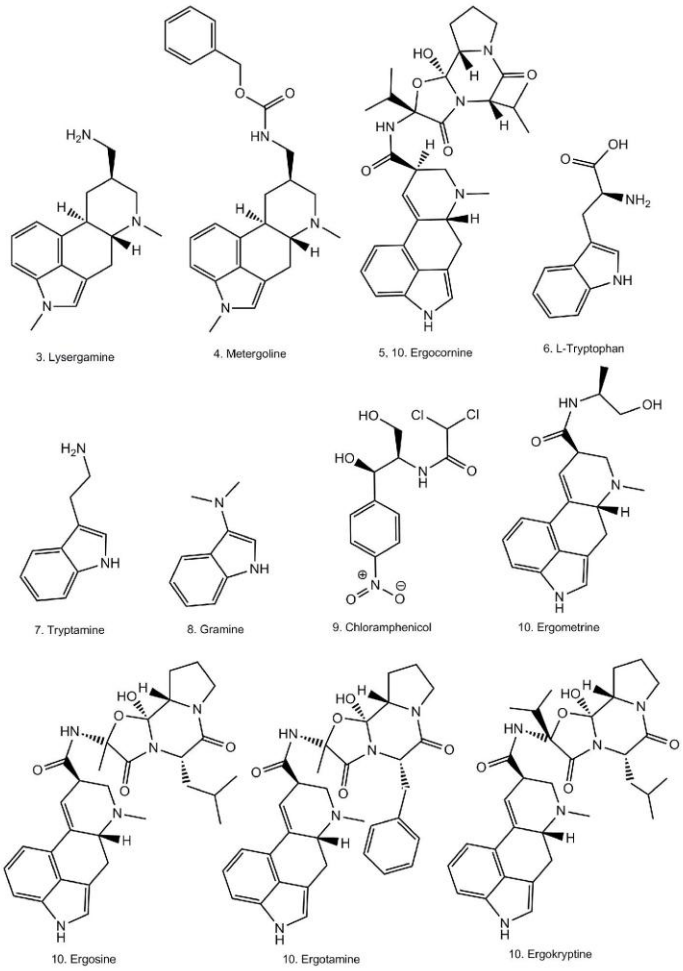


Figure 10. Colorimetric reaction based on gold nanoparticles linked to aptamer M3.2. (1) Reference, no compound added (2) DMF (3) Metergoline (4) Lysergamine (5) Ergocornine (6) L-Tryptophan (7) Tryptamine (8) Gramine (9) Chloramphenicol (10) Ergot contaminated flour sample.

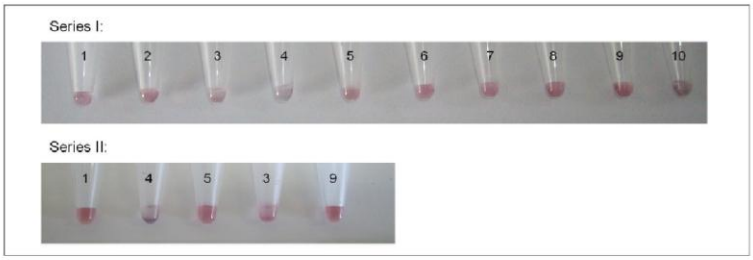


Figure 11. UV-Vis absorbance spectra of samples 1 to 10 from Series I, range from 400 nm to 800 nm.

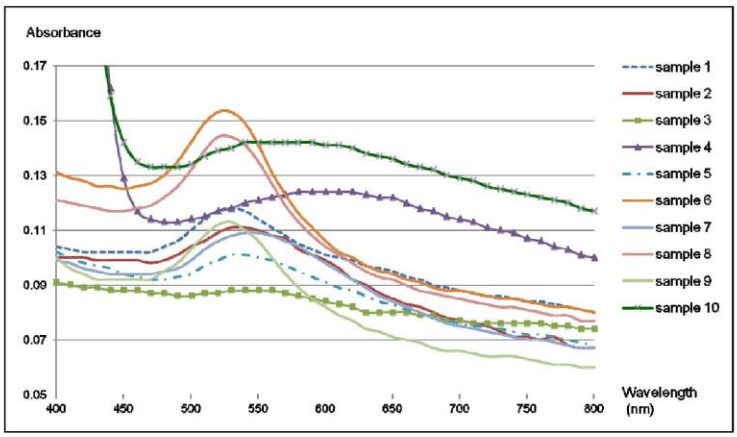


Table 2. Ergot composition of ergot contaminated flour sample (sample 10), determined by LC-MS [34].

Ergot alkaloid	Concentration (µg/kg)	Total epimers	% of ergot alkaloid content
Ergometrine	106	132	4.3
Ergometrinine	26		
Ergosine	1567	1567	51
Ergosinine	<LOQ		
Ergotamine	22	24	0.8
Ergotaminine	2		
Ergocornine	65	84	2.7
Ergocorninine	19		
Ergokryptine	1251	1259	41
Ergokryptinine	8		
Ergocristine	<LOQ	3	0.1
Ergocristinine	3		
Total	3068	3068	100

3. Experimental Section

3.1. Coating of Magnetic Beads with Lysergamine and Metergoline

3.1.1. Production of Lysergamine and Preparation of Lysergamine Coated Beads

In order to obtain a more reactive ergot alkaloid, metergoline was transformed into lysergamine (Figure 3) by hydrogenolysis in a Parr apparatus [20]. In a flask, 50 mg of 10% Pd/C (Sigma Aldrich, Bornem, Belgium) were suspended in 15 mL of methanol and 250 mg of metergoline were added after dissolution in 2 mL of methanol. While stirring, the mixture was sparged with a slow stream of hydrogen gas (2.06 Bar) for 16 h at room temperature (RT). The reaction mixture was filtered through celite and the solvent was evaporated under reduced pressure by using a rotary evaporator. As an oil was obtained, ethyl acetate was added to the product and subsequently evaporated, resulting in a white to light yellow powder of lysergamine. The product resulting from metergoline cleavage was characterized using mass spectrometry in positive mode. The sample was prepared by diluting 2 µL of the reaction mixture in 198 µL of acetonitrile. For the analysis, 5 µL of this solution were injected in a 0.2 mL/min flow of acetonitrile with 0.1% formic acid on an Acquity UPLC system (Waters, Elstree, UK). The mass spectra were recorded using electrospray ionization in positive ion mode using a Xevo TQ MS system (Waters, Elstree, UK).

