New method for determination of β-lactam antibiotics by means of Diffuse Reflectance Spectroscopy using polyurethane foam as sorbent

Vom Fachbereich Chemie
der Universität Duisburg-Essen
(Standort Duisburg)

zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
genehmigte Dissertation

von
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Tag der mündlichen Prüfung:	13.12.2005

The result of these years of work is dedicated to…

The memory of my grandparents, Carlos and Sofia,
whose presence and love I still feel.

My father, Rafael, from whom I learned
honesty and solidarity.

My mothers, Lucia and Sabine for their care and love
in the two historic points of my life.

Rosalia, for her love and staying power, and
because her companionship made it possible for me to move forward.

Bruno and Sofia, for all their embraces,
much needed at the end of every day.

Rafaela, in the nearness and
Tanya and Wladymir in the distance.

Carlos Alberto, for his
unconditional support over the years.

Stella, Vasilis, Apollon and Natali,
my family in Greece, for all the sun we shared together.

Cristobal, who was also a father to me.

Phillipe, in remembrance of The Andes and The Alps.

David, Francisco, Julio, Wilo, Roberto, Dimitris and Armando
for every song, every laughter, every sorrow and every goal we shared together.

Don Lucho, who taught me to plough and sow.

And finally, to Jorge Enrique
for all the bohemian nights.

ACKNOWLEDGEMENTS

I think that this is the hardest section to write, especially because if I have forgotten to mention someone, it can be considered as an offense and not as a slight lapse of mind. Therefore if someone is not mentioned below, my apologies in advance.

First, I express my sincere gratitude to Professor Dr. Heinz-Martin Kuss who gave me the opportunity to be a member in his research group and who guided the development of this research with his suggestions, patience and his wonderful disposition, kindness and humanity.

I particularly want to thank Dr. Evelin Denkhaus and Dr. Ursula Telgheder for their recommendations and unconditional support.

I must acknowledge to Dr. Holger Krohn, Mr. Werner Kaiser, Mr. Gerd Fischer, Dr. Bernd Wermeckes, Mrs. Claudia Ullrich and Mrs. Roswitha Schragmann for their assistance during my time of study.

I especially want to thank my friends I made here: Rajab El-Kailany, Stefan Meisen and Nabil Bader for their advice and solidarity in every moment.

I am particularly grateful to Kirstin Claussen for her help during the writing process of this thesis.

I want to thank Professor Dr. Karl Molt, Professor Dr. Alfred Golloch, Salam Asweisi, Wassef Al Sekhaneh, Khaled Elsherif, Tao Tang, Amila Bilic, Matthias Klug, Paulina Rutarova, Alfred Schlachter, Gregor Aschenbroich, Myint Sein, Nonhlangabezo Mabho, Bülend Bayraktar and Yevgen Berkhoyer for the friendly atmosphere they created throughout my time in the department.

Finally, I am really grateful with Professor Dr. Wibren S. Veeman for his significant corrections and final suggestions.

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ATP	Adenosin tri-phosphate
DNA	Deoxyribonucleic acid
DRS	Diffuse reflectance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
F(R)	Kubelka-Munk value for reflectance
GC	Gas chromatography
HPA	Heteropoly acid
HPMo	Phosphomolybdic acid
HPLC	High performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
MS	Mass spectrometry
PABA	p-aminobenzoic acid
PEC	Predicted environmental concentration
PUF	Polyurethane foam
RF	Retention factor
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RSD	Relative standard deviation
SPE	Solid phase extraction
STP	Sewage treatment plant
TLC	Thin layer chromatography
STP	Sewage treatment plants
UV	Ultraviolet

CHAPTER ONE – INTRODUCTION

2000 B.C. – Here, eat this root
1000 A.D. – That root is heathen.
Here, say this prayer.
1850 A.D. – That prayer is superstition.
Here, drink this potion.
1920 A.D. – That potion is snake oil.
Here, swallow this pill.
1945 A.D. – That pill is ineffective.
Here, take this penicillin.
1955 A.D. – Oops....bugs mutated.
Here, take this tetracycline.
1960 -1999 – 39 more "oops"... Here,
take this more powerful antibiotic.
2000 A.D. – The bugs have won!
Here, eat this root.

Anonymous

If we look at the human history, we can realize that our ability to control infectious diseases is a recent development. The strongest advance in chemotherapy has been the discovery and industrial production of antibiotics, chemical substances, isolated from living organisms as molds and actinomycets, which are able to inhibit the life processes of microorganisms.

Nevertheless, the introduction of the antimicrobial agent into the microbial surroundings represents a change in the cell environment. If all microorganisms are sensitive to the change, then the whole population will be eliminated. But if few members in the population, which were resistant to the effects of the antibiotic, could survive and could reproduce, a new population will grow up. This new population has a different DNA than the original population because they have mutated and this allows the microorganisms to resist the effect of the antibiotic.

Antimicrobial resistance is an inevitable natural biological phenomenon that is exacerbated by the abuse, overuse and misuse of antimicrobials in the treatment of human illness and in animal husbandry, aquaculture and agriculture. Disease – and therefore resistance – also thrives in conditions of civil unrest, poverty, mass migration and environmental degradation where large numbers of people are exposed to infectious diseases with little in the way of the most basic health care. Drug resistance is the most telling sign that we have failed to take the threat of infectious diseases seriously. It suggests that we have mishandled our precious arsenal of disease-fighting drugs, both by overusing them in developed nations and, paradoxically, both misusing and underusing them in developing nations (WHO, 2000).

The use of antibiotics generates wastes with considerable amount of unaltered antibiotic, since the biological absorption of these antimicrobial agents under the metabolic pathways in humans and animals is around 30%; or, what is the same, 70% of an administrated doze is excreted unchanged by the living organism and disposed into the waste water system. From the whole antibiotics used in human medicine and animal veterinary, β-lactam antibiotics reach 65% of the total amount of administrated antibiotics and 72% of the total amount of emitted antibiotics.

Aiming specially to hospitals and husbandry farms where antibiotics are used daily, there is a necessity to perform cheap analytical methods for direct determination in the field, and to keep the expensive laboratory examinations only for confirmatory analysis. Besides, in 1998, the U.S. Geological Survey began to discuss the topic of pharmaceuticals and other organic wastewater contaminants in the environment, stating that there were “limited to no current methods for looking at these emerging compounds”. Although analytical methods to analyze individual compounds exists, the key is to develop methods in which 20-30

compounds can be determined in a single analysis since “if each compound had its own method, it would be so inefficient and expensive” (Erickson, 2002).

There are, of course, fast methods to analyze antibiotics for application in the field, all of them based even on the inhibition of growth of microbial test organisms, ligand assays using biological receptors or antibodies configured in an enzyme-linked immunoassay. These microbiological tests have the advantage to be conducted rapidly, providing an accept/reject decision at the farm but they have the disadvantage to be used in a limited or specific kind of sample and mostly handled only by persons with experience. Fast methods for analyzing antibiotics and for direct determination in the field, based only on a chemical principle, have not yet been applied.

CHAPTER TWO – FUNDAMENTS

"Antibiotic resistance as a phenomenon is, in itself, not surprising. Nor is it new. It is, however, newly worrying because it is accumulating and accelerating, while the world's tools for combating it decrease in power and number."

Joshua Lederberg

2.1 ANTIBIOTICS: THE ANALYTE

2.1.1 ORIGINS AND MAJOR CLASSES

Antibiotics (Greek: *anti* = against and *bios* = life) are chemical substances that inhibit or kill microbes like bacteria and fungi. Antibiotics that stop bacteria from growing are called *bacteriostatic* (e.g. chloramphenicol) while antibiotics that cause bacteria cell death are called *bactericidal* (e.g. penicillin). Antibiotics can be natural products or synthetic chemicals, designed to block some crucial process in microbial cells.

Natural antibiotics are almost all products of secondary metabolic pathways, elaborated when the antibiotic-producing microorganisms enter into stationary phases and face competition for space and nutrients. In that moment, they turn on the genes that encode the antibiotic molecules and use them as chemical weapon to regulate the growth of their neighbors. Then, the antibiotic-producing microorganisms have a selective advantage for growth, including access to nutrients from their dying neighbors, and will have selective pressure to maintain the antibiotic-producing pathways and to turn them on in times of need.

Nevertheless, antibiotic-producing microorganisms need self-protection or autoimmunity mechanisms to protect themselves from the lethal chemical weapons they are producing. In that way, they employ a variety of strategies: the tightly coupled export of the mature antibiotic from the producing cell into the external medium to keep intracellular concentrations low in the producing organism; the antibiotic is exported while still inactive and one step away from the final enzymatic maturation, which happens extracellularly; to alter the structure of their own cell walls, modify the peptidyltransferase component of the protein synthesis machinery on the bacterial ribosomes or produce desensitizing structural mutations in DNA replication enzymes to provide protection from self–destruction.

The major class of antibiotics are natural products or semisynthetic derivatives: β-lactam antibiotics, tetracyclines, aminoglycosides, macrolides, chloramphenicol while three classes are completely synthetic: sulfa drugs, quinolones and oxazolidones. New generations of semisynthetic variants of β-lactam antibiotics and macrolides have been developed to obtain some desired new properties, such as oral bioavailability, increased stability, broader spectrum of activity and efficacy against resistant microbes (Walsh, 2003).

2.1.2 TARGETS AND MODES OF ACTION

Antibiotics inhibit or kill microorganisms by inflicting direct damage upon specific cellular targets, particularly in one of the following ways: they inhibit cell wall biosynthesis; they inhibit protein synthesis; they inhibit nucleic acid synthesis or they inhibit the folic acid biosynthesis (Talaro and Talaro, 2002).

The cell walls of most bacteria contain a rigid girdle of peptidoglycan, which protects the cell against rupture from hypotonic environments.

Active cells must constantly synthesize new peptidoglycan and transport it to its proper place in the cell envelope. β-lactam antibiotics (*penicillins, cephalosporins*) bind with the transpeptidases, the enzymes required to complete these process, causing the cell to develop weak points at growth sites and to become osmotically fragile, where under normal circumstances, the osmotic pressure difference inside the cell, as compared to outside, lyses the cell (Fig. 2.1). It is essential to note that most of these antibiotics are active only in young-growing cells, because old, inactive or dormant cells do not synthesize peptidoglycan (Talaro and Talaro, 2002).

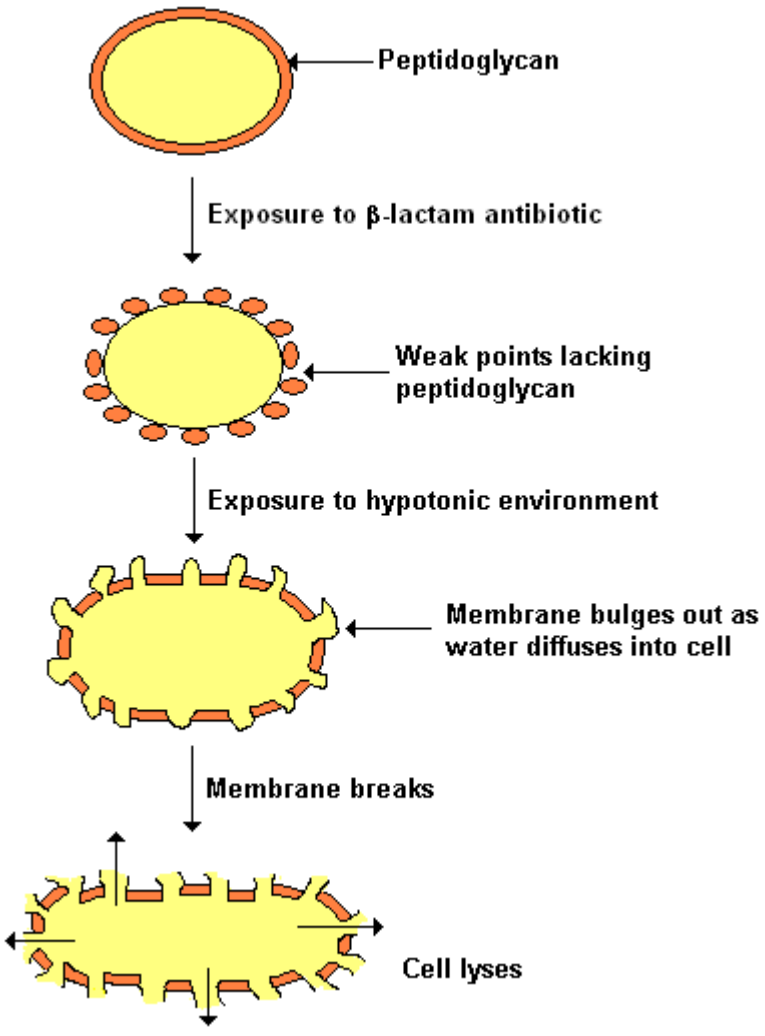


Fig. 2.1 The consequences of exposing a growing cell to a β-lactam antibiotic. (Talaro and Talaro, 2002).

By contrast, vancomycin, a glycopeptide antibiotic does not bind with the transpeptidases but acts directly on the terminal D-alanyl-D-alanine peptide on the peptidoglycan precursors, blocking the transpeptidases reaction. Because the cell wall and its synthesis mechanisms are unique to bacteria, the β-lactam antibiotics have very high specificity and are not toxic to host cells. However, because of the complex structural configurations of these antibiotics, some individuals develop serious antibody-mediated allergies to individual β-lactam compounds after repeated courses of antibiotic therapy (Madigan et al., 2000).

One of the most extensively studied modes of action is that of the sulfonamides. Because these synthetic drugs interfere with an essential metabolic process in bacteria, they represent a model for competitive inhibition. They act as structural or metabolic analogs that mimic the natural substrate of an enzyme and compete for its active site. Sulfonamides are very similar to the natural metabolic compound p-aminobenzoic acid (PABA) required by bacteria to synthesize the coenzyme tetrahydrofolic acid, which participates in the synthesis of purines and certain amino acids.

A sulfonamide molecule has high affinity for the PABA site on the enzyme (Fig. 2.2) and can successfully compete in a chemical race with PABA to occupy those sites. Sulfonamides ultimately cause an inadequate supply of tetrahydrofolic acid for purine production, which invariably halts nucleic acids synthesis and prevents bacterial cells from multiplying. Sulfonamides are valuable in therapy because they inhibit bacteria and certain fungi, but not mammalian cells. Although humans require tetrahydrofolic acid for nucleic acid synthesis as much as bacteria do, humans cannot synthesize it because human cells lack this special enzymatic system. Thus, it is an essential nutrient that must come from the diet, and human metabolism cannot be inhibited by sulfa drugs (Talaro and Talaro, 2002).

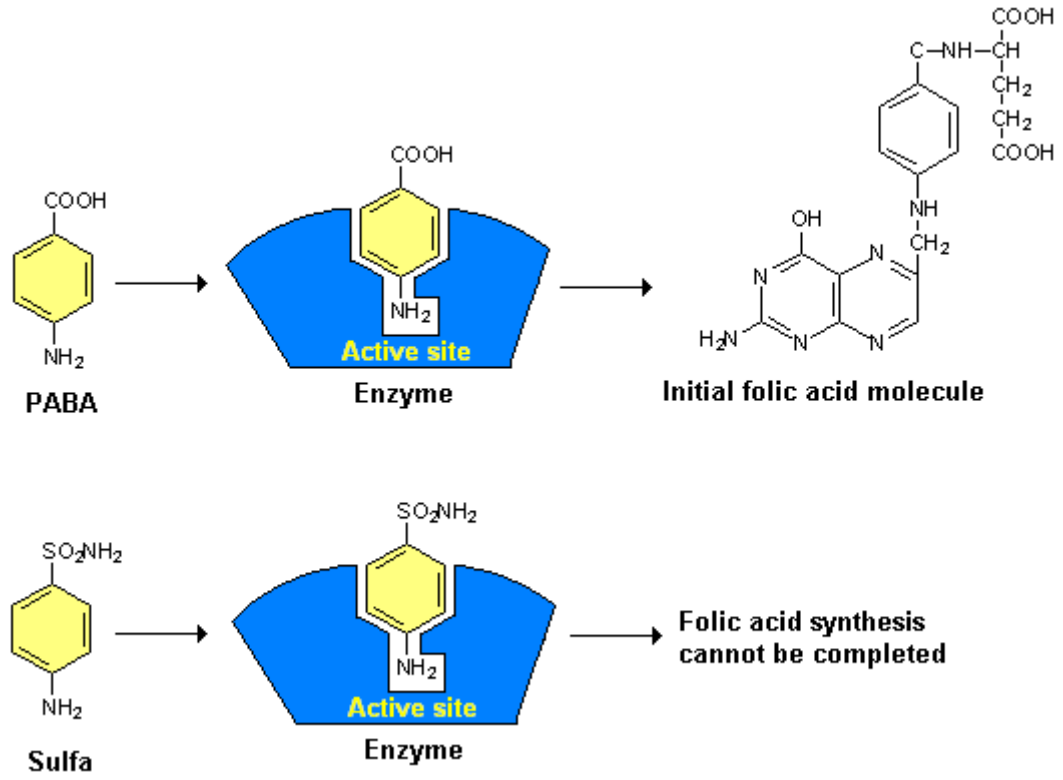


Fig. 2.2 The mode of action of sulfa drugs. (Talaro and Talaro, 2002).

Aminoglycosides (*streptomycin, kanamycin, gentamycin, neomycin*), macrolides (*erythromycin, tylosin*), tetracyclines and chloramphenicol act by inhibiting protein biosynthesis. They block the subunits known as 30S and 50S of the bacterial ribosome and cause the misreading of the mRNA, leading to abnormal proteins. Although human cells also have ribosomes, the ribosomes of eucaryotes are different in size and structure from those of prokaryotes, so these antibiotics usually have a selective action against bacteria (Talaro and Talaro, 2002).

Quinolones antibiotics (*ciprofloxacin, nalidixic acid*) inhibit the nucleic acid biosynthesis. They bind with enzymes *gyrase* and *topoisomerase IV* responsible for coiling and uncoiling the DNA molecule, a necessary process for DNA replication and transcription (Walsh, 2003).

2.1.3 PRODUCTION AND USE

Antibiotics are widely used in human and veterinary medicine for the treatment of several infectious diseases and in agriculture as feed additive for prophylaxis to prevent infection (Oka et al., 1995).

The production of antibiotics for human clinical use, categorized by its economic impact, indicates that β-lactam antibiotics represented 54% (for the year 1994) (Madigan et al., 2000) and 56% (for the year 1995) (Walsh, 2003) of the total worldwide antibiotic market.