3.1.2. Preparation of Lysergamine Coated Magnetic Beads

A suspension of NHS-activated magnetic beads (Invitrogen, Merelbeke, Belgium) was prepared by washing a quantity of 2×10^9 magnetic beads with 0.3 mL of dry DMF and placing them in 0.5 mL of dry DMF. Then, 5 mg of lysergamine were added, after dissolution in 0.4 mL of dry DMF. The mixture was stirred for 24 h at RT. By applying a magnet, the supernatant was removed and a quench solution of 2 µL of ethanolamine in 0.6 mL of dry DMF was added to the magnetic beads and the mixture was stirred for 5 h at RT. After applying a magnet, the supernatant was discarded; the magnetic beads were then washed 5 times with 0.3 mL of DMF and finally placed in 0.4 mL of DMF.

3.1.3. Preparation of Metergoline Coated Magnetic Beads

In order to coat magnetic beads with metergoline, 5 mg (1.24×10^{-5} mol) of metergoline (Sigma Aldrich, Bornem, Belgium) were dissolved in 0.4 mL of dry DMF and placed in a suspension of 2×10^9 NHS-activated magnetic beads (Invitrogen, Merelbeke, Belgium), previously washed with dry DMF and placed in 0.5 mL of dry DMF. The mixture was stirred for 24 h at RT. After applying a magnet, the supernatant containing the excess of metergoline was removed. A solution of 2 µL of ethanolamine in 0.6 mL of DMF was added to the magnetic beads in order to quench the unreacted NHS groups and the mixture was stirred for 4 h at RT. After applying a magnet, the coated magnetic beads were washed 5 times with DMF before being placed in 0.4 mL of DMF.

3.1.4. Preparation of Ethanolamine Coated Beads

Quenched magnetic beads were prepared by adding a solution of 5 µL ethanolamine in 0.6 mL of dry DMF to a suspension of 2×10^9 NHS-functionalized magnetic beads in 0.3 mL of dry DMF. The mixture was stirred for 4 h at RT. By applying a magnet, the supernatant was discarded and the ethanolamine coated magnetic beads were washed 5 times with 0.3 mL of DMF, and were finally placed in 0.4 mL of DMF.

All the coating reactions were characterized by infrared spectroscopy with an FTIR-ATR spectrometer (Vector 22 Brücker, Zürich, Switzerland). For each sample, 80 µL of the coated magnetic bead solutions were taken and after applying a magnetic stand, the solvent was discarded. The coated magnetic beads were washed with ethanol and placed in 10 µL of ethanol. The concentrated solutions of coated magnetic beads in ethanol were evaporated onto the infrared chip for the analysis.

3.2. Selection of DNA Aptamers for Lysergamine and Metergoline

3.2.1. Selection Procedure

The first selection cycle was performed directly with the magnetic beads coated with lysergamine or metergoline. All the other selection cycles were performed after a counter-selection with ethanolamine coated magnetic beads. A counter-selection was not performed for the first selection cycle as the amount of ssDNA recovered, which is in the range of 2% of the amount of library used, would be too low to amplify. However, starting from the second selection cycle, the counter-selection step was really crucial to remove the non-specifically binding aptamers. For the first selection cycle, metergoline or lysergamine coated beads were incubated with the ssDNA library. For this, 15 µg of an 80-base DNA library having the following structure 5'-AGCAGCACAGAGGTCAGATG-N40-CCTATGCGTGCTACCGTGAA-3' (Eurogentec, Seraing, Belgium) was placed in 0.5 mL of binding buffer (100 mM NaCl; 20 mM Tris-HCl pH 7.6; 5 mM KCl; 2 mM $MgCl_2$, $6H_2O$; 1 mM $CaCl_2$, $2H_2O$) with an amount of 1×10^8 metergoline or lysergamine coated magnetic beads, re-suspended in 0.3 mL of binding buffer. The mixture was gently stirred for 30 min at RT. For the following selection cycles, the new pool of ssDNA was first placed in a suspension of 1×10^8 ethanolamine coated magnetic beads in binding buffer. By applying a magnet, the supernatant containing the ssDNA fraction not bound to the ethanolamine beads was taken and placed in a suspension of 0.1 mL of 1×10^8 ergot alkaloids coated magnetic beads in binding buffer. The mixture was gently stirred for 40 min at RT.

3.2.2. Elution of ssDNA

After applying a magnet, the supernatant was discarded and the beads were washed twice with the binding buffer. The ssDNA fraction bound to the ergot alkaloid coated magnetic beads was eluted with 3×0.2 mL of elution buffer (3.5 M urea, 40 mM Tris-HCl, 10 mM EDTA, 0.02% Tween 20) by stirring gently (300 rpm) for 10 min at 80 °C (Eppendorf Thermomixer, Rotselaar, Belgium).

3.2.3. Precipitation of ssDNA

The eluted ssDNA was precipitated by adding 60 μL of 3 M sodium acetate (10% volume of elution buffer) and 1 mL of ice-cold ethanol. This solution was placed at −80 °C for 1 h to ensure complete precipitation. The solution was centrifuged at 13,000 rpm at 4 °C for 20 min in order to concentrate the DNA. The supernatant was discarded leaving approximately 50 μL of solution in the tube. Then, 1 mL of 70% ice-cold ethanol in water was added and the solution was centrifuged at 13,000 rpm at 4 °C for 25 min. Again, the supernatant was discarded leaving 50 μL of solution in the tube and this latter step was repeated twice. The ethanol solution was evaporated in a speed vacuum device at 30 °C for 10 min. The resulting DNA pellet was re-suspended in 15 μL of water (18.2 megohms/cm). The amount of ssDNA obtained from the selection was analyzed with a UV-Vis spectrophotometer (Nanodrop, Erembodegem, Belgium) in order to determine the recovery rate.

3.2.4. Amplification of the selected ssDNA aptamers by PCR

The ssDNA recovered from the selection procedure was amplified by PCR by using a thermostable *Taq* polymerase, PCR buffer, 10 mM dNTP solution and 25 mM $MgCl_2$ solution (Fermentas, St Leon-Rot, Germany). The set of primers used for the PCR: 5'-AGCAGCACAGAGGTCAGATG-3' (forward primer) and 5'-TTCACGGTAGCACGCATAGG-3' (reverse primer), was provided by Eurogentec (Seraing, Belgium). In each vial, 6 μL of the ssDNA solution were placed in 20 μL water (18.2 megohms/cm). Then, the following solutions were added to the DNA template: 5 μL of PCR buffer, 3.5 μL of 10 mM dNTP, 11 μL of 25 mM $MgCl_2$, 0.5 μL of 100 μM solution of forward primer, 0.5 μL of 100 μM solution of reverse primer and 0.8 μL of Taq polymerase (5 U/μL). The mixture was placed in a thermocycler (Mastercycler personal Eppendorf VWR, Leuven, Belgium) with the following temperature profile: heating at 94 °C over 6 min for the initial denaturation step, followed by 18 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 20 s, followed by a final elongation step of 72 °C over 1 min 30 s. The Taq polymerase was added to the PCR reaction mixture after 4 min of the initial denaturation step. A gel-electrophoresis of the PCR product was performed after each PCR. A 2% agarose gel was prepared in 1× Tris-acetate-EDTA (TAE) buffer with 10 μL of ethidium bromide for 100 mL of buffer. In each well of the agarose gel, 20 μL of PCR solution were placed after staining with a loading dye. A 50 base pair DNA ladder was used to check the length of the PCR product.

3.2.5. Preparation of ssDNA from Biotinylated PCR Product

A part of the double-stranded DNA obtained from the PCR was amplified with a reverse biotinylated primer (Eurogentec, Seraing, Belgium) in order to separate the strands of the PCR product by using streptavidin magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen, Merelbeke, Belgium), with a ratio of 0.1 mg of magnetic beads for 1 μg of dsDNA. The streptavidin magnetic beads were washed with 1× binding and washing (B & W) buffer (5 mM tris-HCl pH 7.5, 0.5 mM EDTA, 1 M NaCl) and resuspended in 2× B & W buffer to a concentration of 5 μg/μL. An equal amount of water solution of dsDNA was added to the solution of the streptavidin magnetic beads and the mixture was gently stirred for 25 min at RT. By applying a magnet, the magnetic beads were gathered onto the tube wall and the supernatant was discarded. The beads were washed twice with

1× B & W buffer and re-suspended in 0.15 M NaOH for 5 min at 37 °C in order to release the non-biotinylated DNA strand. After applying a magnet, the solution was placed in a clean vial and immediately neutralized with an equal amount of 0.15 M acetic acid. The DNA was precipitated by adding 10% volume of 3 M sodium acetate and ice-cold ethanol to give a ratio of 70% in the final solution. This solution was incubated at −80 °C for one hour and then centrifuged at 13,000 rpm at 4 °C for 20 min. The solution was discarded; leaving approximately 50 μL at the bottom of the tube and 70% ethanol was added to the DNA pellet. The solution was centrifuged again with the same conditions and the step was repeated. The supernatant was discarded, leaving 50 μL of solution, which was evaporated in a speed vacuum device (Eppendorf Concentrator 5301, VWR, Leuven, Belgium). The ssDNA was re-suspended in water and the concentration was determined by UV-Vis spectroscopy (Nanodrop, Thermo scientific, Erembodegem, Belgium).

3.2.6. Cloning and Sequencing of Aptamers

After each selection cycle, a part of the ssDNA selected was amplified by PCR with non-modified primers and cloned into the vector pCR2.1-TOPO (TOPO TA cloning kit, Invitrogen, Merelbeke, Belgium) and transformed in *Escherichia coli* TOP 10 competent cells. The colonies containing the aptamer sequences were picked and the plasmid DNA was extracted by using a plasmid extraction kit (QIAprep Spin Miniprep kit, Qiagen, Venlo, Netherlands). After DNA purification, the plasmid DNA was sequenced (VIB Genetic Service Facility, Antwerp, Belgium). The secondary structures of the aptamers were determined using Mfold software developed by Zucker [21] in order to calculate the lowest energy levels of the secondary structures formed by the aptamers.

3.3. SPR Analysis of the Complexes of Aptamers and Lysergamine

3.3.1. Preparation of Biotinylated Lysergamine Linker

In order to prepare the biotinylated derivative of lysergamine to be used with streptavidin SPR chip, 6 mg (2.22×10^{-5} mol) of lysergamine were dissolved in 0.25 mL of dry DMF and a solution of 2 mg (3.4×10^{-6} mol) of NHS-PEG4-biotin linker (EZ-Link, Thermo Scientific, Belgium) in 0.25 mL of dry DMF was added. The mixture was stirred for 24 h at RT.

3.3.2. Preparation of Biotinylated Ethanolamine Linker

As a reference material, a NHS-PEG4-linker quenched with ethanolamine was prepared by dissolving 3 mg (4.97×10^{-5} mol) of ethanolamine (Sigma Aldrich, Belgium) in 0.25 μL of dry DMF and adding 2 mg (3.4×10^{-6} mol) of NHS-PEG4-biotin linker dissolved in 0.25 mL of dry DMF. The reaction mixture was stirred for 5 h at RT.

The products obtained from the reaction between the biotinylated linker and lysergamine or ethanolamine were characterized using mass spectrometry by placing 2 μL of the reaction mixtures in 198 μL of acetonitrile. For each sample, 5 μL were injected into a 0.2 mL/min flow of acetonitrile with 0.1% formic acid on an Acquity UPLC system (Waters, Elstree, UK). The mass spectra were recorded using electrospray ionization in positive ion mode on a Xevo TQ MS system (Waters, Elstree, UK).

3.3.3. Coating SPR Chip with Biotinylated Linkers

As streptavidin supports restrict the use of organic solvents, 75 μL from each of the reaction mixtures were diluted in 300 μL of HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid (EDTA), 0.005% surfactant P20), in order to obtain a ratio of 25% of DMF in the final solution, which was then injected into the SPR chip (Biacore GE Healthcare, Diegem, Belgium). In the SPR device (Biacore, GE Healthcare, Diegem, Belgium), a streptavidin chip was used and 120 μL of the two different coating solutions were injected with a flow of 5 μL/min.

After the coating reaction, the HBS-EP buffer was replaced by a continuous flow of binding buffer and the solutions of the different aptamers in binding buffer were loaded onto the coated SPR chip. The lysergamine coated surfaces were regenerated by applying pulses of 5 M urea.

The analysis of the binding of ssDNA aptamers to the lysergamine coated surface was compared with the binding to the ethanolamine coated surface, which was the most relevant reference for this analysis. However, several other references were tested such as a biotin coated streptavidin surface and a non-modified streptavidin surface.