In 1996, about 10200 tons of antibiotics were used in the EU, of which approximately 50% was applied in veterinary medicine and as growth promoters. In 1999, 13288 tons of antibiotics were used in the EU and Switzerland, of which, 65% was used in human medicine, 29% was used in the veterinary field and 6% as growth promoters. Since most growth promoters have now been banned within the EU, only four compounds remain in this group of feed additives. This may explain the decline in the use of antibiotics in animal husbandry compared with human medicine. In the USA, 16200 tons were produced in 2000 of which 70% was used in livestock farming. This is eight times the amount used in human medicine (Kümmerer, 2003).

Reports about the use of antibiotics in both human and veterinary medicine, show that the use of antibiotics keeps a general trend: β-lactam antibiotics are the most frequently prescribed drug for treatment of infections in developed and developing countries. In Norway, for the years 1988 - 1989, penicillins accounted for 38.2% of total antibiotic prescriptions, with phenoxymethylpenicillin (penicillin V) as the most frequently prescribed penicillin with 31.8% (Straand et al., 1998). In 1995 in Sharjah, United Arab Emirates, the prescription of antibiotics was 45% of the total drugs prescribed, with penicillins (most frequently amoxicillin)

with 74% and cephalosporins with 9% of the total antibiotic prescriptions (Hasan et al., 1997).

For the years 1993 to 1999 in England, 90% of drugs prescribed to treat infections were antibiotics, with penicillins as the most commonly prescribed antibiotics and with amoxicillin as the most commonly prescribed penicillin, accounting for 61% of the prescriptions (NPC, 2005). In 1998 in Saudi Arabia, 87.1% of all medical prescriptions for acute respiratory infections contained antibiotics. From the total drugs prescribed, antibiotics accounted for 35.5%, with amoxicillin as the most frequently prescribed antibiotic with 48%, followed by other penicillins with 23% (El-Gilany, 2000).

During 1999-2000 in Babol, Iran, an overall antibiotic prescribing rate of 61.9% was recorded, similar to rates reported in England, 60.7%; Norway, 48.0% and Sudan, 63.0% (Moghadamnia et al., 2002). Respiratory tract infections and sore throat accounted for more than 50% of antibiotic prescription, where penicillins were the main antibiotic class consumed, regardless of clinical situation and age. Cephalosporins and macrolides were also frequently used, particularly to threat respiratory tract infections in adults (Sommet et al., 2004).

The most frequent use of β-lactam antibiotics in veterinary medicine is for the treatment of mastitis, the most common disease affecting dairy cows. Benzylpenicillin (Penicillin G), ampicillin, cloxacillin, dicloxacillin and nafcillin are mainly administered by various routes to treat cows in Japan (Takeba et al., 1998). In Sweden, approximately 20% of the cows are treated each year (Gustavsson, 2003). Nevertheless, information on the use of antibiotics in livestock is severely limited and the lack of data in this field is worse than in human therapy as a result of animal antibiotic use is not being documented (Huang et al., 2001).

2.1.4 EMISSION AND FATE IN THE ENVIRONMENT

After administration, a significant amount of unaltered antibiotic is excreted from the body and therefore, disposed into drainage system. If the antibiotic is not completely removed or deactivated in the sewage treatment plants (STP), the antibiotic will be released into surface water and it will take part of topsoil in fields (Halling-Sorensen et al., 2000). This is a critical point, because normally, there are no STP at all in most hospitals, agricultural livestock farms and whole cities in the countries of the South Cone.

Although little is known about the extent of environmental occurrence, transport and ultimate fate and effects of pharmaceuticals in general (Kümmerer, 2003), it is sure that antibiotics are not bioaccumulative, but their continual input into the environment gives them a persistent quality (Erickson, 2002), added to the effect of low biodegradation rates in soil (Al-Ahmad et al., 1999).

Estimated concentrations of antibiotics in untreated wastewater in USA, including metabolism, show values of 16 µg/L for amoxicillin, 12 µg/L for cephalexin and 2.8 µg/L for penicillin G. These estimations have only considered antibiotics used in human medicine (Sedlak and Pinkston, 2001).

A meticulous study for Germany about the consumption of antibiotics in hospitals and households and their corresponding emission and predicted environmental concentration (PEC) into effluents and municipal sewage shows that β-lactam antibiotics represent 65% (penicillins with 49% and cephalosporins with 16%) of the total amount of antibiotics used and 72% (penicillins with 53% and cephalosporins with 19%) of the total amount of antibiotics emitted into the sewage water system (Fig 2.3) (Kümmerer and Henninger, 2003).

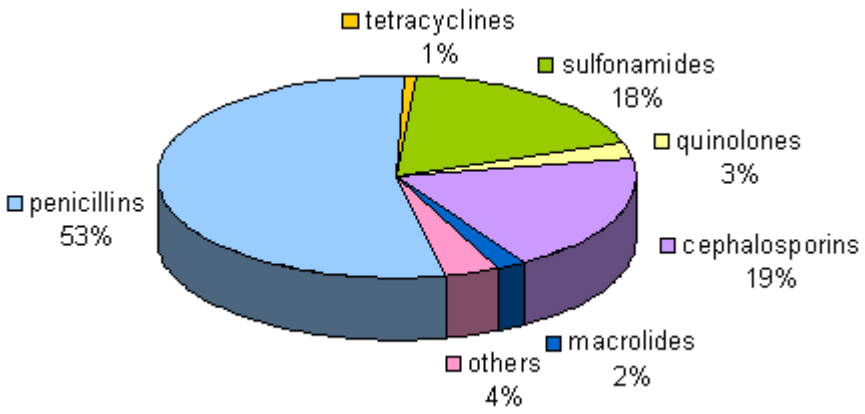


Fig. 2.3 Percent emission of antibiotics into effluents. (Kümmerer and Henninger, 2003).

The emission of antibiotics from hospitals and households into municipal sewage water present an annual PEC about 71 µg/L. Of this, 38.3 µg/L is attributable to penicillins (25.7 µg/L for amoxicillin) and 13.5 µg/L to cephalosporins; while the effluent from four hospitals before reaching municipal sewage presents PEC between 800 and 1100 µg/L, where 400 µg/L is attributable to penicillins and 390 µg/L to cephalosporins (Fig. 2.4). The PEC concentrations correspond entirely to data reported for the analysis of hospital effluent and the influent and effluent of municipal STP. For this reason, the quality of the data is sufficient to permit their use for risk assessment. Besides, the antibiotics used in Germany are most frequently used throughout Europe (Kümmerer and Henninger, 2003).

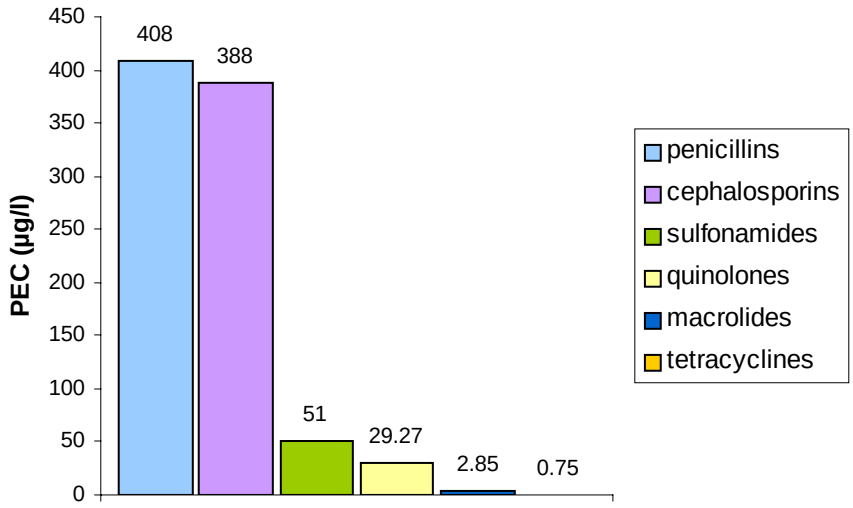


Fig. 2.4 Predicted environment concentrations of antibiotics in hospital effluents. (Kümmerer and Henninger, 2003).

2.1.5 ANTIMICROBIAL DRUG RESISTANCE

Twenty years ago, physicians in industrialized nations believed that infectious diseases were a scourge of the past. Even more, major drug manufacturers turned away from intensive antibacterial research, and concentrated their energies on seeking cures for heart disease, Alzheimer's and other chronic diseases closing the door on further research into new drugs designed to combat bacterial infections. Soon, researchers discovered that pathogens develop resistance to antimicrobials through a process known as natural selection. When a microbial population is exposed to an antibiotic for example, more susceptible organisms will succumb, leaving behind only those resistant to the antimicrobial attack (WHO, 2000).

2.1.5.1 How resistance develops and spreads

The property of antibiotic resistance can be intrinsic as well as acquired. *Intrinsic resistance* exists naturally and has been so since long

time ago before antibiotics were introduced into human life. *Acquired resistance* represents the ability of a microorganism through specific genetic changes to resist the effects of a chemotherapeutic agent to which it is normally susceptible (Sjölund, 2004).

The use of antibiotics led to microbial drug resistance as an adaptive response in which microorganisms begin to tolerate an amount of drug that would ordinary be inhibitory. The development of mechanisms for circumventing or inactivating antimicrobial drugs is due largely to genetic versatility and adaptability of microbial populations (Talaro and Talaro, 2002).

Acquired resistance is driven by two genetic processes in bacteria: *vertical evolution* that involves mutation and selection, and *horizontal evolution* that involves exchange of genes between strains and species. Vertical evolution comes from a spontaneous mutation in the bacterial chromosome which imparts resistance to a member of the bacterial population (Aliaga, 2001).

The large numbers of bacterial cells in a population and the short generation times facilitate the development of mutants (Bacterial DNA replication machinery may produce one error in 10^7 cellular divisions; in replication of a 3 x 10^6 base par genome containing about 3000 genes, that is 0.3 errors per generation. If there are 10^{11} bacteria in a population, then there may be 1000 mutant variants. If the mutations are randomly distributed through the bacteria genome, then 1000 genes, one out of every three, will have a mutation. If one of these confers a selective advantage for survival, e.g., in the presence of a given antibiotic, then the resistant bacterium will be selected for, grow up as its neighbors perish, and take over the culture. This can happen in a matter of days in patients being treated with antibiotics (Walsh, 2003).

Without the selective pressure it is very difficult to fix a mutation in a population, but the influence of the antibiotic spread out the resistant mutant where the selective environment of the antibiotic will kill all non mutants bacteria while the resistant ones are allowed to grow and flourish (Aliaga, 2001).

Horizontal evolution is the acquisition of resistant genes from another microorganism. The mechanism for developing this kind of resistance is variable, but frequently; the bacteria with resistant genes donate these genes to other bacteria through specific exchange processes inherent to bacteria that involves conjugation, transduction and transformation. *Conjugation* consists in a process where the genetic material crosses from donor bacterium to receptor bacterium by means of a physical contact between them. *Transduction* consists in the transference of genetic material from one bacterium to another by means of a bacteriophage[1]. After the infection the viral DNA is inserted in the chromosomes of the bacterium. *Transformation* consists in the acquisition and incorporation of DNA directly from the environment where it has been released from another cell (Aliaga, 2001).

Bacteria can become "resistant" to individual antibiotics by developing specific defense mechanisms which make the antibiotic ineffective. Generally there are three mechanisms that are utilized by bacteria to do this: (1) preventing the antibiotic from binding and entering the organism, (2) producing an enzyme that inactivates the antibiotic or (3) changing the internal binding site of the antibiotic. One way in which bacteria have become resistant to β-lactam antibiotics is by being able to express β-lactamase enzymes, an example of the second type of resistance. There are actually dozens of enzymes, produced by many different bacteria, which are capable of degrading the β-lactam structured antibiotics (Belcher,

[1] A bacteriophage is a virus that infects a bacterium.

2005). Specific examples of bacterial resistance are the mutated TEM and SHV genes, mainly found in strains of *Escherichia coli* and *Klebsiella pneumoniae*, which have developed extended spectrum β-lactamases (Samaha-Kfoury and Araj, 2003).

The antibiotic presence in a population exerts a selective pressure on that population to retain the resistant gene (Sjölund, 2004). Therefore, the indiscriminate and widespread use of antibiotics maintains selective pressure in the environment. The transfer of resistance genes as well as the already resistant bacteria themselves is favored particularly by the presence of antibiotics, even at subtherapeutic concentrations over a long period. Exposure of bacteria to subtherapeutic concentrations, like the use of antibiotics as growth promoters, increases the speed with which resistant bacterial strains are selected. Nevertheless, emergence of resistance is a highly complex process, which is not yet fully understood with respect to the significance of the interaction of bacterial populations and antibiotics (Kümmerer, 2004).

2.1.5.2 The big guns of resistance

The best way to visualize the effects produced by antimicrobial resistance is making a review of real data related with the development of resistance on several infectious diseases for several antimicrobial agents.

a) Pneumonia:

More than any other infectious disease, pneumonia remains the number one killer worldwide. Statistics from 1998 show that 3.5 million people died as a result of this disease, specially in developing countries where poverty and inadequate medical care contribute to high mortality rates. In lab samples, as many as 70% of chest infections due to *Streptococcus pneumoniae* and *Haemophilus influenzae* are resistant to one of the first-line antimicrobials that in the past were effective medications.

Moreover, there is a general confusion of the difference between viral and bacterial respiratory infections because both forms present the same clinical symptoms and they can only be distinguished with laboratory tests, normally expensive and therefore unavailable in many parts of the world. While bacterial infections can kill, treating viral illness with antibiotics is ineffective. Recent studies indicate that for every 100 respiratory infections, only 20% require antibiotic treatment. This means that 80% of patients are treated with unnecessary medications thereby leading drugs directly into the sight lines of resistance (WHO, 2000).

b) Diarrhea:

This kind of infection has claimed the life of more than 2.2 million people in 1998. *Shigella dysenteriae*, a high dangerous bacterium that kills adults and children alike, is resistant to almost every available drug. Without treatment, death can follow within days of infection. Ten years ago, a shigella epidemic could easily be controlled with co-trimoxazole, a drug cheaply available as generic form. Today, nearly all shigella are non-responsive to the drug, while resistance to ciprofloxacin, the only viable medication left, appears to be just around the corner. Shigella is rare in developed countries, and thus, not a pressing concern to pharmaceutical companies favouring higher returns on research and development (WHO, 2000).

c) Malaria:

Malaria is a mosquito-borne disease that killed 1.1 million people in 1998. With an estimation of 300 to 400 million new cases globally each year, malaria promises to be a pre-eminent threat to development in endemic regions. Like other diseases once considered banished to the geopolitical margins, malaria is reappearing in areas of the world formerly deemed disease-free. In the year 1999, a report from WHO warned of “a serious risk of uncontrollable resurgence of malaria” in Europe owing to

civil disorder, global warming, increased irrigation –canals are important breeding grounds for mosquitos– and international travel. In the United Kingdom, 1000 new cases of malaria are imported each year from malaria-endemic countries. In the former USSR, weakening public infrastructures have triggered large-scale epidemics in central Asian republics, while in Turkey numbers have increased tenfold since the disease was believed nearly defeated in 1989. Resistance to chloroquine, the former treatment of choice, is now widespread in 80% of the 92 countries where malaria continues to be a major killer, while resistance to newer second and third-line drugs continues to grow. Unfortunately, many of these new drugs are not only expensive and have serious side effects, but most will be eventually rendered ineffective by the malaria organism's complex epidemiology and facility for rapid mutation. Mefloquine resistance emerged in South-East Asia almost as soon as the drug became a treatment option (WHO, 2000).

d) Leishmaniasis:

It is an insect-borne disease, which is showing resistance to the highly toxic, heavy metal-based antimonials at rates of 64% in some developing nations. Currently, visceral leishmaniasis – otherwise known as Kala-azar – afflicts 500000 individuals each year in 61 countries in Central and South America, East Africa and India. The sandfly-transmitted parasite attacks the spleen, liver and bone marrow and is characterized by fever, severe weight loss and anaemia. Left untreated, the disease is fatal. Drug-resistant leishmaniasis results when treatment courses are too short, interrupted, or consist of poor-quality or counterfeit drugs. Once infected, victims remain vulnerable to potentially fatal flare-ups throughout their lifetime. As with most infectious diseases, resistant strains flourish in areas where poverty is high, surveillance is low and treatment frequently inconsistent due to limited medical access, inadequate diagnosis, the availability of black-market drugs, and political discord. Active monitoring procedures that could reveal the true extent of the disease are hindered by

lack of available funds and civil unrest. In one study, WHO researchers conducting a house-to-house search discovered that the actual rate of infection was 48 times higher than the one which had been initially reported. In the state of Bihar in north-western India, up to 70% of Leishmania cases are non-responsive to current treatments, while in Bangladesh, Brazil – and particularly Sudan (where 90% of all cases originate), resistance continues to grow (WHO, 2000).

e) Gonorrhoea:

This infection is one example of how antimicrobial abuse has propelled a once-curable nuisance into a potentially life-threatening contagion. The development of antimicrobial resistance in gonorrhoea is one of the major health care disasters of the 20th century. Of the sexually transmitted infections, gonorrhoea is the most resilient with a resistance rate that continues to outstrip new treatment strategies. Gonorrhoea resistance firstly showed up in Ground Infantries (GI) during the Vietnam war and is now entrenched around the globe with multi drug resistance strains appearing in 60% of those infected each year. In most of South-East Asia, resistance to penicillin has been reported in nearly all strains at a rate of 98% overall. Newer, more expensive drugs – notably ciprofloxacin – are likewise showing an increasing failure rate (WHO, 2000).

2.1.6 ANALYTICAL METHODS TO DETERMINE B-LACTAM ANTIBIOTICS

2.1.6.1 Screening methods

Based on legal regulations stating that no food (meat, poultry, eggs, fish, shellfish, milk) should contain antibiotics, the necessity to analyze for residues of antibiotics used as feed additives or veterinary medicine has developed screening techniques that are relatively simple, rapid and inexpensive and permit a large number of samples to be analyzed with high reliability. Typical limits of detection (LOD) of commercially available

screening tests used for detecting β-lactam antibiotics in milk are between 5 to 10 μg/L (Oka et al., 1995). Mostly, screening tests are based on:

a) Microbial inhibition:

The microbial inhibition uses antibiotic-sensitive strains of bacteria as test organisms, e.g. *Bacillus stearothermophilus var. calidolactis C-953* for testing ampicillin and *Micrococcus luteus ATCC 9341* for testing penicillin G. This kind of test uses a disk loaded with the sample to be analyzed, which is placed on an agar plate that contains the antibiotic-sensitive strain of bacteria that is incubated for a specified period of time, depending on the specific assay. If the zones around the disk are free of bacterial growth, the sample contains antibiotic. These bioassays are effective in detecting antibiotic residues, but they present susceptibility to interfering substances which may yield false positives.

b) Radioimmunoassay (RIA):

RIA is based on the competition between an isotopically labeled analyte and an unlabeled analyte for a binding site on an antibody. The presence of large amounts of unlabeled analyte results in less radioactivity being bound to the antiserum. A comparison of the ratio of the bound to free labeled analyte with that obtained from a series of standards permits the quantification of the analyte in unknown samples. The RIA technique involves three steps. The first step requires the preparation of the three components: the radiolabeled (^{14}C, ^{3}H, or ^{125}I) antigen (Ag* - antibiotic), the unlabeled antigen (Ag – antibiotic from the sample) and the antibody (Ab) with high avidity for the antigen. The second step involves an equilibrium reaction in which the labeled or unlabeled antigen reacts with the antibody to produce an antibody combined with a labeled antigen (Ag*Ab), or an antibody combined with an unlabeled antigen (AgAb). The final step is to remove the unbound antigen from the solution. Because antibody or antigen reactions are stoichiometric, the determination of

either bound radiolabeled or unbound radiolabeled antigens will result in the direct calculation of the amount of antibiotic.

c) Enzyme-linked immunosorbent assay (ELISA):

Elisa differs from RIA in that the antigen or antibody is labeled with an enzyme instead of a radioactive isotope. After an enzymatic reaction, there is a development of a chromatic or fluorescent product, which can be measured spectrophotometrically and quantificated as an indication of analyte concentration. Nevertheless, most ELISA techniques applied to the analysis of antibiotics use some preliminary sample treatment before applying the sample in a suitable form for the test. Even though the protocol for conducting ELISA test would appear quite simple to a trained analyst, feedlot operators prefer to use microbial inhibition assays instead of ELISA for field tests, even when the ELISA test is a simple card test format.

d) Bioluminescent immunoassay:

Bioluminescent immunoassay is based on the use of luciferin-luciferase enzyme system to measure the ATP that is coming from bacteria as a result of light emission during the bioluminescent reaction. In this method, ATP is coupled to a hapten. When anti-hapten specific antibodies react with ATP-hapten conjugate, the light-generating reaction catalyzed by luciferase, is diminished. The luminescence intensity produced by this reaction is proportional to the extracted ATP concentration.

Although screening methods provide fairly reliable test results, are relatively simple, inexpensive and require simple equipment, they lack compound specificity and are, at the best, semi-quantitative, and therefore, they are suitable only when solely qualitative information is desired, e.g. screening the presence of penicillins (Moore and Nygren, 2004).

e) Biosensors:

A technological improvement of screening methods has been the development of biosensors, an analytical device in which a biological material, capable of specific chemical recognition, is in intimate contact with a physico-chemical transducer to give an electric signal. A biosensor has a basic configuration comprising three components: the biological element, normally an enzyme; the transducer, which can be an electrode, transistor, optical device or thermistor; and the electronic component for processing and interpretation of the electric signal to display the result (Higgins, 1988).

Enzymes are frequently used as the biological component in biosensors due to their high specificity for certain substrates. The enzymatic product is measured and this can be accomplished by a number of mechanisms, *e.g.* temperature, change in pH, and optical detection, but amperometric detection is by far the most commonly applied transducer. Despite the high specificity and selectivity achieved by enzymes, enzyme biosensors are sometimes impractical because the enzyme may be unstable, expensive, require a co-factor or be difficult to purify (Gustavsson, 2003).

Several biosensors have been developed for the analysis of β-lactam antibiotics, e.g. carboxypeptidase as binding protein site for penicillin G using surface plasmon resonance (SPR) as optical detector (Gustavsson, 2003); 7-aminocephalosporanic acid linked to glucose oxidase as binding protein site for penicillin G using an amperometric transducer (Setford et al., 1999); *Escherichia coli* bacteria cells immobilized on agar membrane for cephalosporins using a pH electrode as detector (Garcia et al., 1998); penicillinase optodes for penicillin G and ampicillin using a diode array spectrophotometer as detector (Polster et al., 1995).

2.1.6.2 Spectrophotometric methods

Native β-lactam antibiotics are colorless and do not have chromophores with significant UV adsorption properties. In that way, applications for determining β-lactam antibiotics using spectrophotometry are based in suitable derivatizations to obtain colored compounds for measurements in the visible range. Several applications using spectrophotometry for determination of β-lactam antibiotics are shown in Table 2.1; nevertheless, all of these spectrophotometric methods have been developed for analysis of β-lactam antibiotics only in pure forms or in pharmaceutical preparations.

Table 2.1 Spectrophotometric determinations of β-lactam antibiotics

Antibiotic	Reagent	Determination range (μg/ml)	Reference
Cefaclor	Ammonium paramolybdate	10-100	Issopoulos, 1989b
Cefazolin		40-400	
Cefotaxime		40-400	
Cefoxitin		25-250	
Cefamandole		40-400	
Amoxicillin	4-nitrophenol	1-24	Amin et al., 1994
Amoxicillin	3-methylbenzo-thiazolin-2-hydrazone	0.6-12	Revanasiddappa et al., 1999,
Amoxicillin	Diazotized benzocaine	2.0-16	El-Ashry et al., 2000
Cefadroxil		0.8-12	
Ampicillin	Mo-SCN	1.5-77.5	Mohamed, 2001
Dicluxacillin		3.0-75.0	
Flucloxacillin		1.5-79.0	
Amoxicillin		7.5-85.0	
Ampicillin	Pyrocatechol violet	0.2-28	Amin, 2001
Amoxicillin		0.2-25	
Cloxacillin		0.2-22	
Dicloxacillin		0.2-38	
Flucloxacillin		0.2-44	
Cefoperazone	Ce (IV)	5-30	Salem and Saleh, 2002
Cefadroxil		5-30	
Cefprozil		5-30	
Amoxicillin		5-30	
Ampicillin	Folin-Ciocalteu	8-38	Ahmad et al., 2004
Amoxicillin		10-34	
Carbenicillin		12-60	

2.1.6.3 Chromatographic methods

The procedure to detect antibiotics is usually performed in two steps: first a screening test, and second, the samples found positive are confirmed by a chemical method. A confirmatory method has to be able to detect which molecule is present in the sample and to quantify it (Ghidini et al., 2002).

High Performance Liquid Chromatography (HPLC) with ultraviolet (UV) detector has been applied for the routine analysis of antibiotics. This technique has been more accepted than gas chromatography (GC) because the latter is complicated, time consuming and require suitable volatile derivatives. Moreover, seems quite difficult to develop a universal derivatization procedure suitable for the whole analyte group, because they show different properties concerning the number and kind of functional groups (Hirsch et al., 1998). However, when the peak of a target antibiotic has appeared on the LC chromatogram, HPLC-UV methods lack qualitative information being necessary to ensure the identification of the observed peak. (Oka et al., 1995) (Ghidini et al., 2002).

In 2002, the European Commission presented the decision 2002/657/EEC that states: “Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods” (Ghidini et al., 2002) (Bogialli et al., 2004). On such a way, High Performance Liquid Chromatography coupled to a Mass Spectrometer (HPLC-MS) is the ideal technique to separate, identify and quantify several chemical compounds and it has been used to analyze β-lactam antibiotics in food, biological and some environmental samples like: milk (Riediker et al., 2001) (Bruno et al., 2001); tissues (Fagerquist and Lightfield, 2003) (Bogialli et al., 2004); urine (Türk et al., 2003); river water (Castiglioni et al., 2004), hospital sewage water (Lindberg et al., 2004). The application of HPLC-MS technique is mostly preceded by the use of solid-phase extraction (SPE) for

clean up and/or preconcentration of analytes from the matrix. Under these conditions, absolute limits of quantitation (LOQ) for amoxicillin and ampicillin in sewage water were 37 μg/L and 33 μg/L, respectively (Lindberg et al., 2004). Using more sophisticated instruments like HPLC-MS-MS, limits of quantitation of 20 ng/L has been obtained for penicillin G and penicillin V (Hirsch et al., 1998).

2.2 POLYURETHANE FOAM: THE SORBENT MATERIAL

Polyurethane foam (PUF) presents significant interest in analytical chemistry due to its special characteristics as sorbent material: high efficiency, versatility, chemical and mechanical stability, resistance to organic solvents, relatively low cost and wide availability. The unique sorption property of this polymer is a combination of various hydrophilic and hydrophobic centers and the reactive terminal groups (Dmitrienko et al., 2003).

2.2.1 MANUFACTURING PROCESS

The manufacturing process, shown in Fig. 2.5, involves two important reactions: the first one, between an isocyanate and a polyol to form the urethane group, and the second, between water and isocyanate, responsible for foam formation by the liberation of carbon dioxide, as an *in situ* blowing agent (Braun et al., 1985).

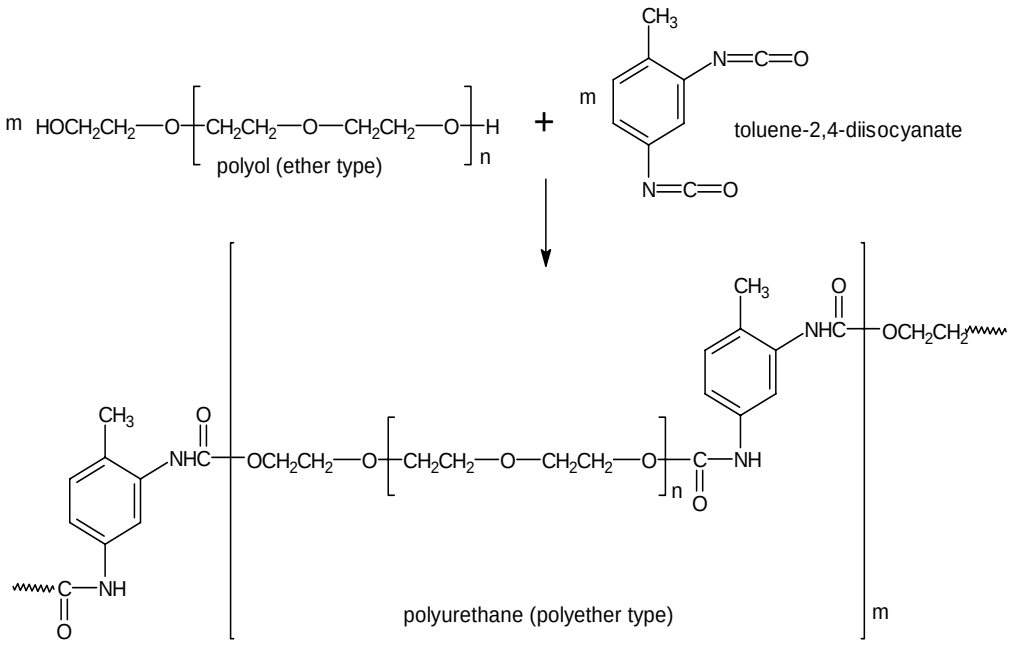


Fig. 2.5 Preparation of polyurethane foam.

Polyethylene glycol, polypropylene glycol and propylene oxide adduct of glycerol are typical polyols used for the production of PUF. Glycerin adduct having greater than 90% secondary hydroxyl terminal groups and a molecular weight of 3000 g/mol is the most widely used polyol (polyether type) for the production of polyurethane foam (Braun et al., 1985).

The preparation of polyethylene glycol, polypropylene glycol and propylene oxide adduct of glycerol is shown in Fig. 2.6.

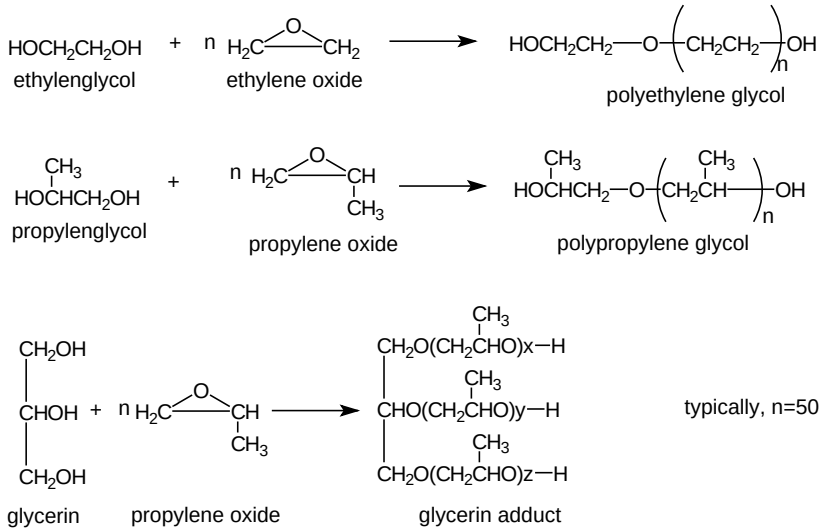


Fig. 2.6 Preparation of polyglycols.

The most widely used isocyanate is toluene diisocyanate, usually employed as a mixture of the 2,4 and 2,6 isomers in proportion 80/20, respectively, as shown in Fig. 2.7.

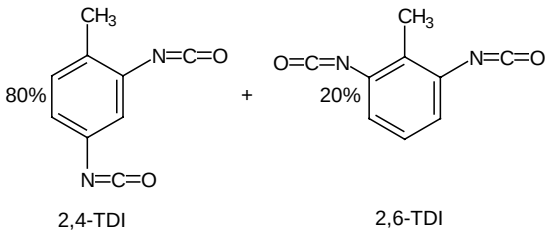


Fig. 2.7 Toluene diisocyanate composition.

PUF can be prepared in soft, flexible and rigid forms; and the choice of the polyol has a major effect on these foam properties. Flexible foams (low cross-link density) are prepared from polyols of moderately high molecular weight and low degree of branching (polyethylene or polypropylene glycol), while rigid foams (high cross-link density) are prepared from highly branched polyols of low molecular weight (glycerin adduct) (Braun et al., 1985).

Depending on the relationship between the rate of the macromolecule growth and the rate of gas evolution in the preparation process, the cell walls of the polymer are either broken or not. As a rule, flexible PUF have an open-cell structure and rigid PUF have a closed-cell structure (Dmitrienko and Zolotov, 2002).

2.2.2 CHARACTERISTICS AND PROPERTIES

PUF is a light material with a range of bulk density between 15 and 300 kg/m^3. It presents enhanced stability against thermo-oxidative destruction up to 220 °C. It is not altered by acids and bases up to certain concentration: hydrochloric acid up to 6 M, sulfuric acid up to 2 M, nitric acid up to 2 M, sodium hydroxide up to 2 M, ammonium hydroxide up to 2 M; and solvents like benzene, carbon tetrachloride, chloroform, diethyl ether, acetone, ethyl acetate, hexane and alcohols. Nevertheless, it is dissolved by concentrated sulfuric acid and hot arsenic (III) chloride, destroyed by concentrated nitric acid and oxidized by alkaline potassium permanganate (Braun et al., 1985).

The diversity of functional groups in the polymer chains of PUF creates favorable conditions for the formation of intermolecular bonds with different energies having substantial influence on the physico-chemical properties of the material. An important kind of hydrogen bond arises from the interaction of hydrogen atoms of the proton-donating urethane groups with the oxygen atoms of the proton-withdrawing ether groups. Studies of PUF using IR spectrophotometry have shown that most of the NH groups in PUF are involved in hydrogen bonds with the oxygen of the polyether chain. Due to these strong hydrogen bonds which form in the PUF a continuous three-dimensional network, a special structural feature appears: the ability of the network to be destroyed and reconstructed after heating, treatment with solvents or mechanical stress. In case of pronounced deformations, the secondary bonds are destroyed while the primary (chemical) bonds remain intact. Evidently, the ability of PUF to

repair the defects arising upon deformations can be explained by the relative easy network destruction and subsequent restoration. Therefore, after multiple compression, PUF is softened and its initial strength is restored after a certain resting time (Dmitrienko and Zolotov, 2002).

2.2.3 SORPTION MECHANISMS BY PUF

Over the years, different possible sorption mechanisms by polyurethane foam have been proposed, according to the nature of the adsorbed species.

In the first reported study about sorption with PUF, the solvent extraction mechanism was proposed, based on the fact that most of the substances sorbed by PUF are those that can be extracted from aqueous solutions by diethyl ether. The substances strongly adsorbed are free molecules with high polarisability, such as iodine, chlorine, bromine, aromatic compounds, dithizonates, thiocyanates, diphenylcarbazones and univalent anions with high polarisability, such as $AuCl_4^-$ $TlCl_4^-$, $FeCl_4^-$. Moreover, since the foam is a polyether, it should take up protons from aqueous acids and then would need to adsorb anions to maintain electrical neutrality (Bowen, 1970).

This mechanism is supported on the relatively high sorption capacity of PUF, calculated on the basis of sorption isotherms of metal halide complexes with relative low specific surface area. Besides, the extraction of metal acid complexes by PUF increases in the presence of alkali metals, indicating that a salting-out effect, typical of liquid extraction, is observed also with PUF. Finally, PUF presents low anion-exchange capacity due to the electron-donating nitrogen and oxygen atoms from PUF can be protonated. Nevertheless, due to the basicity constant of these groups are relative low, PUF can exhibit anion-exchange properties only in highly acid solutions (Dmitrienko and Zolotov, 2002).

From the point of view of solvent extraction, the long chain portions of PUF act much as if they were the analogous liquid monomers in solvating the sorbed species, while the urethane groups are largely inactive in the process. This simple description has been widely accepted and it is enough to account for observed sorption of non-polar or even moderately polar compounds, but it is not possible to apply it for metal-thiocyanate complexes because if it assumed that a neutral protonated species like $H_2Co(SCN)_4$ is being formed and extracted by the PUF under these conditions, then it is forced to deduce that the conjugate anion $[Co(SCN)_4]^{2-}$ is more strongly basic than OH^-. However, basicity of this order is not at all typical of any anionic metal complexes and, moreover, it was observed that extraction drops abruptly in more acid solutions rather than increasing as might be expected. Therefore, it is concluded that hydrogen ions cannot be involved in the extraction of cobalt-thiocyanate species in particular, nor of other metal complex ions, from neutral or basic solutions (Hamon et al., 1982).

A sorption mechanism that does not necessarily require protonation of polyurethane foam and by which anionic metal complexes may be sorbed is the cation-chelation mechanism. According to this mechanism, specific sites of the polymer are able to complex with metallic cations. The identification of the specific sites in the PUF that are responsible for complexation was performed using two different kinds of PUF: polyether and polyester types, both of them based in polyethylene copolymer and polypropylene copolymer. The results indicated that the polyether PUF type was several orders of magnitude more efficient than the polyester PUF type. Nevertheless, this behavior is completely different from that of monomeric solvent analogues, which generally show esters to be superior to ethers as metal ion extractants and indicates also the inadequacy of a simple solvent extraction mechanism. The results related with the copolymer type have shown that PUF based on polyethylene copolymer presented better efficiency. In this case, the difference in efficiency cannot

be attributed to electron densities on the respective oxygen atoms since the mildly electron-donating character of methyl group in polypropylene copolymer would lead to expect higher basicity and therefore stronger complexation. The fact that the opposite behavior results suggests that steric limitations imposed by the methyl group must be more important than any induced electronic effects and consequently, specific geometry plays an important role in the process (Hamon et al., 1982).