3.4. Colorimetric Reaction with Aptamer Linker to Gold Nanoparticles

The procedure developed by Liu and Lu [31,32] was followed with the aptamer selected for ergot alkaloids. The following labeled DNA sequences (Eurogentec, Belgium) were used in the colorimetric reaction: 5'-ACTCATCTGTGAAGAGAAGCAGCACAGAGGTCAGATGTCCGTCAGCCCCGATC GCCATCCAGGGACTCCCCCCTATGCCTATGCGTGCTACCGTGAA-3' (modified aptamer M3.2), 5'-TCACAGATGAGT-C3-SH-3' (3'-SH oligo) and 5'-SH-C6-TGCTGCTTCTCT-3' (5'-SH oligo). In a microcentrifuge tube were placed 1.6 μL of 1 mM 3'-SH oligo and 7.4 μL of water. In a second microcentrifuge tube, 1.6 μL of 1 mM 5'-SH oligo was placed in 7.4 μL of water. Then, 1 μL of 500 mM acetate buffer (pH 5.2) and 1.5 μL of tris(2-carboxyethylphosphine) TCEP-HCl (Perbio Science, Erembodegem, Belgium) were added to each tube to activate the thiol moiety. After 1 h of activation, 3 mL of 15 nm colloidal gold solution were added to each tube containing TCEP-activated oligos and the mixtures were gently shaken. The tubes were stored in a drawer for 24 h. Then, 30 μL of 500 mM Tris acetate buffer (pH 8.2) were added dropwise to each tube, followed by the addition of 300 μL of 1 M NaCl. The tubes were stored for another day. The functionalized particles were centrifuged at 13,000 rpm for 15 min at RT. The supernatant was discarded and the particles were dispersed in 200 μL of 100 mM NaCl buffer. The two solutions of functionalized gold nanoparticles were mixed together. Then, 10 μL of 10 μM modified aptamer M3.2 were added and the mixtures were incubated at 4 °C overnight. The aggregates were centrifuged at 2000 rpm for 1 min at RT. The supernatant was discarded and the aggregates were dispersed in 500 μL of 300 mM NaCl buffer. For the optimization of salt concentration, 50 μL of the aggregates solution were taken for each analysis. The phase transition of the aptamer-gold nanoparticle aggregates was studied by recording the UV absorption at 260 nm as a function of the temperature with different NaCl concentrations (from 150 mM to 300 mM) in order to find the appropriate NaCl concentration for which the UV-Vis absorption increases sharply at RT. An optimal concentration of 250 mM NaCl was found, as the melting temperature obtained with this parameter was the closest to RT (~23 °C). For the final test,

two series of samples with different concentrations of gold nanoparticles solutions were prepared: a concentrated solution (Series II), in which 30 μL of aggregates solution were taken and the NaCl concentration adjusted to 250 mM by adding 100 mM NaCl buffer; and a more diluted series of 40 μL of solution of gold nanoparticles diluted twice (Series I), with a final NaCl concentration of 250 mM. For the specificity test, each compound was prepared in a solution of DMF with a concentration of 50 mM and 1 μL of each of the solutions was added to the test tubes containing the aptamer-gold nanoparticles system. The sample of ergot contaminated flour was obtained by adding 300 mg of contaminated flour sample, provided by the European Food Safety Authority (sample EFSA/82/RE), to 500 μL of DMF and vortexing for 1 min. In this way, the ergot alkaloids were extracted from the flour sample and 1 μL of the solution was used for the test. A preliminary test with DMF only was also performed and did not show any effect on the aptamer-gold nanoparticles system. The UV-Vis spectra of the samples from Series I were measured with a Synergy Mx UV-Vis spectrophotometer (Biotek, Bad Friedrichshall, Germany), and were analyzed using Gen5 software.

4. Conclusions

In order to produce a recognition element for ergot alkaloids which can be included in a biosensor, aptamers were selected for two ergot alkaloids, metergoline and lysergamine, by using the iterative selection procedure SELEX. It was possible to isolate, amplify and sequence aptamers binding to metergoline and lysergamine. The complexes of the selected aptamers and lysergamine were characterized by SPR. The selected aptamers have dissociation constants in the nanomolar range, the best one reaching 44 $nmol^2/L^2$ for aptamer M3.2. This aptamer possesses two common motifs of C bases, predicted to be two binding sites. The mathematical analysis confirmed the presence of two binding sites with a lower B_{max} and a lower K_d than the other aptamers tested. This aptamer, which has a high sensitivity for lysergamine, is the first one to our knowledge reported for ergot alkaloids. A colorimetric reaction could be achieved with aptamer M3.2 linked to gold nanoparticles and a specific color change was observed in the presence of lysergamine and small ergot alkaloids. This system can be developed further with the construction of a dipstick test using lateral flow diffusion. Other transducing techniques (electrochemical, piezoelectric, optical, *etc.*) can be used with this aptamer as recognition element for the development of different sensing formats. We believe in the potential of this aptamer for applications in the future regarding the detection of small ergot alkaloids.

Acknowledgments

This study was funded by the Federal Public Service of Health, Food Chain Safety and Environment (FOD) project Ergot RF6204.

The authors thank the Hercules foundation for funding the mass spectrometer.

Tom Rauws (University of Antwerp) is acknowledged for his support in organic synthesis.

We also greatly appreciated the help of Norbert Hancke (University of Antwerp) for the mass spectrometry analyses.

Finally, the authors would like to express their gratitude to the editor of the journal in charge of the submission of this work and the unknown reviewers for their help in the elaboration of this manuscript and their contribution to its scientific correctness.

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Advances in Bioscience and Biotechnology, 2014, 5, 692-698
Published Online July 2014 in SciRes. http://www.scirp.org/journal/abb
http://dx.doi.org/10.4236/abb.2014.58082

Scientific Research

Aptamer-Based Extraction of Ergot Alkaloids from Ergot Contaminated Rye Feed

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Received 27 April 2014; revised 5 June 2014; accepted 9 July 2014

Abstract

Ergot alkaloids are mycotoxins which can be found in food based on cereal-crops, due to a contamination of plants by fungi of the genus *Claviceps*. The ingestion of ergot contaminated cereal crops can lead to a severe poisoning known as ergotism. For food and feed safety purposes, the extraction of ergot alkaloids from ergot contaminated flour was investigated. For the specific recognition of ergot alkaloids, DNA aptamer ligands specially selected for ergot alkaloids were grafted onto silica gel in order to construct a specific solid phase extraction system. The aptamer-functionalized silica gels were used to extract ergot alkaloids from a contaminated rye feed sample. The presence of ergot alkaloids eluted from the aptamer-functionalized silica gels was analyzed using LC-QTOF-MS. By using this simple system, it was possible to specifically extract ergosine, ergokryptine and ergocornine from an ergot contaminated rye feed sample. This aptamer-based extraction tool shows the applicability of aptamers for the specific extraction of toxins or natural compounds from turbid matrices in a one-step procedure.