The study of sorption process of organic dyes by PUF show that when PUF was exposed to a solution of dye containing lithium or potassium chloride, the sorption increased for both, polyether and polyester PUF types. This suggests that the dye was forced into the less polar organic foam phase by addition of salt. In another test, when the polarity of the dye was increased by means of the number of sulphonated groups, the sorption decreased. Although these results support a solvent extraction mechanism, there are some inconsistencies: some polar dyes were better adsorbed than non polar dyes, some dyes that were reasonable well sorbed by PUF were found to be poorly extracted by diethyl ether, anionic dyes were better sorbed by polyether PUF while cationic dyes were better sorbed by polyester PUF. This last observation is consistent with the cation chelation mechanism. Therefore, the sorption process of dyes by PUF can be explained by a dual-mode sorption model: solvent extraction-cation chelation, model which offers and excellent description of the observed behavior (Werbowesky and Chow, 1996).

An additional sorption mechanism has been proposed for the sorption process of aromatic organic compounds containing groups capable to form hydrogen bonds with the polyurethane foam. A particular case is presented with the complete family ortho, meta and para nitrophenol, where the special characteristic of o-nitrophenol is the ability to form strong intramolecular hydrogen bonds. These three compounds were sorbed with polyether and polyester PUF; the sorption of o-nitrophenol in

both PUF types was almost similar and was also the lowest one, while the sorption of the other two phenols was four times higher with polyether PUF. Therefore, the solvent extraction mechanism, modified by hydrogen bonding wherever applicable, may explain the extraction of organic compounds by PUF (Schumack and Chow, 1987).

2.2.4 ANALYTICAL APPLICATIONS

PUF is used for separation, preconcentration and quantification of inorganic and organic species from water and air. The determination of the target analyte can be made directly on the polymer or after elution with an suitable solvent. For direct determinations on the solid material the methods normally used are diffuse reflectance spectroscopy, luminescence and X-ray fluorescence, while, after elution of the compounds from the PUF, spectrophtometry, chromatography, atomic absorption and atomic emission are normally used (Dmitrienko and Zolotov, 2002).

The sorption procedure can be done using three different methods: (1) unloaded (non-modified) PUF for direct sorption of chemical species; (2) loaded PUF, where a suitable reagent is previously immobilized for subsequent specific sorption of the analyte of interest and (3) chemisorption, where the terminal toluidine groups of the PUF are used as reactive species against the desired analyte.

2.2.4.1 Unloaded PUF for sorption of inorganic species

The sorption on unloaded PUF of around 50 elements of the periodic table has been studied. Mostly, PUF adsorbs the elements as metal acid complexes, ion associates or metal chelates. The time required for sorption equilibrium depends on the nature of complex, e.g. several hours for metal chloride complexes to 10 – 30 minutes for thiocyanate complexes. The

sorbed metal can be quantitatively desorbed from PUF using organic solvents as acetone, methanol, ethanol (Dmitrienko and Zolotov, 2002).

Several applications using unloaded PUF for sorption of elements are shown in Table 2.2.

Table 2.2 Sorption of elements by unloaded PUF

Element	Sorbable form	Sorption time	Recovery	Reference
Au	Cyanide Complex	5 min	95%	Braun and Farag, 1983
La	Thiocyanate complex	60 min	85%	Farag et al., 1994
Al		50 min	20%	
Mo(VI)		45 min	98%	
Ga		50 min	30%	
W(VI)		20 min	95%	
Ga	Chloride complex	60 min	n.d.	Carvalho et al., 1995
Co	Thiocyanate complex	10 min	100%	Carvalho et al., 1996
Au	Chloride complex	50 hours	97%	Oleschuk and Chow, 1996
Zn	Thiocyanate complex	5 min	98%	Santiago de Jesus et al., 1998
Mo(V)	Thiocyanate complex	10 min	99%	Costa et al., 2001
Fe	Thiocyanate complex	3 min	98%	Cassella, 2002

Moreover, unloaded PUF is able to adsorb metal chelates such as: iron (Bhattacharya et al., 1990), cadmium (Chakrabarti and Roy, 1997) and europium (Beltyukova and Balamtsarashvili, 1995) with 1,10-phenanthroline; yttrium with 8-hydroxyquinoline (Beltyukova et al., 1995); platinum with dithizone (Kundu and Roy, 1992). Nevertheless, the sorption of metal chelates is mostly made with loaded PUF.

2.2.4.2 Unloaded PUF for sorption of organic species

The use of unloaded PUF has found wide application in sorption of organic compounds, especially those recognized as environmental pollutants in water and air. Since these organic compounds are present in very low levels (μg/L to ng/L), large volumes of sample are necessary to flow through a column packed with PUF. Once collected on the PUF, the pollutants are eluted either by simple elution or Soxhlet extraction with an organic solvent, normally hexane and acetone. After the appropriate volume reduction, the recovered compounds are analyzed with the suitable technique, normally, gas chromatography, spectrophotometry and HPLC (Braun et al., 1985).

Several organic pollutants extracted by unloaded PUF have been reported: carbaryl from water (R > 93%) (Cassella et al., 2000); polycyclic aromatic hydrocarbons from water: naphthalene (R > 83%), phenanthrene (R >97%), anthracene (R > 98%), pyrene (R > 99), acenaphthene (R > 92%), fluorene (R > 96%) and fluoranthene (R > 99%) (Dmitrienko et al., 2002a); chlorinated organic compounds from air: heptachlor (R > 100%), aldrin (R > 100%), endosulfan (R > 90%), chlordane (R > 92%), dieldrin (R > 80%), endrin (R > 100%), DDT (R > 94%) (Nerin et al., 1995); organophosphorus insecticides from water: chlorpyrifos (R > 93%), malathion (R > 92%), diazinon (R > 95%) (El-Shahawi et al., 1995); acaricides from water: dicofol (R >93%) and bromopropilate (R > 95%) (El-Shahawi and Aldhaheri, 1996).

Unloaded PUF is also able to adsorb organic dyes. The sorption efficiency of these compounds is markedly different, depending on the nature, structure and polarity of the dyes. Non-sulphonated dyes present larger distribution coefficients in comparison with the sulphonated ones; and being the number of sulphonate groups related with the polarity, is possible to state that PUF adsorb better less polar compounds (Chow et al., 1990).

2.2.4.3 Loaded PUF

PUF is able to adsorb substantial amounts of chemical compounds, giving the possibility to modify the PUF to improve the sorption properties and preserving the required hydrodynamic characteristics of the material. The loading step must be made before the sorption step; for that, the polymer is treated with an organic reagent which will be physically immobilized in the material due to the hydrophobic character of PUF. In the practice, the reagent to be loaded is dissolved in an appropriate solvent, normally a highly volatile organic solvent, and then the foam is immersed on it. After equilibrium, the foam is removed and the solvent is gradually evaporated. There is also the possibility to use non-volatile organic solvents with extracting properties. These solvents have a dual function: to dissolve efficiently the reagent and to act as plasticizers for the polymer. Normally, tributylphosphate is the most used plasticizer (Dmitrienko and Zolotov, 2002).

Applications using loaded PUF are shown in Table 2.3.

Table 2.3 Sorption of elements and organic compounds by loaded PUF

Analyte	Loaded reagent	Sorption time	Recovery	Reference
Co	tri-octylamine	30 min	98%	Braun and Huszar, 1973
Ag	dithizone	40 min	95%	Braun and Farag, 1974a
Hg	dithizone	60 min	90%	Braun and Farag, 1974b
Ni	dimethylglyoxime	60 min	98%	Lee and Halmann, 1976
Phenol o-clorophenol o-nitrophenol m-cresol o-cresol	tri-octylamine	120 min	96% 95% 95% 97% 97%	El-Shahawi, 1994
U	tri-octylamine	60 min	97%	Toker et al., 1998

Analyte	Loaded reagent	Sorption time	Recovery	Reference
As	diethyldithiocarbamate	30 min	100%	Atanasova et al., 1998
Co			98%	
Se			100%	
Cr			100%	
Pb			100%	
Zn			97%	
Cu			100%	
Mn			99%	
Cd			96%	
Sb			100%	
Sn			94%	
Tl	9,10-phenanthraquinone monoethyl thiosemicarbazone	30 min	98%	Abou-El-Sherbini et al., 2003

2.2.4.4 Chemisorption

A chemisorption process occurs when the target analyte is sorbed by means of a chemical reaction with the sorbent and then, takes part of it. In the case of PUF, the terminal toluidine groups are reactive species towards typical reactions of monomeric aromatic amines: oxidation by free chlorine, diazotization by nitroso acid, azo coupling with 4-nitrophenyldiazonium tetrafluoroborate and condensation with formaldehyde (Dmitrienko et al., 2000a).

The typical chemisorption reactions of toluidine groups of PUF are shown in Fig. 2.8.

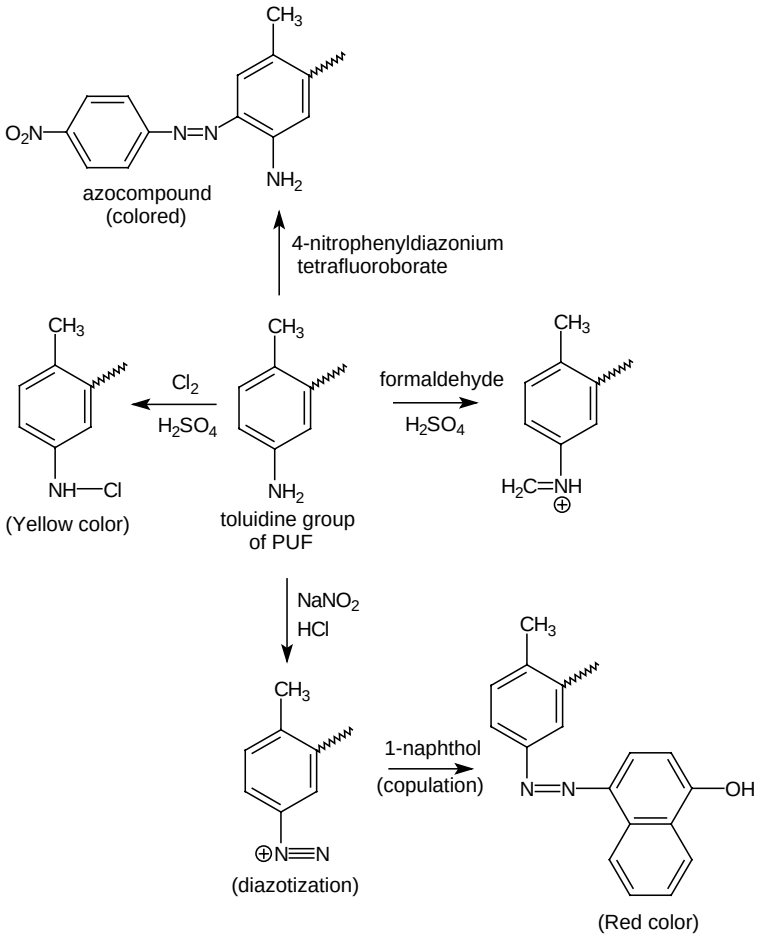


Fig. 2.8 Chemisorption process on PUF.

When the resultant product of a chemisorption process presents color, as occurs in most of the cases, the color intensity can be measured using an analytical technique known as diffuse reflectance. Some applications using this combination have been reported: determination of free active chlorine in water (range of quantitation between 0.2-6 μg/ml) (Dmitrienko et al., 2000b), determination of 1-naphthol and 2-naphthol in water using diazotised PUF (range of quantitation between 0.3-1.2 μg/ml and 0.2-1.5 μg/ml, respectively) (Dmitrienko et al., 2002b), determination of gallic acid in wine using diazotized PUF (range of quantitation between 0.2-6 μg/ml) (Dmitrienko et al., 2002c), determination of nitrite in water (range of quantitation between 0.1-4 μg/ml) (Dmitrienko et al., 2002b).

2.2.5 MICROBIOLOGICAL APPLICATIONS OF PUF

The main application of PUF in microbiology is the immobilization of microorganisms or enzymes for their use in biocatalytic process. In the case of enzymes, the immobilization not only makes easier to carry out the enzymatic reaction under large-scale conditions but also helps to stabilize the enzyme against denaturation (Madigan et al., 2000).

The possibility to immobilize microorganisms on PUF allows to use this methodology in bioremediation process, such as the adsorption and degradation of oil films on surface water by means of oil-degrading yeast cells of *Yarrowia lipolytica*, where the results indicate that about one ton of spilled oil on surface water can be adsorbed by 100 kg or 1 m^3 of PUF (Oh et al., 2000).

Another microorganism immobilized on PUF used to degrade hydrocarbons in water is the micro-alga *Prototheca zopfii*, system that can degrade 39.7 mg of hydrocarbons per hour and per liter, biodegradation rate 2-fold increased than others used (Yamaguchi et al., 1999). Biodegradation of chlorophenols has been studied immobilizing *Phanerochaete chrysosporium* in PUF, obtaining up to 90% of degradation of chlorophenols after 12 days of inoculation at 39 °C (Choi et al., 2002).

In another study using *Pseudomonas sp.* for degradation of naphthalene, the immobilized cells tolerated higher concentrations of naphthalene and were able to degrade the naphthalene more quickly than the free cells. Even more, the PUF-immobilized cells were reused 45 times over a period of 90 days without losing naphthalene-degrading activity (Manohar et al., 2001).

2.3 DIFFUSE REFLECTANCE SPECTROSCOPY: THE MEASURING TECHNIQUE

In transmission spectroscopy, the spectral properties of a solution are determined by measuring the monochromatic light transmitted by the solution. By taking advantage of the transparent nature of the medium under investigation, it is possible to measure the absorption of dissolved substances as a function of the wavelength. However, difficulties appear when an attempt is made to obtain similar information with turbid or colloidal systems where light-scattering phenomena results in substantial losses of energy. Moreover, the technique becomes entirely unsuitable when one wishes to determine the absorption spectra of substances adsorbed on solid surfaces. It is in this point that diffuse reflectance spectroscopy makes an unique contribution (Frei et al., 1975).

2.3.1 SPECULAR AND DIFFUSE REFLECTANCE

Since we were children we are familiarized with specular reflectance: the reflectance produced by a mirror, where the reflected radiation occurs at the surface without transmission through the material. Diffuse reflectance concerns radiation that penetrates the material surface and subsequently reappears at the surface suffering partial absorption and multiple scattering within the internal surface (Frei et al., 1975). Fig. 2.9 shows these two kinds of reflection.

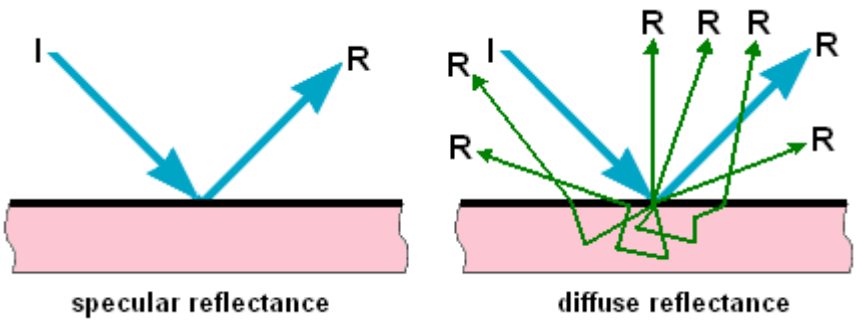


Fig. 2.9 The two kinds of surface reflectance: specular and diffuse.

Measurements of specular reflectance are not of much importance in the field of colorimetry because materials that exhibit purely specular reflection are not often encountered (MacAdam, 1985). In contrast, diffuse reflectance has wide applications in the paper, paint, dye, textile, printing, ceramics and pharmaceutical industry, where the measurement of color is important in routine quality control processes (Frei et al., 1975).

2.3.2 KUBELKA–MUNK THEORY

The theory treating diffuse reflection is that developed by Kubelka and Munk. This theory describes the reflectance of a sample as a function of the light absorption (K) and the light scattering (s) in the sample, where the absorption of the light is proportional to the concentration of colorant used while the scattering of light is a function of the material that supports the colorant (Berger-Schunn, 1994).

For the application of the Kubelka-Munk theory, four assumptions are necessary. The first is that the extent of the horizontal layer is so large relative to its thickness that the light diffused horizontally out of the edges of the sample can be neglected relative to the light moving perpendicular to the layer front. The second assumption is that the material in the layer under study is homogeneous in its composition for the entire distance through which the light passes. The third assumption is that the light incident on the top of the layer is so perfectly diffused that all points on the surface receive equal irradiation. The final assumption is that the top of the sample has the same index of refraction as the medium (typically air) in contact with the sample (Brittain, 1995).

For an infinitely thick opaque layer, in practice achieved with a layer thickness of a few mm, the Kubelka-Munk function states:

$$F(R_\infty) = \frac{(1-R_\infty)^2}{2R_\infty} = \frac{k}{s} \qquad \text{Eq. 2.1}$$

where:

R_∞ is the diffuse reflectance of the layer expressed as a decimal fraction.

k is the molar absorption coeficient.

s is the scattering coefficient, caused by the supporting material.

If s remains constant, a linear relationship should be observed between $F(R_\infty)$ and k. This has been confirmed for weakly absorbing materials where the contribution of regular reflectance is small. When the reflectance of a sample mixed with a non- or low-absorbing powder is measured against the pure powder, the light absorption phenomenon occurs exactly in the same manner as it does in a solution and consequently, the fundaments of the Beer's law can be applied. In that way, the Kubelka-Munk function can be expressed as:

$$F(R_\infty) = \frac{(1-R_\infty)^2}{2R_\infty} = 2.303\frac{\varepsilon c}{s} \qquad \text{Eq. 2.2}$$

where:

ε is the molar extinction coefficient

c is the molar concentration

Since $F(R_\infty)$ is proportional to the molar concentration under constant experimental conditions, the Kubelka-Munk equation in this form is comparable to the Lambert-Beer law of transmission spectroscopy (Frei et al., 1975).

2.3.3 Optimum concentration range for analysis

Even with the systems that conform to the Kubelka-Munk equation, reflectance analysis is of limited use at high and low concentrations. At high concentrations of absorbing material, so little radiation is reflected

that the sensitivity of the spectrophotometer becomes inadequate. At the other end of the concentration spectrum, the instrument reading error becomes disproportionately large compared with the quantity being measured. This situation can be visualized with the aid of Fig. 2.10, which represents percent reflectance (*%R*) as a function of concentration.