Keywords

Single-Stranded Nucleic Acid Aptamer, Aptamer-Functionalized Silica Gel, Ergosine, Ergokryptine, Ergocornine

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How to cite this paper: Rouah-Martin, E., Maho, W., Mehta, J., De Saeger, S., Covaci, A., Van Dorst, B., Blust, R. and Robbens, J. (2014) Aptamer-Based Extraction of Ergot Alkaloids from Ergot Contaminated Rye Feed. *Advances in Bioscience and Biotechnology*, **5**, 692-698. http://dx.doi.org/10.4236/abb.2014.58082

1. Introduction

Ergot alkaloids are biologically active compounds mainly produced by fungi of the genus *Claviceps* [1] [2], which can grow on more than 400 plants and grasses; mainly forage and leading cereal crops worldwide such as wheat or sorghum [1] [2]. *Claviceps* fungi produce ergot alkaloids as secondary metabolites, which are toxic to humans and animals. Ergot alkaloids are based on a four-member ring, ergoline, which is mainly responsible for their biological activity [3]. Ergot alkaloids are undesirable in food and feed, as they can lead to a poisoning known as ergotism [4]. However, a few ergot alkaloids exhibit interesting medicinal properties [5], and are produced on purpose by fungal contamination of cereal crops or produced by saprophytic cultures for instance [6] [7]. Chemical extraction techniques were developed in order to isolate ergot alkaloids [8]-[10], and to quantify them [11]-[15]. However, these techniques are destructive for the samples and cannot be applied for the decontamination of ergot alkaloids from food and feed stuff. Therefore, other techniques were recently elaborated for the removal of ergot alkaloids from food and feed; such as molecularly imprinted polymers (MIPs) [16] [17]. Also, biological recognition elements were developed for ergot alkaloids, such as antibodies [18] [19] and aptamers [20]. These different recognition elements can be immobilized onto solid supports in order to obtain specific solid phase extraction systems. Different types of solid supports can be used, such as organic polymers or inorganic oxides for instance. Silica gel is interesting for this type of applications because it presents a high thermal and chemical stability and enables a wide variety of chemical modifications through the silanol groups dispersed on its surface [21]. The covalent coupling of organic molecules and biomolecules can be done by chemical pretreatments of silica particles with organosilanes, such as dichlorodimethylsilane (DDS) [22] [23], 3-chloropropyltrimethoxysilane (CTS) [24] [25], or 3-aminopropyltriethoxysilane (APTS) [26]. In the present study, we report the functionalization of silica particles with two different aptamers specific to ergot alkaloids, in order to construct a specific solid phase extraction system. The aptamer-functionalized silica gel was tested with a raw extract of contaminated rye flour. The presence of ergot alkaloids eluted from the aptamer functionalized silica gel was determined by quadrupole-time-of-flight mass spectrometry (LC-MS-QTOF) analysis. It was observed that the aptamer-functionalized silica gel could successfully extract three main ergot alkaloids from the sample; namely ergosine, ergokryptine and ergocornine. The use of DNA-grafted silica gels has already been reported in sensing assays [26]-[29] and in extraction systems [30] [31]. However, the realization of aptamer-functionalized silica gel for the specific extraction of ergot alkaloids is reported for the first time in the present article.

2. Materials and Methods

2.1. Choice of Single-Stranded DNA Aptamers

An aptamer, named aptamer M3.2, having a dissociation constant of 44 $nmol^2/L^2$, was previously selected for the ergoline group and was reported in our previous research article [20]. The original aptamer M3.2 consists of 80 bases: but in this experiment, only a fragment of 52 bases containing the predicted binding sites was used for the functionalization of silica gel, taking care of keeping the same conformation of the binding sites. The following 3'-aminated version of the shortened aptamer M3.2 was used 5'-GGTCAGATGTCCGTCAGCCCCGATCGCCATCCAGGGACTCCCCCCTACTGCC-3'-NH_2. Another aptamer selected for ergot alkaloids, named L5.5 and having a dissociation constant of 660 nmol/L, was also tested in this study. The 3'-aminated aptamer L5.5 was used, having the following sequence 5'-AGCAGCACAGAGGTCAGATGGGCAGGATACAACACGTTACTATCCACTCTGCACCGGCGGCCTATGCGTGCTACCGTGAA-3'-NH_2. The secondary structures of aptamers M3.2 and L5.5, determined by using IDT Oligoanalyzer software [32], are shown in **Figure 1**.

2.2. Silica Functionalization with Aminated DNA

A narrow particle range silica gel of 10 - 20 µm (Merck, Germany) was used to prepare the aptamer-functionalized silica gel. For each sample, 300 mg of silica gel were placed in Eppendorf tubes.

Sample 1 was kept as unmodified silica gel in order to be used as a reference material. Sample 1 was washed two times with 500 µL of ultrapure MQ water and placed in 500 µL of MQ water.

The silica gels of Samples 2 and 3 were activated by a treatment with DDS, in order to enable functionalization with aminated molecules [22]. The chemical functionalization of DDS activated silica gel with aminated

DNA is represented in Figure 2. The silica gels of Samples 2 and 3 were first washed two times with 200 µL of diethyl ether and then placed in 500 µL of 5% diethyl ether solution of DDS (Sigma-Aldrich, Belgium), and the solutions were gently stirred for 30 min at room temperature (RT). Then, the solutions were poured in centrifugation columns and the solutions were centrifuged at 13,000 rpm during 1 min. The silica gels were washed two times with 300 µL of methanol and centrifuged in centrifugation columns. Finally, the silica gels were washed three times with 300 µL of MQ water and placed in 300 µL of MQ water.

For the preparation of Sample 2, 0.63 mg of shortened aptamer M3.2 (40 nmol) (Eurogentec, Belgium) was placed in 200 µL of MQ water. Sample 3 was prepared by placing 1.6 mg of 3'-aminated aptamer L5.5 (65 nmol) (Eurogentec, Belgium) in 200 µL of MQ water. The two solutions of 3'-NH_2 modified aptamers were mixed with the solutions of DDS treated silica, and the mixtures were gently stirred for 24 h at RT. The solutions were then centrifuged at 13,000 rpm during 2 min. The recovered silica gels were washed three times with 500 µL of MQ water and finally placed in 500 µL of MQ water.

2.3. Sample Preparation

A sample of ergot contaminated rye feed having a known concentration of ergot alkaloids was used for the extraction of ergot alkaloids. A sample of 0.5 g of the ergot contaminated flour sample was placed in 5 mL of 0.1

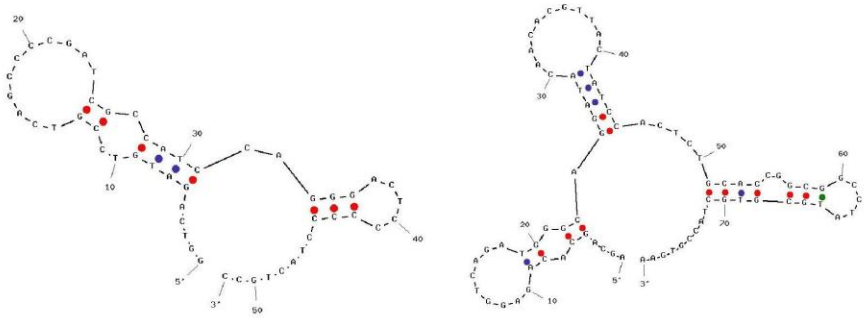

Figure 1. Secondary structures of shortened version of aptamer M3.2 (left) and aptamer L5.5 (right).

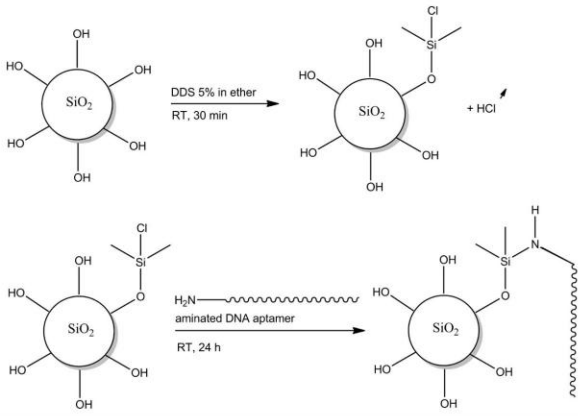

Figure 2. Chemical representation of silica gel functionalization with aminated DNA using DDS as silane-coupling agent.

M HCl and gently stirred for 1 h at RT. Then, the solution was transferred to Eppendorf tubes, which were centrifuged at 13,000 rpm for 2 min at RT. The supernatant was taken and placed in clean vials.

2.4. Specific Extraction of Ergot Alkaloids Using Aptamer-Functionalized Silica Gels

For each silica sample, 500 µL of rye feed extract were used. The reference sample was prepared by adding 500 µL of rye feed extract to the solution of unmodified silica gel. Sample 2 was prepared by mixing 500 µL of rye feed extract with the solution of aptamer M3.2 functionalized silica gel. Finally, Sample 3 was prepared by mixing 500 µL of rye feed extract with the solution of aptamer L5.5 functionalized silica gel. The three solutions were gently mixed for 1 h at RT. After centrifugation in centrifugation tubes, the supernatants were discarded, and the silica gels were washed three times with 500 µL of MQ water. For the elution step, the silica gels were recovered and placed in clean Eppendorf tubes with 500 µL 0.1 M HCl, and were then gently stirred at 70˚C for 15 min. The silica gels were centrifuged in centrifugation tubes at 13,000 rpm for 1 min and the supernatants were kept for the LC-QTOF-MS analysis.

2.5. LC-QTOF-MS Analysis

Ergot alkaloids were separated and identified by LC-QTOF-MS having a 1290 series High Pressure Liquid Chromatography (HPLC) system coupled to a 6530 quadrupole-time-of-flight mass spectrometer (QTOF-MS) (Agilent Technologies). Chromatographic separation was achieved using a Zorbax Eclipse Plus C18 column (100 mm × 2.1 mm × 1.8 µm, Agilent Technologies) and the following mobile phase composition: water 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Gradient elution was as follows: initial 5% B, linear change of B to 99%, from 0 to 5.0 min followed by isocratic elution at 99% B from 5.0 to 8.0 min, then linear change of B% to 5% until 8.1 min, then maintained at 5% B until 12.0 min in order to re-equilibrate the column. The flow rate was 0.3 mL/min and the injection volume 5 µL. Total run time was 12.0 min. The column was kept at a constant temperature of 45˚C. The QTOF-MS was run in ESI positive mode scanning m/z from 50 to 1000 amu at a scan rate of 3 spectra/s. An Agilent Jetstream source was used with the following parameters: gas temperature 275˚C, gas flow 8 L/min, nebulizer pressure 40 psi, sheath gas temperature 325˚C, sheath gas flow 11 L/min. Nozzle, capillary, fragmentor, and skimmer voltages were set to 0 V, 3500 V, 110 V, and 65 V, respectively. The instrument was calibrated during run times by monitoring positive ions with m/z 121.0508 and 922.0098. The Mass Hunter (Agilent Technologies) software was used for data acquisition and processing.

3. Results and Discussion

The ergot alkaloid content of the sample of ergot contaminated rye feed given by the LC-MS analysis performed by Diana Di Mavungu *et al.* [33] is shown in **Table 1**. The total ergot alkaloid concentration of this sample given by this analysis was 1081 µg/kg, representing a moderately high contaminated ergot sample. Ergotamine, ergosine, ergocornine and ergokryptine were the main ergot alkaloids of this sample according to this study.

The ergot alkaloids eluted from the silica gels were analyzed by LC-QTOF-MS analysis. LC-QTOF-MS technique was used because it provides a high mass accuracy (typically 2 to 10 ppm error) and a good resolution (>20,000), which is necessary to generate a formula for an unknown compound with a given m/z value. Another advantage of the LC-QTOF-MS is its ability to fragment compounds, which provides high resolution mass spectra of the product ions allowing the confirmation of the generated formula. The screening of the six main ergot alkaloids was based on a self-made comma separated value (csv) database. The accurate masses given with four digits were compared to the theoretical monoisotopic masses, with the experimental error given in ppm.

In the LC-QTOF-MS analysis of the samples after extraction using the aptamer-functionalized silica gels, the following data were analyzed: the presence of the six main ergot alkaloids, the presence of the ergot alkaloid precursor fragment at m/z 223.1283, and finally the presence of other compounds than ergot alkaloids. The samples were analyzed in positive ion mode; and the hydrogen atom mass was subtracted in the final results. **Table 2** shows the compounds found in the three samples tested. In Sample 1, resulting from the elution from non-functionalized silica gel, no ergot alkaloid could be found, neither the ergot alkaloid precursor fragment. No other compound was detected in this sample. In Sample 2, resulting from the elution from aptamer M3.2 functionalized silica gel, two ergot alkaloids, were found: ergosine at m/z 547.2874 (theoretical m/z 547.2794) and ergokryptine 575.3085 (theoretical m/z 575.3107). The fragment at m/z 223.1283 was found for both the ergot

Table 1. LC-MS analysis of the ergot alkaloid content of the ergot contaminated rye sample [12] [33].

Ergot alkaloid	Concentration (µg/kg)
Ergometrine/inine	50
Ergosine/inine	416
Ergotamine/inine	337
Ergocornine/inine	126
Ergokryptine/inine	126
Ergocristine/inine	26
Total ergot alkaloids	1081

Table 2. LC-QTOF-MS analysis of the ergot alkaloid content after elution from silica gels.