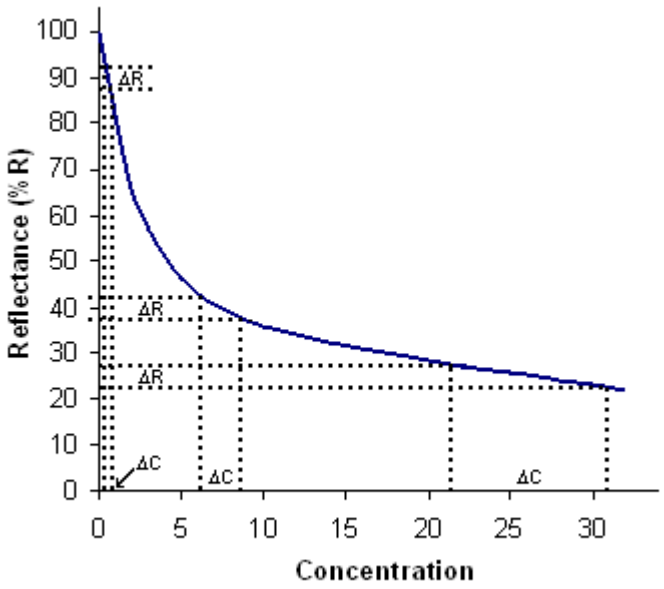


Fig. 2.10 Percent reflectance as function of concentration.

When an arbitrary error amounting to ΔR is plotted at 25%R, 40%R and 90%R, it is readily apparent that the corresponding absolute error in terms of concentration ΔC is greatest at 25%R. At the other extreme, 90%R, the absolute concentration error, the smallest of the three, approaches in magnitude the quantity being measured. In the both cases, large relative errors will be present in the determination. Therefore, there must be a point where these two effects can be balanced in the way to reduce the relative error to a minimum. For systems exhibiting no deviation from the Kubelka-Munk equation, the optimum conditions for maximum accuracy can be deduced calculating the relative error dc/c. In that way, Eq. 2.2 can be written in the form:

$$c = \frac{s}{2.303 * \varepsilon * 2} * \frac{(1-R_\infty)^2}{R_\infty} \quad \text{Eq. 2.3}$$

Differentiating Eq. 2.3 yields:

$$dc = \frac{s}{2.303 * \varepsilon * 2} * \frac{2(1-R_\infty)(-dR_\infty)R_\infty - (1-R_\infty)^2 dR_\infty}{R_\infty^2} \quad \text{Eq. 2.4}$$

$$dc = \frac{s}{2.303 * \varepsilon * 2} * \frac{(2-2R_\infty)(-R_\infty dR_\infty) - (1-2R_\infty + R_\infty^2)dR_\infty}{R_\infty^2} \quad \text{Eq. 2.5}$$

$$dc = \frac{s}{2.303 * \varepsilon * 2} * \frac{-2R_\infty dR_\infty + 2R_\infty^2 dR_\infty - dR_\infty + 2R_\infty dR_\infty - R_\infty^2 dR_\infty}{R_\infty^2} \quad \text{Eq. 2.6}$$

$$dc = \frac{s}{2.303 * \varepsilon * 2} * \frac{R_\infty^2 dR_\infty - dR_\infty}{R_\infty^2} \quad \text{Eq. 2.7}$$

$$dc = \frac{s}{2.303 * \varepsilon * 2} * \frac{(R_\infty^2 - 1)dR_\infty}{R_\infty^2} \quad \text{Eq. 2.8}$$

The relative error in concentration is obtained dividing Eq. 2.8 by Eq. 2.3:

$$\frac{dc}{c} = \frac{(R_\infty + 1)dR_\infty}{(R_\infty - 1)R_\infty} \quad \text{Eq. 2.9}$$

Eq. 2.9 may be used to determine the relative error in concentration when the uncertainty in reflectance measurement is known. It is assumed that the uncertainty in reading the reflectance, dR_∞, is ± 1% (Frei et al., 1975). Therefore:

$$\frac{dc}{c} = 0.01\frac{R_\infty + 1}{(R_\infty - 1)R_\infty} \quad \text{Eq. 2.10}$$

The accurate value of reflectance at which the relative error is minimized is obtained by differentiating Eq. 2.10 and setting the derivative equal to zero:

$$d\left[0.01 * \frac{R_\infty + 1}{(R_\infty - 1)R_\infty}\right] = 0 \qquad \text{Eq.2.11}$$

$$0.01 * \frac{dR_\infty * (R_\infty - 1)R_\infty - (2R_\infty dR_\infty - dR_\infty) * (R_\infty + 1)}{((R_\infty - 1)R_\infty)^2} = 0 \qquad \text{Eq.2.12}$$

$$0.01 * \frac{dR_\infty * (R_\infty^2 - R_\infty) - (2R_\infty^2 dR_\infty + 2R_\infty dR_\infty - R_\infty dR_\infty - dR_\infty)}{((R_\infty - 1)R_\infty)^2} = 0 \qquad \text{Eq.2.13}$$

$$0.01 * \frac{R_\infty^2 dR_\infty - R_\infty dR_\infty - 2R_\infty^2 dR_\infty - 2R_\infty dR_\infty + R_\infty dR_\infty + dR_\infty)}{((R_\infty - 1)R_\infty)^2} = 0 \qquad \text{Eq.2.14}$$

$$0.01 * \frac{-R_\infty^2 dR_\infty - 2R_\infty dR_\infty + dR_\infty)}{((R_\infty - 1)R_\infty)^2} = 0 \qquad \text{Eq.2.15}$$

$$R_\infty^2 + 2R_\infty - 1 = 0 \qquad \text{Eq.2.16}$$

The positive solution of the Eq. 2.16 indicates that the minimum relative error in c, or the optimum value for reflectance measurements, occurs at a reflectance value of 0.414:

$$R_\infty = \frac{-2 \pm \sqrt{2^2 - 4 * 1 * (-1)}}{2 * 1} = \frac{-2 \pm \sqrt{8}}{2} = \frac{-2 \pm 2\sqrt{2}}{2} = -1 \pm \sqrt{2}$$

$$R_\infty = -1 + \sqrt{2} = -1 + 1.4142 = 0.4142$$

This can be appreciated in Fig. 2.11, where the minimum in the curve corresponds to 41.4 %R and the ideal range concentration for analysis using diffuse reflectance lies between 20 % and 65 % R (Frei et al., 1975).

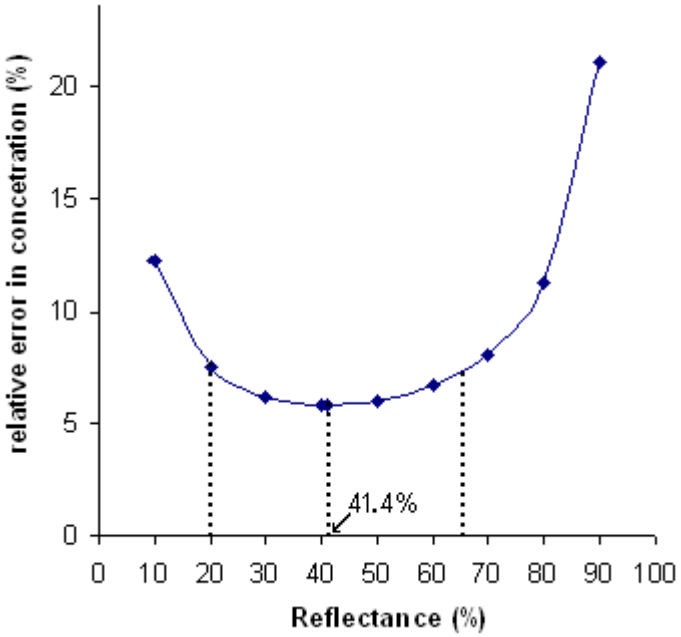


Fig. 2.11 Percent relative error in concentration as function of percent reflectance.

2.3.4 MEASUREMENT GEOMETRY

The elimination of specular reflection is an important consideration in the design of instruments and the preparation of samples for the measurement of diffuse reflectance since the contribution of the specular reflectance distorts diffuse reflectance spectra, which are usually less structured than transmission spectra (Frei et al., 1975).

Two kinds of measuring techniques are used to achieve elimination of specular reflection: spherical illumination system and 45° illumination system.

The spherical illumination system consists in a sphere that has its inner wall coated with a highly reflecting material, normally MgO or $BaSO_4$, to minimize absorption of radiation. The function of the sphere is to collect all diffuse reflected light and to transport it to the detector while specular reflection is completely eliminated (Berger-Schunn, 1994).

The 45° illumination system consists in a device where the incident light beam reaches the sample at 45°, while the diffuse reflected light is measured at 0° with the normal. These two systems are shown in Fig. 2.12.

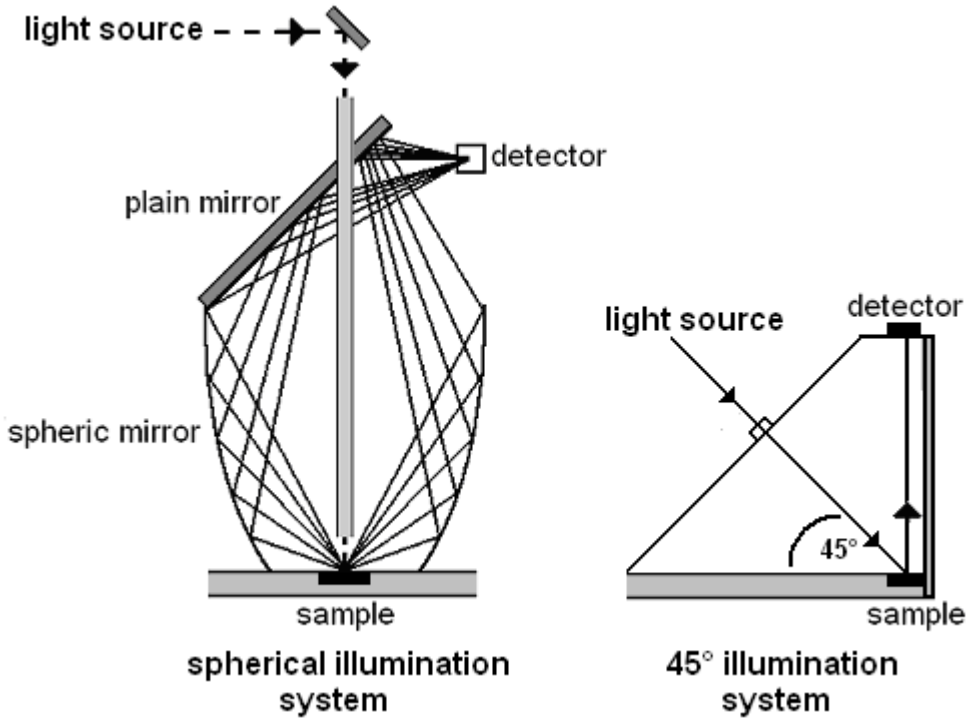


Fig. 2.12 Measurement geometry for diffuse reflectance.

2.3.5 ANALYTICAL APPLICATIONS

Diffuse reflectance spectroscopy is an objective way to measure the color on the surface of a solid material that can be applied only to the analysis of substances which are either colored or can be converted into a colored species by the use of a suitable chromogenic reagent.

Several analytical applications have been reported for different solid materials such as: cellulose for determination of palladium after colorimetric reaction with 4-(2-pyridylazo)resorcinol (Gordeeva et al., 2002); silica gel for determination of vitamins (Frodyma and Lieu, 1967); poly(vinylpyrrolidone) for determination of iodine and iodide (Arena et al., 2002); polystyrene-divinylbenzene for determination of copper (II), nickel

(II), iron (III) and chromium (VI) after colorimetric reaction with diethyldithiocarbamate, nioxime, 8-hydroxy-7-(6-sulfo-2-naphtylazo)-5-quinolin sulfonic acid and cetylpyridinium chloride, respectively (Fritz et al., 2003).

The use of DRS using PUF as solid material has presented analytical applications for inorganic and organic analytes, such as nitrite, nitrate, 1-naphtol, 2-naphtol (Dmitrienko et al., 1997a) (Dmitrienko et al., 2002b), gallic acid (Dmitrienko et al., 2002c), free chlorine (Dmitrienko et al., 2000b), ascorbic acid (Dmitrienko et al., 1998), phosphate (Abbas, 2003).

2.4 PHOSPHOMOLYBDIC ACID: THE COLORIMETRIC REAGENT

Phosphomolybdic acid belongs to the chemical species known as heteropolyacids (HPA), inorganic species with the general formula $H_nX_xM_mO_y$, where X is usually P^V, As^V, Si^{IV} and Ge^{IV}; and M is usually V^V, Mo^{VI} and W^{VI}; all of them, in their highest oxidation states (Pope, 1983).

2.4.1 STRUCTURE AND PROPERTIES

The structure of a typical heteropolyacid where the *heteroatom* (X) and the *addenda atom* (M) are in the proportion 1:12 are known as Keggin-type HPA. A structural model of the Keggin-type anion $(XM_{12}O_{40})^{3-}$ is illustrated in the Fig. 2.13: four oxygen atoms form a central tetrahedron of heteroatom X, and twelve terminal and twenty-four bridged oxygen atoms form twelve octahedral of addenda atoms M (Izumi et al., 1992).

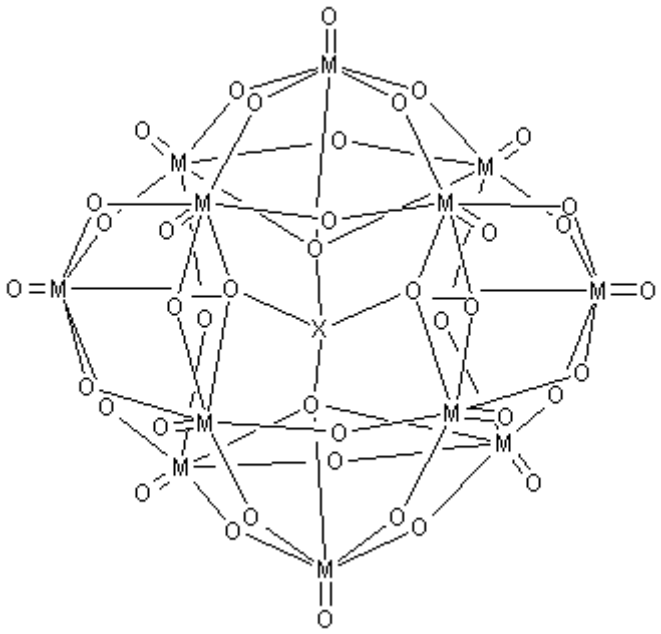


Fig. 2.13 Structure of a Keggin-type heteropoly acid.

The majority of the chemical, technological and medical applications using HPA are based on the Keggin-type HPA, being phosphotungstic acid ($H_3PW_{12}O_{40}$), phosphomolybdic acid ($H_3PMo_{12}O_{40}$), silicotungstic acid ($H_4SiW_{12}O_{40}$) and silicomolybdic acid ($H_4SiMo_{12}O_{40}$) the most representative HPA, where two-thirds of the applications are based on them (Katsoulis, 1998).

The most important properties of the Keggin-type HPA include water and air stability, large size diameter (6 – 25 Å), high molecular weight (1000 – 10000 g/mol), high solubility in water but also in organic solvents as alcohol, ether, and ketone, complete dissociation in water, thermal stability and photoreducibility. HPA of molybdenum present an additional special characteristic: high oxidizing ability, where the color of oxidized form is different form the reduced form. In the other side, HPA of tungsten are not susceptible to reduction (Izumi et al., 1992) (Katsoulis, 1998).

2.4.2 ANALYTICAL APPLICATIONS

Heteropoly acids have found application in medicine, as antiviral and antitumoral agents (Rhule et al., 1998); biochemistry, where phosphotungstic acid has been used for decades as precipitants for proteins and also as stain for electron microscopy (Pope, 1983); organic chemistry, where heteropoly acids are used as catalysts for a broad variety of reactions (Izumi et al., 1992); and different others applications such as corrosion resistant coatings, dyes-pigments-inks, recording materials, capacitors, ion selective membranes, wood pulp bleaching, processing of radioactive wastes and more (Katsoulis, 1998).

In analytical chemistry, the application of molybdenum heteropoly acids is centered in their redox properties to form *heteropoly blues*, a chemical species formed after reacting the HPA with a reducing agent, where the addenda atom (molybdenum in this case) suffers a partial reduction and forms a compound with mixed valences of Mo^{V} and Mo^{VI} (Pope, 1983).

The official photometric method for determination of phosphorus is based on the *in situ* formation of phosphomolybdic acid and its subsequent reduction by ascorbic acid; method that allows to measure phosphorus in the range 0.01 – 0.5 mg/L (Chau, 1984). A corollary of these application represent the determination of ascorbic acid using silicomolybdic acid (Dmitrienko et al., 1998).

A remarkable application of phosphomolybdic acid has been the determination of thiols in petroleum (Karr, 1954) and in natural gas. Although phosphomolybdic acid was not suitable for development of a colorimetric method for thiols in natural gas, the method can be used for quantitative determinations of low concentrations of thiols in solutions (Knight and Verma, 1979).

Based on this last property, phosphomolybdic acid has been used successfully in the spectrophotometric determination of β-lactam antibiotics such as penicillins: ampicillin, amoxicillin and carbenicillin (Ahmad et al., 2004); cephalosporins: cephalexin, cephradine, cephazolin, cefaclor, cefoxitin, cephamandole and cefotaxime (Issopoulos, 1988b); and cefadroxil, cefapirin, ceforanide and cefuroxime (Issopoulos, 1989a). These determinations are based on the reduction of phosphomolybdic acid by the thiols generated after the acid hydrolysis of β-lactam antibiotics.

The generation of thiols from β-lactam antibiotics is produced under acid medium, being penicillamine the characteristic degradation product of penicillins (Fig. 2.14). This has been confirmed by different analytical methods and for different penicillins: from penicillin G by chemical screening (Abraham et al., 1943); from penicillin G, ampicillin and cloxacillin by direct titration (Grime and Tan, 1979); from penicillin G by HPLC (Kessler et al., 1981).

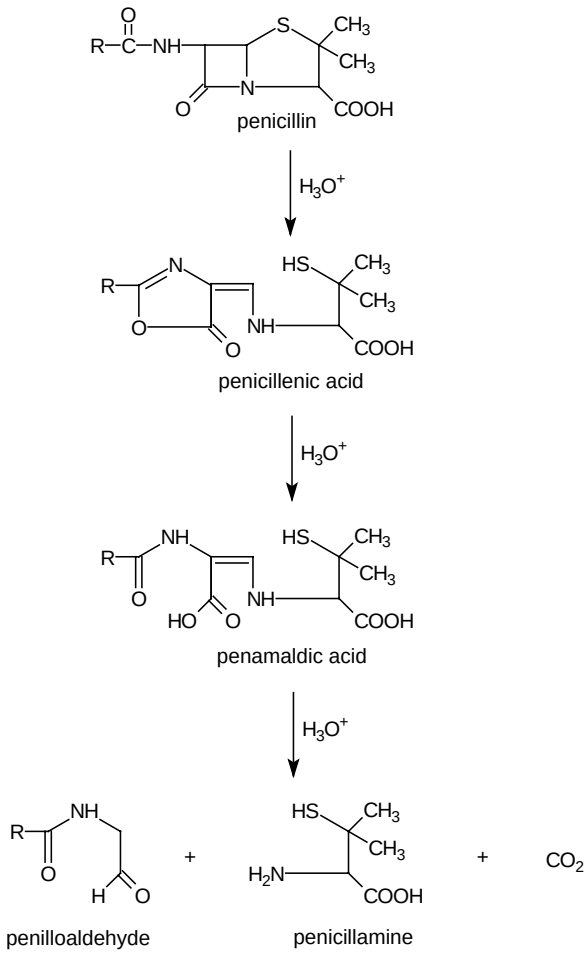


Fig. 2.14 Acid hydrolysis of a penicillin (Grime and Tan, 1979).

In the case of cephalosporins, the acid hydrolysis of cephalexin does not give as final product a thiol but a thiolactone. Nevertheless, the intermediate product that forms the thiolactone is a thiol (Fig. 2.15) (Dinner, 1977).

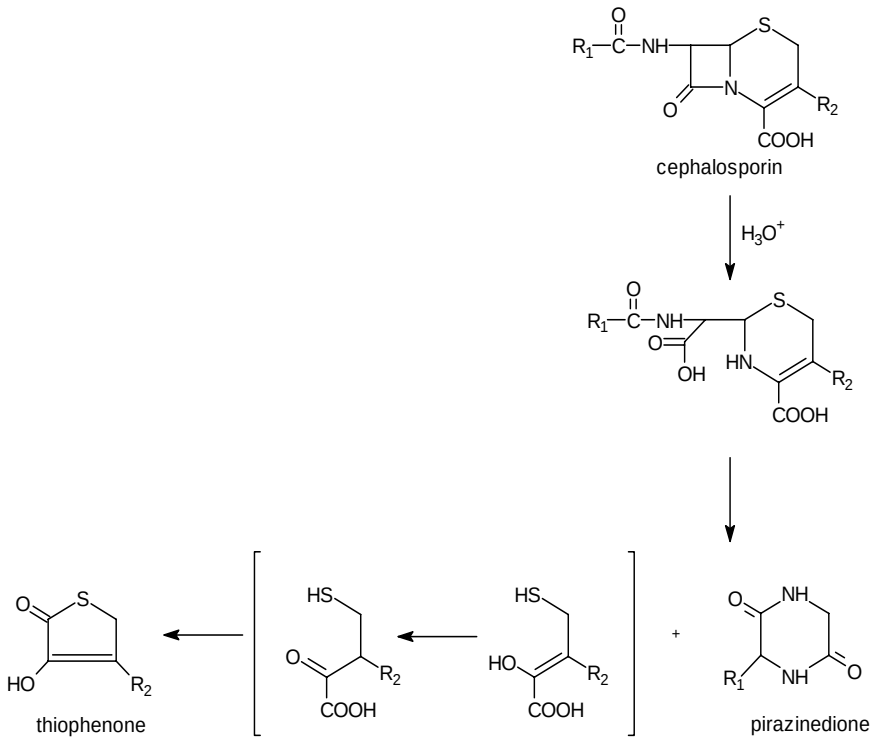


Fig. 2.15 Acid hydrolysis of a cephalosporin (Dinner, 1977).

2.4.3 SORPTION OF PHOSPHOMOLYBDIC ACID BY POLYURETHANE FOAM

The oxidized and reduced forms of phosphomolybdic acid can be quantitatively adsorbed on PUF from aqueous solutions, in a similar process to the extraction with diethyl ether. Although the extraction mechanisms are not completely established, the sorption of phosphomolybdic acid by PUF is only possible under acid medium. This fact suggests that the protonation of ethereal oxygen atoms of the polymer in acid medium results in the formation of an oxonium-type salt, which can extract the HPA by an anion-exchange mechanism (Khan and Chow, 1983). Moreover, an additional criteria indicates that hydrogen-bond interactions are also involved in the sorption process of HPA by PUF, due to PUF-ester type are not so efficient as PUF-ether type for extracting HPA from acid medium (Dmitrienko et al., 1997b).

The adsorption isotherms based on the Langmuir theory indicate that for both, oxidized and reduced forms of phosphomolybdic acid, the

sorption capacity on PUF is practically the same (Dmitrienko et al., 1997b).

2.5 ADSORPTION: THE PHYSICAL-CHEMICAL PHENOMENON

2.5.1 CHARACTERISTICS

Adsorption is the process of trapping atoms or molecules (adsorbate) that are incident on a solid surface (adsorbent). Adsorption is a spontaneous process that occurs when the interaction between the adsorbate and adsorbent reduces the enthalpy of the surface ($\Delta H_{ads} < 0$) (McQuarrie and Simon, 1997).

In the adsorption process, the entropy change of the adsorbate, ΔS_{ads}, is necessarily negative since the adsorbed species are more ordered than when they are not adsorbed due to the loss of at least one degree of translational freedom. The spontaneity of the adsorption process requires that the Gibbs free energy, ΔG, also be negative. Based on the fact that the enthalpy change ΔH_{ads} accompanying physical adsorption is always negative, physical adsorption is always an exothermic process, as shown by equation 2.16 (Lowell and Shields, 1984).

$$\Delta H_{ads} = \Delta G + T\,\Delta S_{ads} \qquad \text{Eq. 2.16}$$

The amount of adsorbed substance is proportional to the surface of adsorbent, consequently, to obtain a high level of adsorption, the adsorbent surface must be as big as possible. Therefore, good adsorptive potentials will be found only in materials with highly developed surfaces, such as substances with a porous or spongy structure, or substances divided in fine particle size (Wedler, 1976).

There is a convenient mathematical idealization that asserts that a cube of edge L possesses a surface area of 6 L^2, and that a sphere of radius r

exhibits 4 πr^2 of surface. However, under microscopic examinations, all real surfaces are not a perfect plane of molecules, but contain imperfections such as terraces, steps, pores and other surface imperfections (Fig. 2.16). These surface imperfections will always create real surface area greater than the corresponding geometric area (Lowell and Shields, 1984).

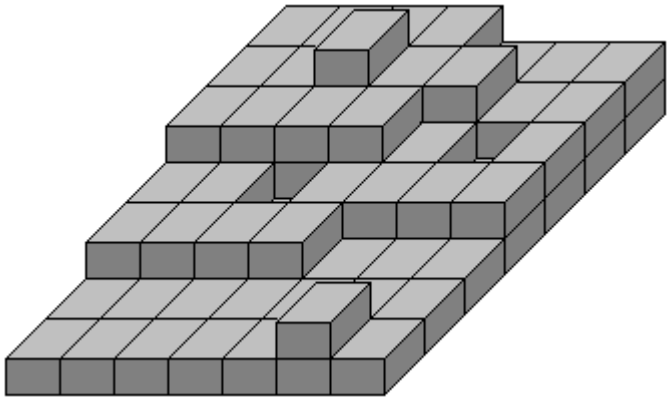


Fig. 2.16 The structural imperfections of a solid surface.

There are two types of adsorption processes that need to be distinguished: physical adsorption and chemical adsorption (chemisorption). In physical adsorption, the binding forces arise from van der Waals forces, dipole-dipole interactions and London dispersion forces. In chemisorption, covalent chemical bonds are formed between the atoms or molecules of the surface and the atoms or molecules of the adsorbed substance (Mortimer, 2000).

Because chemisorption occurs through chemical bonding, the presence of thermal energy is often necessary, since most chemical reactions are associated with an activation energy. An important factor related to chemisorption is that the adsorbed molecules are localized on the surface. On the other side, physical adsorption is accompanied by low heat of adsorption without structural changes occurring on the surface. Physical adsorption may lead to surface coverage by more than one layer since the

adsorbate is not restricted to specific sites and, therefore, free to cover the entire surface. At elevated temperatures physical adsorption does not occur since less or no activation energy is required; therefore, at low temperature the equilibrium is rapidly achieved. In contrary to chemisorption, physical adsorption is a fully reversible process (Lowell and Shields, 1984).

2.5.2 ADSORPTION ISOTHERMS

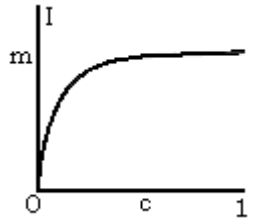
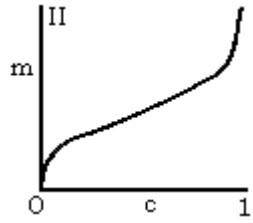
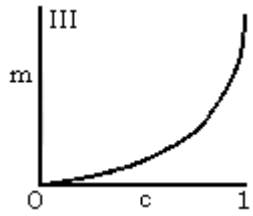
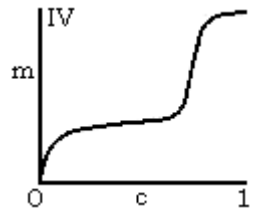
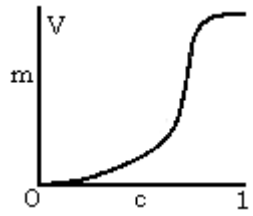
When a solution is in contact with the active surface of a solid, an equilibrium is established for the adsorption and desorption process of the species present in the solution. At constant temperature, the relationship between the concentration of a given species in solution and the amount that is adsorbed can be expressed as an equation or as a plot. The line so obtained is called the *adsorption isotherm* (Pecsok et al., 1976).

The adsorption isotherms indicate the dependence of the quantity adsorbed at the equilibrium point in function on the concentration of substance in a gas or liquid phase, at fixed temperature. The study of adsorption isotherms is important because they allow to determine the equilibrium constant for the adsorption-desorption process, the specific surface area available for adsorption, and the enthalpy of adsorption (McQuarrie and Simon, 1997).

2.5.2.1 Classification of adsorption isotherms

The shape of all adsorption isotherms studied using nitrogen as adsorbate, fit into one of the five different types shown in Table 2.4. Each one of them reflects some unique condition and occurrence (Lowell and Shields, 1984) (McCash, 2004) (Sing, 1989).

Table 2.4 Types of adsorption isotherms

Isotherm type	Characteristic
	Limited to only few molecular layers. It is encountered in chemisorption where the asymptotic approach to a limiting quantity indicates that all the surface sites are occupied. In physical adsorption it is typically encountered with microporous[2] adsorbents, which once filled with the adsorbate leave no external surface for additional adsorption. Characteristic of Langmuir isotherm.
	Represents unrestricted multilayer adsorption on a non-porous or macroporous adsorbents The inflection point occurs near the completion of the first adsorbed monolayer. In this point, a second layer is formed and therefore, the amount adsorbed is increased. The process continues with the formation of higher layers. At saturation, the number of adsorbed layers becomes infinite. Characteristic of Brunauer-Emmett-Teller isotherm (BET).
	It is associated with multilayer formation from the beginning of the adsorption process where the adsorbate interacts greater with the adsorbed layer than with the adsorbent surface. Similar as isotherm type II, the process continues with the formation of higher layers and at saturation, the number of adsorbed layers becomes infinite.
	It is related with capillary condensation taking place in mesopores. Presents considerable interest in the study of catalysis because an increase in concentration to a certain level results in a different coverage when compared to the coverage formed by decreasing the concentration to the same level.
	Results from monolayer formation where the adsorbate and adsorbent present weak interactions.

[2] IUPAC classification : micropores have width not exceeding 2 nm.
mesopores have width between 2 nm and 50 nm.
macropores have exceeding 50 nm.

2.5.2.2 The Langmuir isotherm

The simplest theoretical model of adsorption is based on the Langmuir theory that assumes that the adsorbed molecules do not interact with another, that the enthalpy of adsorption is independent of the surface coverage and that there are a finite number of surface sites where a molecule can be adsorbed. In a general way, an adsorption process can be represented by:

$$A + surface\ site \underset{k_d}{\overset{k_a}{\rightleftharpoons}} A_{ads}$$

$$K = \frac{k_a}{k_d} = \frac{[A_{ads}]}{[A][surface\ site]} \qquad \text{Eq. 2.17}$$

The surface contains a set of sites at which molecules of A can be adsorbed forming only a single layer of molecules on the surface. The fraction of the surface sites occupied by adsorbed A molecules is denoted by Θ. The adsorption is assumed to be an elementary process so that the rate of adsorption is of first order, where the concentration of A molecules ($[A]$) in the fluid phase is proportional to $1 - \Theta$, the fraction of surface sites available for adsorption (McQuarrie and Simon, 1997).

$$\text{Rate of adsorption} = k_a \bullet [A] \bullet (1-\Theta) \qquad \text{Eq. 2.18}$$

But, the desorption is also assumed to be an elementary process, so that:

$$\text{Rate of desorption} = k_d \bullet \Theta \qquad \text{Eq. 2.19}$$

At equilibrium, the rate of desorption equals the rate of adsorption:

$$k_d \bullet \Theta = k_a \bullet [A] \bullet (1 - \Theta) \qquad \text{Eq. 2.20}$$

Solving this equation for Θ, the Langmuir Adsorption Isotherm is obtained:

$$\Theta = \frac{k_a[A]}{k_d + k_a[A]} = \frac{K[A]}{1 + K[A]} \qquad \text{Eq. 2.21}$$

Here, K is the concentration equilibrium constant, and has the units of reciprocal concentration (L/mol). The value of K can be determined from the Langmuir isotherm (Fig. 2.17) by calculating the value of the concentration of A corresponding to Θ = 1/2. When $[A] = K$, the surface adsorption sites are half occupied.

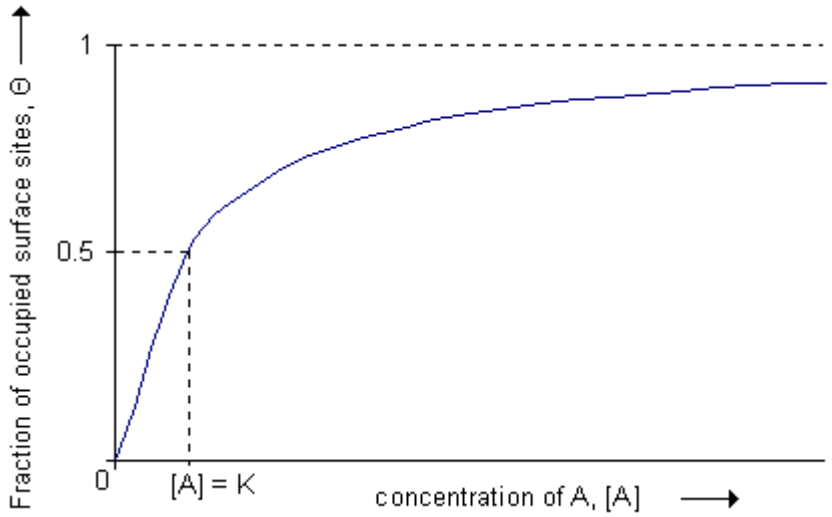


Fig. 2.17 The Langmuir isotherm.

Since the value of Θ is not directly measurable but the mass adsorbed is proportional to Θ; normally, a graph of the mass adsorbed is used for plotting the isotherms. The location of the asymptote corresponds to the value where m_{ads} = 1, but accurately locating the asymptote on the graph is difficult due to experimental errors, and therefore, it is desirable to make a linear plot using $1/m_{ads}$ vs. 1/[A], as shown in Fig. 2.18 (Mortimer, 2000).

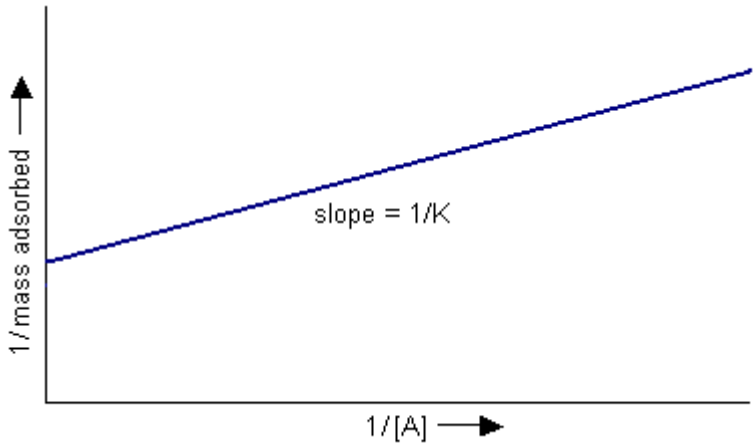


Fig. 2.18 Linear plot of Langmuir isotherm.

In practice, the equation used for calculating the Langmuir adsorption isotherm is expressed as indicated in Eq. 2.22 (Mortimer, 2000).

$$\frac{1}{m_{ads}} = \frac{1}{K[A]V_m} + \frac{1}{V_m} \qquad \text{Eq. 2.22}$$

Where:

m_{ads} = adsorbed mass of species A in the solid material

[A] = concentration of species A in the solution

K = concentration equilibrium constant

V_m = limit sorption capacity

CHAPTER THREE – OBJECTIVES

The scientist: He will spend thirty years in building up a mountain range of facts with the intent to prove a certain theory, then he is so happy in his achievement that as a rule he overlooks the main fact of all—that his accumulation proves an entirely different thing.

Mark Twain

Specially for developing countries, the necessity to find alternative methods of analysis to be used for direct application in fields has vital importance, due to the difficulty to find accessible laboratories and where the lack of modern sophisticated instrumentation increases the cost in a way that routine analysis are difficult to be done in a systematic way. However, alternative methods can be easily used everywhere, keeping the use of laboratories for confirmatory analysis.

3.1 GENERAL OBJECTIVES

The general purpose of the present study was to develop a simple, inexpensive, precise and sensitive chemical analytical procedure to determine β–lactam antibiotics, having as application the analysis of environmental samples. The development of the method was established avoiding the use of complicated materials, contaminating solvents and chemical reagents, sophisticated and expensive instrumentation, microorganisms or enzymes.

3.2 SPECIFIC OBJECTIVES

- To search for a chemical colorimetric reaction useful for the determination of β-lactam antibiotics that yields a stable colored product proportional to the amount of antibiotics.

- To adsorb the colored product obtained onto polyurethane foam and to evaluate the color intensity directly on the solid material using diffuse reflectance spectroscopy.