Sample	Ergot alkaloids	Retention time (min)	Theoretical monoisotopic mass	Determined monoisotopic mass	Score mass	Differential mass (ppm)	Fragment m/z 223.1283
1 Elution silica	None						
2 Elution aptamer M3.2	Ergosine	5.55	547.2794	547.2874	98.11	−1.88	Yes
	Ergokryptine	5.7	575.3107	575.3085	92.05	3.88	Yes
3 Elution aptamer L5.5	Ergosine	5.59	547.2794	547.2797	99.88	−0.48	Yes
	Ergokryptine	5.8	575.3107	575.3112	99.71	−0.72	Yes
	Ergocornine	5.669	561.2951	561.2916	81.13	6.2	Yes

alkaloids detected. No other compound or impurity were found, or were present at a very low level not detectable by this analysis. In Sample 3, resulting from the elution from aptamer L5.5 functionalized silica gel, three ergot alkaloids were found: ergosine at m/z 547.2797 (theoretical m/z 547.2794), ergokryptine at m/z at 575.3112 (theoretical m/z 575.3107) and ergocornine at m/z 561.2916 (theoretical m/z 561.2951). The ergot alkaloid precursor fragment at m/z 223.1283 was found for the three ergot alkaloids detected in the sample. No other compound was detected in this sample as well. The experiment was repeated three times and gave similar results. These results show that the presence of aptamers in the silica gel allowed the specific extraction of ergot alkaloids from the sample. However, the other ergot alkaloids present in the sample; such as ergotamine, ergometrine and ergocristine; were not retained by the aptamer-functionalized silica gels, or were present at a very low level not detectable by this analysis.

In this study, it was observed that aptamers selected for ergot alkaloids were highly specific, having a molecular recognition restricted to a few related chemical compounds within the ergot family group. In comparison to other biorecognition elements developed for ergot alkaloids, similar specificity was obtained with antibodies [34]. Having a similar molecular affinity towards ergot alkaloids, aptamers present several advantages over antibodies, such as their chemical robustness and their animal-friendly synthetic production. In comparison to MIPs, it was observed that aptamers were more specific and had a more restricted range of recognition [16] [17]. Depending on the use of the extraction system, it can be said that MIPs are suited for a general extraction of ergot alkaloids, while aptamers can be used for a more specific extraction of certain ergot alkaloids. It is also possible to envisage a more complete extraction system by mixing different aptamer-functionalized silica gels. This would allow the modulation of the extraction process to a more or less important range of molecules within the ergot alkaloid family. Concerning the extraction solution, only 0.1 M HCl was used; which can be easily neutralized by a 0.1 M basic solution. This study shows the applicability of aptamers for extraction purposes, offering a new range of possibilities for the specific extraction of toxins, natural compounds or pollutants from food or environmental matrices.

4. Conclusion

Aptamer-functionalized silica gels were realized in order to extract ergot alkaloids from ergot contaminated rye

feed. The ergot alkaloids eluted from the aptamer-functionalized silica gels were measured by LC-QTOF-MS. It was shown that the aptamer-functionalized silica gels could successfully be used to extract three ergot alkaloids from the sample, namely ergosine, ergokryptine and ergocornine. The other compounds present in the sample were not retained by such a system. The DNA aptamers were neither denatured by the silica functionalization, nor by the acidic solution used for the extraction of ergot alkaloids. Although aptamers were mainly developed for sensing purposes, this study shows that it is also possible to use aptamers for the specific extraction of compounds. This type of system can be easily applied for food clean-up, extraction of toxins or contaminants from various environmental or food samples, as well as the specific extraction of natural compounds from turbid matrices.

Acknowledgements

This study was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment (FOD) project Ergot RF6204. The authors thank Dr. Abdelkrim Azmi for his help and support.

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Biosensors and Bioelectronics 26 (2010) 1178–1194

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Review

Recent advances in recognition elements of food and environmental biosensors: A review

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ARTICLE INFO

Article history:
Received 30 April 2010
Received in revised form 8 July 2010
Accepted 11 July 2010
Available online 17 July 2010

Keywords:
Recognition elements
Biosensor
Phage
Nucleic acid
Molecular imprinted polymer (MIP)

ABSTRACT

A sensitive monitoring of contaminants in food and environment, such as chemical compounds, toxins and pathogens, is essential to assess and avoid risks for both, human and environmental health. To accomplish this, there is a high need for sensitive, robust and cost-effective biosensors that make real time and *in situ* monitoring possible. Due to their high sensitivity, selectivity and versatility, affinity-based biosensors are interesting for monitoring contaminants in food and environment. Antibodies have long been the most popular affinity-based recognition elements, however recently a lot of research effort has been dedicated to the development of novel recognition elements with improved characteristics, like specificity, stability and cost-efficiency. This review discusses three of these innovative affinity-based recognition elements, namely, phages, nucleic acids and molecular imprinted polymers and gives an overview of biosensors for food and environmental applications where these novel affinity-based recognition elements are applied.

Contents

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doi:10.1016/j.bios.2010.07.033

Chapter Number X

The use of phages and aptamers as alternatives to antibodies in medical and food diagnostics

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1. Introduction

In the post-genomic and proteomic era, there is a better understanding of important physiological components such as DNA, RNA, proteins and small biological molecules, all of which have proven to be the mediums of disease progression. Identification and investigation of disease-specific biomarkers in the initial stage of a disease can greatly increase accuracy in diagnosis, treatment and even prevention. This approach offers great potential to significantly reduce disease-related mortality rates. There is an increasing need in the medical field for rapid, cheap and reliable diagnostic systems in order to detect all the well-known and recently identified biomarkers for different diseases. This identification is not a trivial exercise because these disease biomarkers are present in minute quantities in physiological conditions such as the bloodstream or body fluids which are often contaminated with many other compounds that can hinder detection. Apart from biomarker diagnosis, another area of concern for human health has been food contamination. Trading of contaminated food between countries and high population mobility increases the potential for outbreaks and health risks posed by microbial pathogens and toxins in food. Food safety has become a global health goal. Periodic toxin and microbiological analyses of food samples are important to diagnose and prevent problems related to health and food safety. However, food-borne pathogens are mostly present in very low numbers among various other microorganisms, making their detection difficult. To be able to detect these disease carriers and biomarkers in their natural conditions, highly sensitive as well as specific recognition elements are required. It is necessary to develop detection techniques that are reliable, fast, easy, sensitive, selective, cost-effective and also suitable for real time, *in situ* monitoring. Such techniques to detect pathogens and biomarkers would not only improve clinical success rates but also offer a great commercial advantage to the medical field and the food industry.

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Selection and Characterization of PCB-Binding DNA Aptamers

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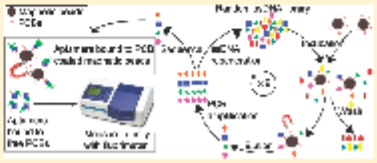
Supporting Information

ABSTRACT: Polychlorinated biphenyls (PCBs) are persistent organic pollutants (POPs) that resist natural degradation and bioaccumulate in nature. Combined with their toxicity, this leads them to cause cancer and other health hazards. Thus, there is a vital need for rapid and sensitive methods to detect PCB residues in food and in the environment. In this study, PCB-binding DNA aptamers were developed using PCB72 and PCB106 as targets for aptamer selection. Aptamers are synthetic DNA recognition elements which form unique conformations that enable them to bind specifically to their targets. Using in vitro selection techniques and fluorometry, an aptamer that binds with nanomolar affinity to both the PCBs has been developed. It displayed high selectivity to the original target congeners and limited affinity toward other PCB congeners (105, 118, 153, and 169), suggesting general specificity for the basic PCB skeleton with varying affinities for different congeners. This aptamer provides a basis for constructing an affordable, sensitive, and high-throughput assay for the detection of PCBs in food and environmental samples and offers a promising alternative to existing methods of PCB quantitation. This study therefore advances aptamer technology by targeting one of the highly sought-after POPs, for the first time ever recorded.