- To study the effect of possible interferences and to use solid phase extraction to clean up the sample.

CHAPTER FOUR - EXPERIMENTAL SECTION

After a certain high level of technical skill is achieved, science and art tend to coalesce in esthetics, plasticity and form. The greatest scientists are always artists as well.

Albert Einstein

4.1 INSTRUMENTATION AND EQUIPMENT

A diode-array X-dap spectrophotometer (IKS optoelektronik GmbH, Germany) was used for all absorbance and reflectance measurements. In order to develop a measurement, the instrument was coupled by optical fiber with one of the following attachments: a reflection head with 0°/45° geometry for measurements of diffuse reflectance on solid material, and a 10 mm path length cell holder for measurements of absorbance on solutions. The spectrophotometer was controlled via PC by means of XLAB software, version 3.11. The measurements of solutions were performed using a 10 mm light path quartz cell (100.QS, Hellma, Germany). Flow-through measurements were done using a 10 mm light path flow quartz cell (178.010.QS, Hellma, Germany) coupled with a Minipuls 3 peristaltic pump (Gilson, France).

The weight of reagents was determined using suitable electronic balances: Mettler-Toledo AE 240 (sensitivity 0.0001 g to 40 g) and Sartorius M2P (sensitivity 0.001 to 2000 mg).

The pH measurements were done using a Sension156 portable multiparameter meter (HACH, USA) with pH electrode 51935-00 (HACH, USA).

For diffuse reflectance measurements, a home-made horizontal turning device (25 rpm as slowest speed) was prepared using a mechanical stirring motor (Type RW 18; IKA-Werke GmbH & Co. KG, Germany) coupled with the sample holder.

For sorption processes, a home-made vertical shaking device (25 rpm as slowest speed) was prepared using a mechanical stirring motor (Type RW 18; IKA-Werke GmbH & Co. KG, Germany) coupled with 25 universal clamps aligned in parallel one by one. Each clamp was used to hold a reactor tube with capacity up to 60 ml.

For chemical reactions carried out under temperature, a water bath system controlled by a Haake D1 thermostat was used.

4.2 MATERIALS

Polyurethane foam polyether type in three different bulk densities was used:

Table 4.1 Types of polyurethane foam used

PUF name and code	Density (kg/m^3)	Manufacturer
Contipur 1621	15.0	Contitech, Germany
Contipur 3550	33.5	Contitech, Germany
Lastilux 5525	55.0	DUMO, Belgium

The preparation of chemical solutions was done using necessary laboratory glassware from borosilicate Blau-Brand (Brand, Germany). Special borosilicate glass columns with 3 cm diameter x 13 cm high were used as reactors. The capacity of each reactor was 60 ml, excluding the glass stopper.

Commercial solid phase extraction cartridges used for testing the removal of possible interferences were:

Table 4.2 Solid Phase Extraction cartridges

Name	Sorbent material[3]	Manufacturer
Bakerbond C18 Polar Plus	Octadecylsilane bonded to Silica Gel, 500 mg	J.T.Baker
Bakerbond C18	Octadecylsilane bonded to Silica Gel, 500 mg	J.T.Baker
Chromabond Easy	Polar modified polystyrene-divinylbenzene copolymer, 500 mg	Macherey-Nagel
Strata X	Patent-pending polystyrene-divinylbenzene copolymer, 500 mg	Phenomenex
Isolute ENV+	Hydroxylated polystyrene-divinylbenzene, 500 mg	IST
SepPak tC18 plus	Trifunctional silane	Waters

4.3 CHEMICAL REAGENTS, SOLVENTS AND WATER

All solutions were prepared using deionized water, purified by Elix3 - MilliQ System (Millipore, USA).

Table 4.3 Chemical reagents used

Name	Company	Quality	Purity
Acetone	Fluka	Puriss. p.a.	> 99.5 %
Methanol	Merck	P.a, ACS, ISO	≥ 99.8 %
Ethanol	Fluka	Puriss. p.a.	≥ 99.8 %
Hydrochloric acid	Riedel-de Haen	Puriss. p.a.	≥ 37 %
Sulphuric acid	Fluka	Puriss. p.a.	≥ 95 %
Phosphoric acid	Fluka	Puriss. p.a.	≥ 85 %
Phosphomolybdic acid	Riedel-de Haen	Puriss. p.a.	---
Silicomolybdic acid	Aldrich	---	---
Folin-Ciocalteu Reagent	Fluka	---	2 N respect to acid
L(+)-Cysteine	Riedel-de Haen	Biosynth.	≥ 99.0 %
DL-Penicillamine	Fluka	Puriss. p.a.	≥ 99.0 %
L(+)-Ascorbic acid	Riedel-de Haen	Analytical reagent	≥ 99.7 %
Stannous chloride	Fluka	Pure	≥ 98.0 %

[3] Description of sorbent material as stated by the manufacturer.

Standards of benzylpenicillin (Penicillin G), phenoxymethylpenicillin (penicillin V), amoxicillin, ampicillin, cefotaxime and cefuroxime were friendly donated by the Institute for Energy and Environmental Technology, IUTA, Germany, and used as received.

Table 4.4 Chemical reagents used only for evaluating possible interferences

Name	Company	Quality	Purity
L-aspartic acid	Fluka	Ultra	≥ 99.5 %
O-acetylsalicylic acid	Fluka	Purum	≥ 99.0 %
barbituric acid	Aldrich	---	99.0 %
benzaldehide	Bayer	Rein	---
caffeine	Fluka	Purum	≥ 99.0 %
chloramphenicol	Gerbu	Pharma	≥ 99.0 %
citric acid	J.T.Baker	Baker	---
m-cresol	Fluka	Pract.	≥ 98.0 %
o-cresol	Merck	For synthesis	98.0 %
p-cresol	Riedel-de Haen	Chem. pure	99.0 %
L-cystine	Riedel-de Haen	Biosynth.	99.0 %
β-D-fructose	Calbiochem	---	99.9 %
D(+)-galactose	Sigma	Ultra	≥ 99.0 %
glycine	Merck	P.a.	≥ 99.7 %
D(+)-glucose	Riedel-de Haen	Extra pure	---
L-glutamic acid	Fluka	Micro-select	≥ 99.5 %
hydrazine sulfate	Aldrich	ACS reagent	≥ 99.0 %
hydroquinone	Fluka	Puriss p.a.	≥ 99.0 %
hydroxylamine HCl	Fluka	Puriss p.a.	≥ 98.0 %
L-hydroxyproline	Merck	For biochemistry	99 %
hippuric acid	Aldrich	---	98 %
humic acids sodium salt	Janssen	Tech.	---
lactose	Merck	---	---
D(+)-maltose	Fluka	Ultra	≥ 99.0 %
DL-methionine	Merck	---	---
4-nonylphenol	Fluka	Techn.	~ 85 %
oxalic acid	Riedel-de Haen	Analytical reagent	≥ 99.5 %

Name	Company	Quality	Purity
paracetamol	Merck	Ph Eur	≥ 99.0 %
phenol	Bayer	---	---
pirocatechol	Merck	For synthesis	> 99.0 %
3,4-dimethylphenol	Fluka	Purum	≥ 98.0 %
L-phenylalanine	Fluka	Micro-select	≥ 99.0 %
L-proline	Fluka	Ultra	≥ 99.5 %
propionaldehyde	Fluka	Purum	98 %
sacarose	Merck	---	---
salicylic acid	Merck	For synthesis	≥ 98.0 %
streptomycin sulfate	Gerbu	DAB 10	≥ 99.5 %
5-sulphosalicylic acid	Merck	P.a.	≥ 99.5 %
sulfanilamide	Fluka	Purum	≥ 98.0 %
tetracycline HCl	Gerbu	Pharma	≥ 99.4 %
thioacetamide	Merck	P.a.	≥ 99.0 %
thiosulphate sodium	Fluka	Puriss p.a.	≥ 99.0 %
thiourea	Riedel-de Haen	Extra pure	99 %
L-tryptophan	Fluka	Micro-select	≥ 99.5 %
L-tyrosine	Fluka	Micro-select	≥ 99.0 %
urea	Merck	P.a.	≥ 99.0 %
uric acid	Merck	For biochemistry	≥ 99.0 %

4.4 PROCEDURES

4.4.1 PREPARATION OF PUF DISKS

Blocks of polyurethane foam of all three bulk densities were cut in sheets of thickness 5 mm using a proper vertical saw for foam. Then, the sheets were immersed in liquid nitrogen and using a cork borer, disks with diameter of 20 mm were prepared. For each bulk density, the disks were weighted one by one and separated in 10 lots, according to the distribution: *average weight* ± (0.0001, 0.0002, 0.0003, 0.0004, 0.0005) g. This was done with the purpose to reduce the error due to the adsorbed amount of any analyte is proportional to the mass of adsorbent. In corresponding experiments, only disks of the same lot were used. All PUF

disks were firstly washed with 1.0 M HCl, secondly with pure water until the washings are acid free and third with acetone. The disks were air-dried and then stored in brown-stoppered flasks.

4.4.2 PREPARATION OF ANALYTICAL SOLUTIONS

Analytical solutions were prepared placing 40 ml of water in 10 glass reactor tubes. Aliquots of 0, 20, 50, 100, 200, 400, 600, 800, 1000 and 1200 µl of stock antibiotic solution (100 µg ml^{-1}) were added into each reactor and then 0.1 ml of 1.0 M HCl to set the pH to 2.50, followed by 1.0 ml of 0.01 M phosphomolybdic acid. The tubes were heated in a water bath at 90 °C for 60 minutes. After the heating step, the reactors were cooled to room temperature and 0.5 ml of 1.0 M HCl were added to set the pH to 2. A PUF disk previously soaked in 0.01 M HCl was added to each reactor and the air bubbles were removed using a glass rod. The reactors were closed and mechanically shaken for 90 minutes. The disks were removed, squeezed between filter paper and air-dried. Finally, the diffuse reflectance of each disk was measured, using a white PUF disk to setup the reflectance to 100%.

4.4.3 SOLID PHASE EXTRACTION

The clean up of possible interferences was made using the corresponding SPE cartridge coupled with a flow through cell and by means of UV detection. Each cartridge was first conditioned with methanol until no change in UV signal was achieved. Then, 0.1 M HCl was added through the cartridge until no change in UV signal was achieved. After that, the acidified sample (pH = 1.0 with HCl) was loaded and the change in UV signal was used to determine the behaviour of the cartridge. Once the sample was loaded, the cartridge was washed with 0.01 M HCl and then, the analyte was eluted using methanol - 0.1 M HCl [1:1]. In the same way, the change in UV signal was used to evaluate the elution efficiency.

CHAPTER FIVE - RESULTS AND DISCUSSION

What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on.

Jacques Cousteau

5.1 SPECTRA

5.1.1 ABSORPTION SPECTRA

The absorption spectra of phosphomolybdic acid (aqueous solution) in its oxidized form (before reaction) and reduced form (after reaction[4]) are shown in Fig. 5.1.

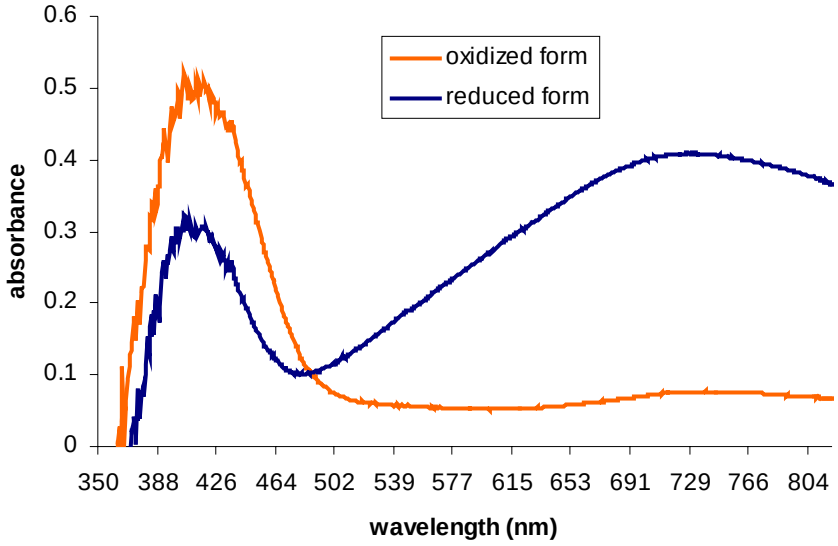


Fig. 5.1 Absorbance spectra of phosphomolybdic acid.

[4] Stannous chloride was used as reducing agent.

The oxidized form has yellow color and presents the maximum of absorbance at 410 nm while the reduced form has blue color and presents the maximum of absorbance at 730 nm.

The diminution of absorbance at 410 nm indicates less concentration of oxidized form of phosphomolybdic acid as result of the corresponding reduction to form heteropoly blues, and therefore, the increment of absorbance at 730 nm. The proportion of the absorbance increment at 730 nm is stronger than the proportion of absorbance diminution at 410 nm due to the extinction coefficient of the heteropoly blues must be higher than its similar of the oxidized form of phosphomolybdic acid.

5.1.2 ABSORPTION VS. DIFFUSE REFLECTANCE SPECTRA

The absorption spectra of individual solutions of the heteropoly blues formed after reaction between each antibiotic and phosphomolybdic acid indicate that the maximum absorption peak for all solutions occurred at 705 nm, as is shown in Fig. 5.2.

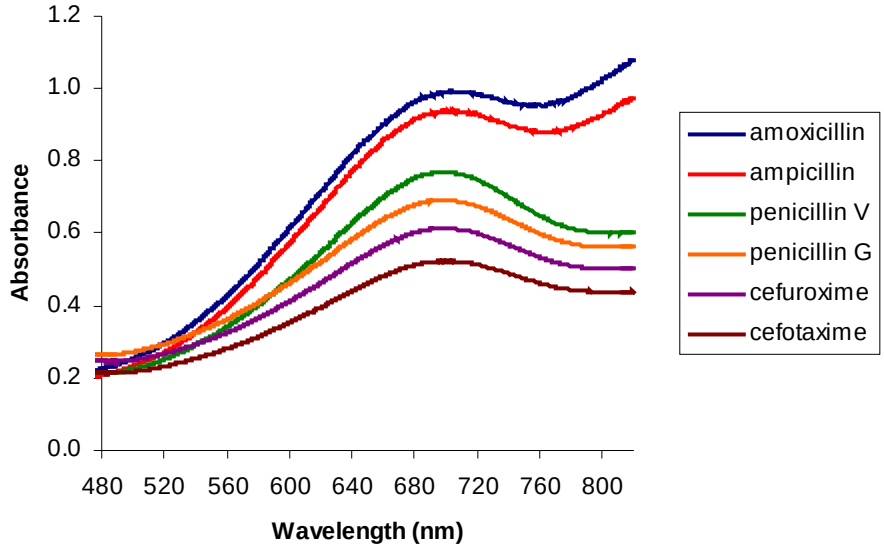


Fig. 5.2 Absorbance spectra of heteropoly blues in solution.

The heteropoly blues of each individual solution was adsorbed on a PUF disk and the diffuse reflectance was measured. The diffuse reflectance spectra, presented as Kubelka-Munk function F(R) in Fig. 5.3, show that the maximum peak occurred at 720 nm.

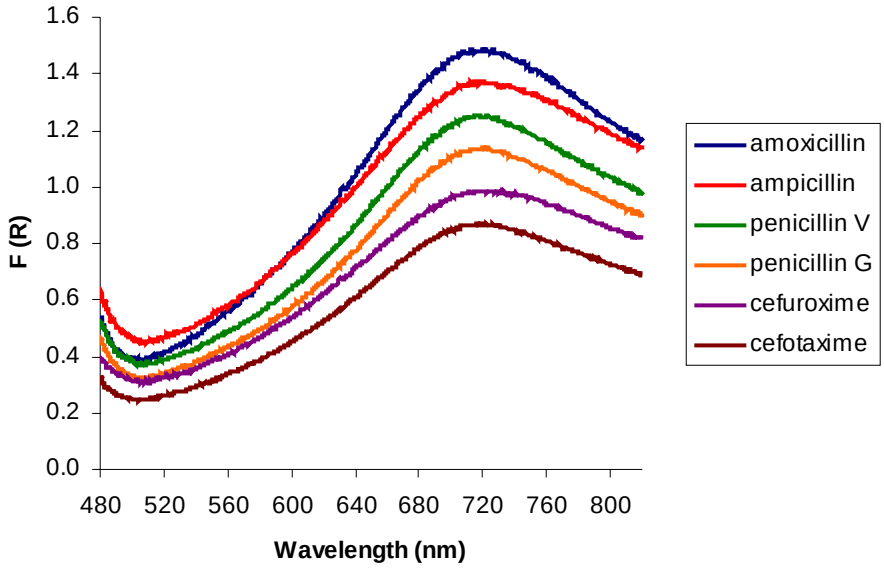


Fig. 5.3 Diffuse reflectance spectra of heteropoly blues adsorbed on PUF.

The shifting from 705 nm to 720 nm occurs normally when comparing absorbance of an analyte in solution versus the diffuse reflectance of the same analyte adsorbed on a solid material. This phenomena is due to the analyte responsible of color is trapped on the solid material and its vibrational structure is strongly suppressed.

5.2 EVALUATION OF INSTRUMENTAL PARAMETERS

Beetween the several instrumental parameters that were necessary to evaluate to obtain the best conditions for sensibility and reproducibility in all corresponding measurements, the reflectance geometry and the way how to make the measurements took important attention, as decribed as follows:

5.2.1 REFLECTANCE MEASUREMENT GEOMETRY

The head attachment used to measure diffuse reflectance was evaluated in its two possible ways: the first, with the incident light beam at 0° and the detector at 45°, and the second, in the opposite position, that is, the incident light beam at 45° and the detector at 0°; using PUF of all three bulk densities (Fig. 5.4).

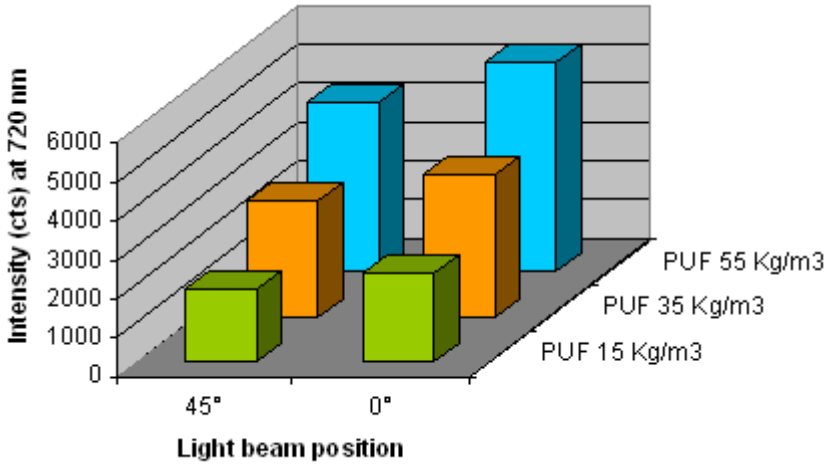


Fig. 5.4 Detector response in function of incident light beam position and PUF density.