Polychlorinated biphenyls (PCBs) are a group of 209 individual congeners that belong to a broad family of man-made organic chemicals known as chlorinated hydrocarbons. They were widely used as lubricants and coolants in a variety of industrial and electrical applications, because of their nonflammability, chemical stability, high boiling point, and electrical insulating properties.[1] However, these very characteristics make them resistant to natural degradation and persistent in the environment,[2] and therefore they have been classified as persistent organic pollutants (POPs), along with several other compounds.[3] Their natural propensity to bioaccumulate and biomagnify, combined with their toxicity, leads them to cause cancer[4] and other adverse health effects on the immune system, reproductive system, nervous system, and endocrine system.[5] Even though production was banned more than two decades ago in several countries (76/403/EEC which refers to the directive of the council of the European community), trace levels of PCBs are still present in the environment, continuing to pose a major threat to public health.[6] The possible sources of exposure to these ubiquitous environmental pollutants include consuming food and water containing PCBs, breathing air with PCBs, and occupational health exposures.[7] However, the most common source of exposure is from eating PCB-contaminated foods such as fish, eggs, red meat, poultry, milk, and cheese.[8] The Food and Drug Administration (FDA) has designated tolerance levels for PCBs that range between 0.2 and 3 ppm in food, and these residue limits are used to determine whether or not a food is safe to consume. Sensitive and cost-effective monitoring systems that enable rapid screening for PCBs in food and environmental samples are urgently needed to ensure food safety and to minimize risks for human and environmental health.

Currently, suspicious samples are routinely tested by gas chromatography−mass spectrometry (GC-MS) methods which are sensitive techniques[9] but have some limitations such as the extensive costs incurred from sophisticated equipment and highly trained personnel. The conventional biosensors, which use enzymes or antibodies as recognition elements,[10] are more cost-effective but lag in terms of stability and production time. Moreover, antibodies are obtained by immunizing animals and therefore, besides raising ethical concerns, they also cannot always be generated for small toxic agents or nonimmunogenic analytes. The limitations of these existing techniques have therefore propelled considerable research efforts toward the advancement of rapid, accurate, and economic alternative recognition elements. Aptamers, which are nucleic acid ligands, fulfill the current research requirements for affinity molecules and have therefore surfaced as promising alternatives. This class

Received: November 8, 2011
Accepted: December 14, 2011
Published: December 14, 2011

Journal of Biotechnology 155 (2011) 361–369

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

In vitro selection and characterization of DNA aptamers recognizing chloramphenicol

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ARTICLE INFO

Article history:
Received 31 December 2010
Received in revised form 2 May 2011
Accepted 24 June 2011
Available online 4 August 2011

Keywords:
DNA aptamers
Antibiotics
Chloramphenicol
Detection
Molecular recognition

ABSTRACT

Chloramphenicol (Cam), although an effective antibiotic, has lost favour due to some fatal side effects. Thus there is an urgent need for rapid and sensitive methods to detect residues in food, feed and environment. We engineered DNA aptamers that recognize Cam as their target, by conducting *in vitro* selections. Aptamers are nucleic acid recognition elements that are highly specific and sensitive towards their targets and can be synthetically produced in an animal-friendly manner, making them ethical innovative alternatives to antibodies. None of the isolated aptamers in this study shared sequence homology or structural similarities with each other, indicating that specific Cam recognition could be achieved by various DNA sequences under the selection conditions used. Analyzing the binding affinities of the sequences, demonstrated that dissociation constants (K_d) in the extremely low micromolar range, which were lower than those previously reported for Cam-specific RNA aptamers, were achieved. The two best aptamers had G rich (>35%) nucleotide regions, an attribute distinguishing them from the rest and apparently responsible for their high selectivity and affinity ($K_d \sim 0.8$ and 1 μM respectively). These aptamers open up possibilities to allow easy detection of Cam via aptamer-based biosensors.

1. Introduction

Veterinary drugs are often administered to farm animals for therapeutic and prophylactic purposes (Prescott, 2008; Winckler and Grafe, 2001). However, only a small percentage of the ingested antibiotics are metabolized by the animals, leaving a significant proportion to be either accumulated in their tissues or excreted and emitted into the environment (Boxall, 2010). The occurrence of antibiotics in the environment endorses antibiotic resistance and raises food safety issues since antibiotic resistance can be transmitted to humans via the food chain (Girardi and Odore, 2008). One such antibiotic is chloramphenicol (Cam). Cam inhibits translation, by binding prokaryotic ribosomes and blocking the peptide bond formation (Jardetzky, 1963). Although an effective antimicrobial drug, Cam has lost favour due to serious side effects such as aplastic anemia (Burton et al., 1988), leukaemia (Smith et al., 2000) and gray baby syndrome (Mulhall et al., 1983). To protect consumer health, maximal residue limits of veterinary medicinal products in foodstuffs of animal origin have been established according to European Union regulation (Regulation (EU) No. 37/2010). More specifically, in the case of Cam, it is a forbidden substance, and a minimum required performance limit of 0.3 μg/kg (Decision 2002/657/EC) has been designated, in particular as a decision limit for products coming from outside the European Union.

For fast and sensitive detection of Cam residues in food, feed and environment, chemically analytical methods do exist (Gantverg et al., 2003), but they have limitations in terms of costs incurred by the sophisticated equipments and the highly trained personnel required. The conventional affinity-based assays, which use enzymes or antibodies as recognition elements (Fodey et al., 2007), are more cost-effective, but lag in terms of detection time. Thus, there is a pressing need for rapid, accurate and economic alternative recognition elements. Aptamers fulfil these requirements and have therefore emerged as a viable option. Aptamers are artificial nucleic acids ligands (DNA or RNA) able to specifically recognise a given target, ranging from small molecules (Mann et al., 2005) to whole cells (Cerchia and de Franciscis, 2010). The name *aptamer* derives from the Latin word *aptus* meaning *to fit*, and the Greek word *meros* meaning *part* or *portion*, referring to the folding properties of single-stranded nucleic acids, responsible for their specific three-dimensional structures. This flexibility of aptamers results in

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doi:10.1016/j.jbiotec.2011.06.043