The incident light beam positioned at 0° in function to the sample gave higher instrumental response. When an incident light beam reaches the sample in a perpendicular way, all the light has the same contact and interaction with the sample surface, but if the light reaches the sample in other form, as in this case at 45°, an attenuation effect occurs at the contact place, and therefore, less light is reflected.

The higher the PUF density, the higher the detector response, as result of more amount of light reaching the detector. PUF of higher density has more compact surface and less porosity and then, the light suffers less internal scattering and attenuation.

5.2.2 SAMPLE ROTATION

Using a 0°/45° geometry, only the light reflected in one direction (45° in this case) is detected. This reflected light is not always homogenous because the material presents some imperfections like hills, holes, canals, etc.

The reflectance spectra obtained in static mode present strong differences between them, making the analytical calculation not an easy task. Reflectance spectra of the same sample, obtained in a static mode for five different measurements, which were made simultaneously, are shown in Fig. 5.5, where it is possible to see the lack of reproducibility achieved.

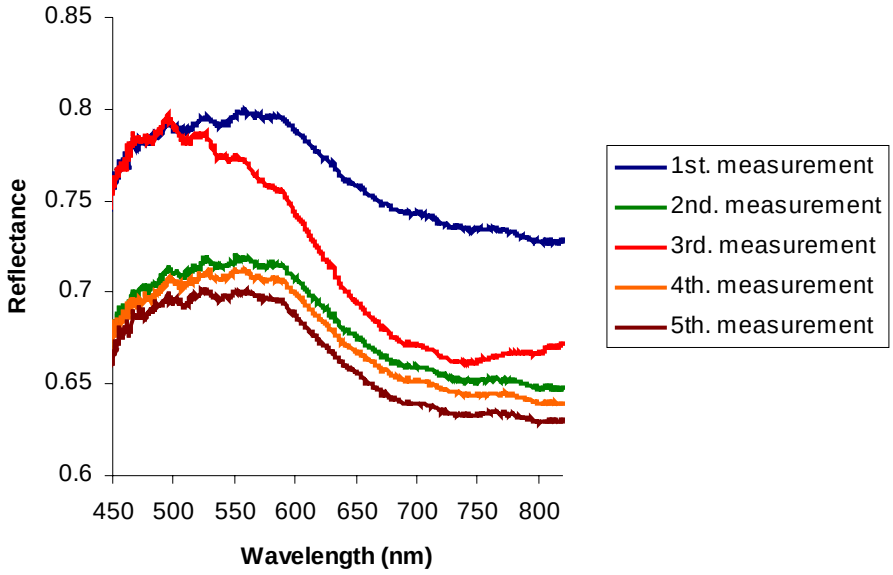


Fig. 5.5 Reflectance spectra performed under static mode.

An interesting solution was found to solve the previous problem. The sample under analysis was rotated during all the time of measurement. With 25 rpm the reflectance spectra did not show differences between them. Higher speed of rotation is not necessary. The only important aspect to take care is to not allow the external light to come in. Reflectance

spectra obtained while the sample was rotated are shown in Fig. 5.6, where is possible to visualize the strong reproducibility possible to be achieved at the moment to obtaining the spectra.

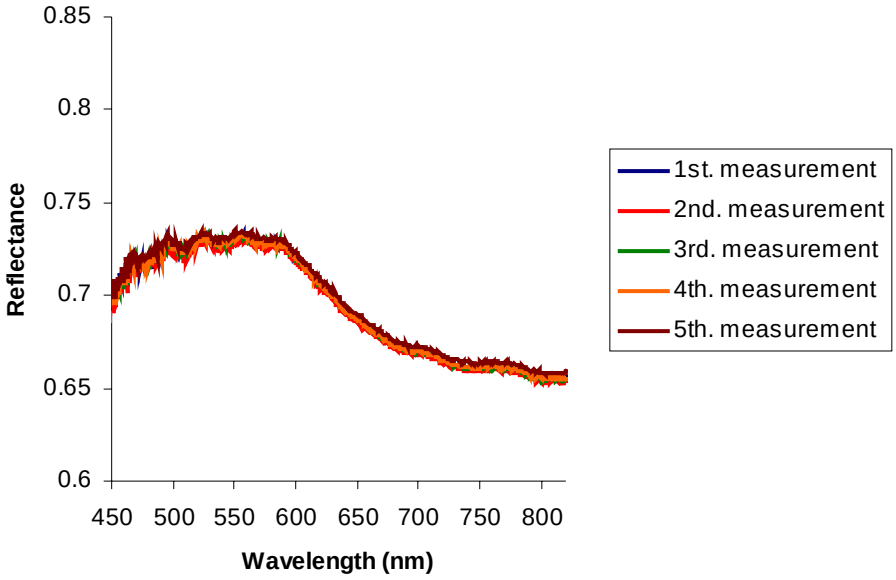


Fig. 5.6 Reflectance spectra performed under rotational mode.

5.3 EVALUATION OF ANALYTICAL PARAMETERS

Several analytical parameters were studied to obtain the best development of heteropoly blues and subsequent adsorption on PUF: concentration of phosphomolybdic acid, pH, mineral acid type, heating time.

5.3.1 DEVELOPMENT OF HETEROPOLY BLUES IN FUNCTION OF pH AND MINERAL ACID TYPE

The reactivity of amoxicillin with phosphomolybdic acid at different pH was evaluated using three different mineral acids as medium: hydrochloric acid, sulfuric acid and phosphoric acid. The results (Fig. 5.7) show that the maximum development of heteropoly blues is achieved at pH 2.5 with

hydrochloric acid. Therefore, this was the mineral acid selected for all following experiments.

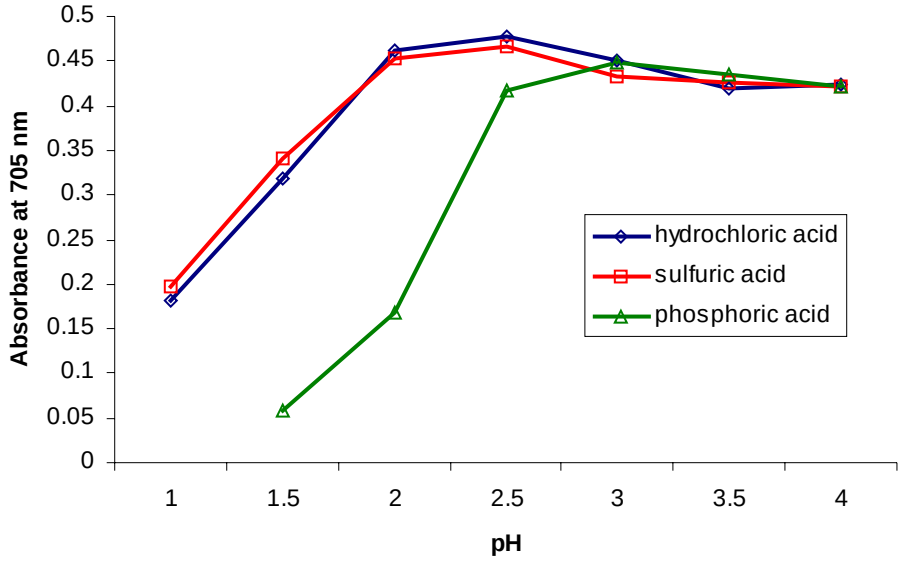


Fig. 5.7 Reactivity of amoxicillin with phosphomolybdic acid in function of pH. $C_{amox.}$ = 25 µg/ml; C_{HPA} = 0.25 mM; T_{react} = 90 °C ; $time_{react}$ = 60 min.

5.3.2 DEVELOPMENT OF HETEROPOLY BLUES IN FUNCTION OF THE CONCENTRATION OF PHOSPHOMOLYBDIC ACID.

Phosphomolybdic acid at different concentrations and also at three different pH was tested to find the maximally coloured PUF disk. Phosphomolybdic acid was prepared at five different concentrations between 0.05 mM and 1.0 mM. Three different pH values: 1.5, 2.5 and the natural value of the phosphomolybdic acid solution were tested. The pH was adjusted with 1.0 M HCl and amoxicillin was the antibiotic used. As is shown in Fig. 5.8, 0.25 mM phosphomolybdic acid at pH 2.5 gave the best result and was selected for all determinations.

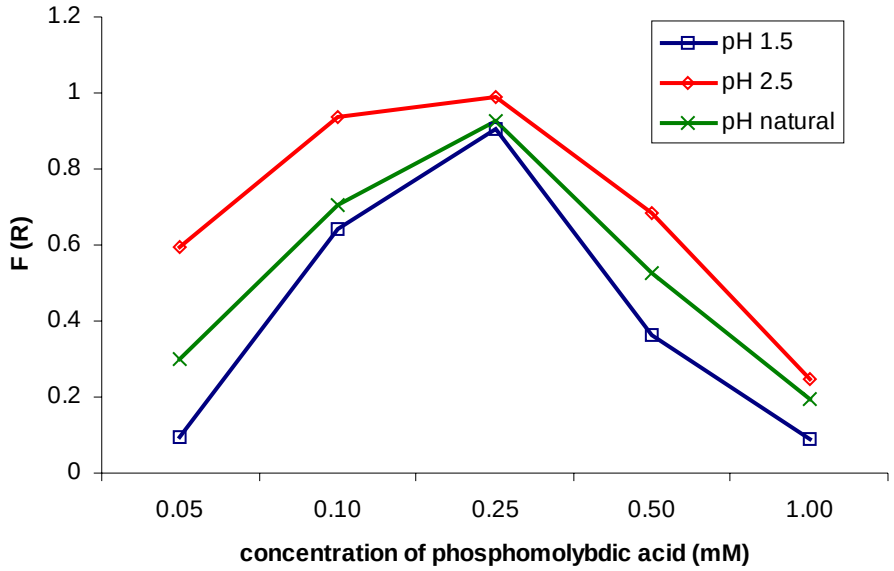


Fig. 5.8 Reactivity of amoxicillin in function of the concentration of phosphomolybdic acid. $C_{amox.}$ = 2.0 µg/ml; T_{react} = 90 °C ; $time_{react}$ = 60 min; T_{ads} = 20 °C; $time_{ads}$ = 90 min; m_{PUF} = 0.052 g; $density_{PUF}$ = 35 kg/m³; λ_{DRS} = 720 nm.

5.3.3 DEVELOPMENT OF HETEROPOLY BLUES IN FUNCTION OF TIME

All antibiotics were tested to find the necessary time for reacting with phosphomolybdic acid. As shown in Fig. 5.9, minimum 60 minutes are needed for all solutions to reach the maximal absorbance value using a temperature of 90 °C suministrated by means of a water bath. Higher temperatures can be used to accelerate the reaction but the temperature at 90 °C is suitable for applications that can be developed at different places not located near to the sea level, as occurs, for instance, in South America Andeans.

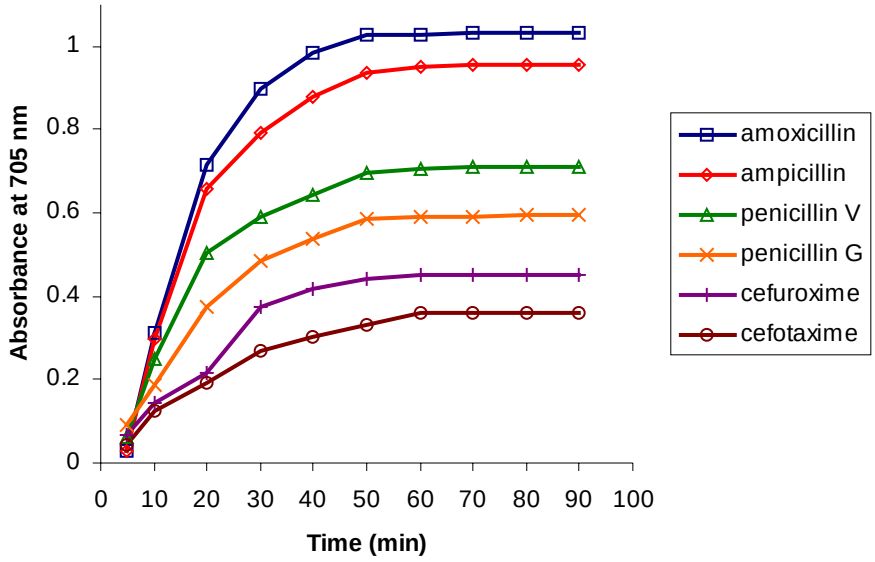


Fig. 5.9 Development of heteropoly blues for all antibiotics in function of heating time. $C_{antib.}$ = 50 µg/ml; C_{HPA} = 0.25 mM; T_{react} = 90 °C.

5.3.4 SORPTION OF REDUCED PHOSPHOMOLYBDIC ACID IN FUNCTION OF TIME

The time necessary for the sorption process was evaluated using two different concentrations of phosphomolybdic acid: 0.25 mM and 0.1 mM and PUF of 35 kg/m^3. As we can see in Fig. 5.10, the minimum sorption time necessary to adsorb the reduced phosphomolybdic acid on one PUF disk is 60 minutes, but an ideal time is 90 minutes, and this was the time used in all sorption steps.

In a previous study about sorption of phosphomolybdic acid by PUF (Dmitrienko et al., 1997b), only 30 minutes were necessary to reach the equilibrium point. The difference can be explained in the fact that the concentration of phosphomolybdic acid used in that study was 10 times higher that the one used here. At higher concentration of analyte, faster equilibrium point is achieved.

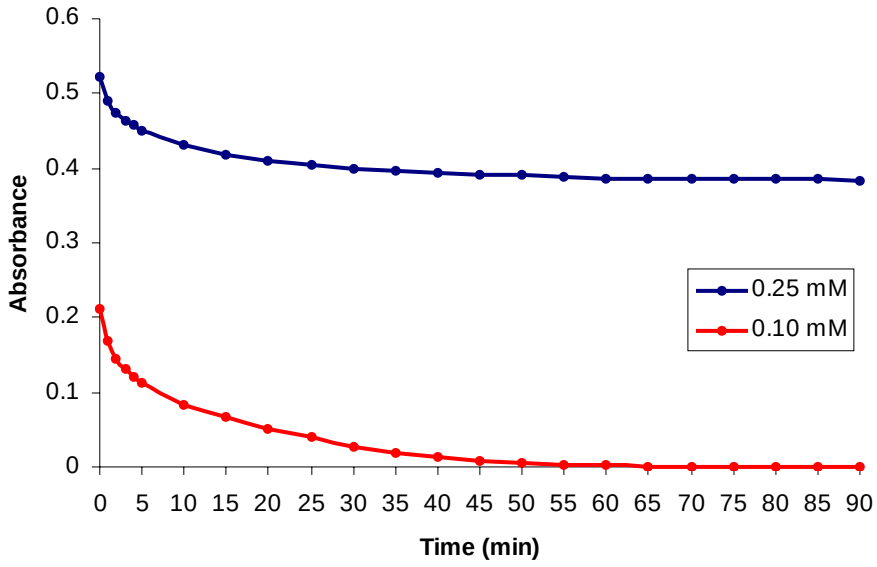


Fig. 5.10 Sorption of reduced phosphomolybdic acid in function of time. V_{HPA} = 125 ml; T_{ads} = 20 °C; m_{PUF} = 0.057 g; $density_{PUF}$ = 35 kg/m³.

5.4 ADSORPTION ISOTHERMS

The adsorption isotherms of the oxidized and the reduced forms of phosphomolybdic acid using PUF of 35 kg/m³ are shown in Fig. 5.11. It is possible to conclude that the reduced form is adsorbed better than the oxidized form. In our case, this is useful because we are interested to adsorb the reduced form of phosphomolybdic acid and, in principle, any amount of oxidized phosphomolybdic acid might present competition for adsorbing sites.

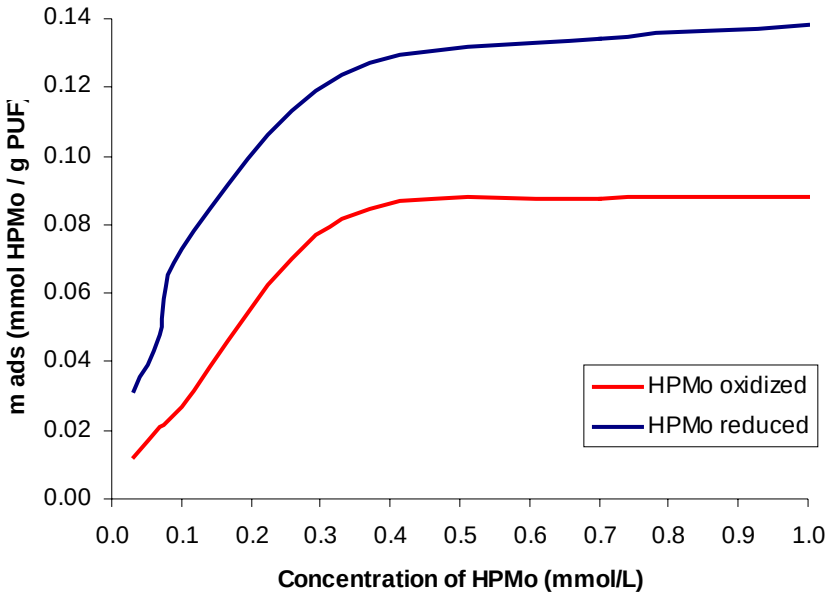


Fig. 5.11 Adsorption isotherm of phosphomolybdic acid by PUF. V_{HPA} = 40 ml; T_{ads} = 20 °C; $time_{ads}$ = 90 min; m_{PUF} = 0.054 g; $density_{PUF}$ = 35 kg/m³.

5.4.1 LANGMUIR ISOTHERM

Based on the classification of adsorption isotherms studied in sub-chapter 2.5.2.1, the shapes shown in Fig. 5.11 suggest that the sorption process of phosphomolybdic acid by PUF can be described using the Langmuir isotherm. Therefore, the Eq. 2.22 takes the form:

$$\frac{1}{m_{ads}} = \frac{1}{K[HPMo]V_m} + \frac{1}{V_m} \qquad \text{Eq. 5.1}$$

Where:

m_{ads} = adsorbed mass of phosphomolybdic acid by PUF

K = concentration equilibrium constant

[HPMo] = concentration of phosphomolybdic acid

V_m = limit sorption capacity

The plot obtained using Eq. 5.1 is shown in Fig. 5.12.

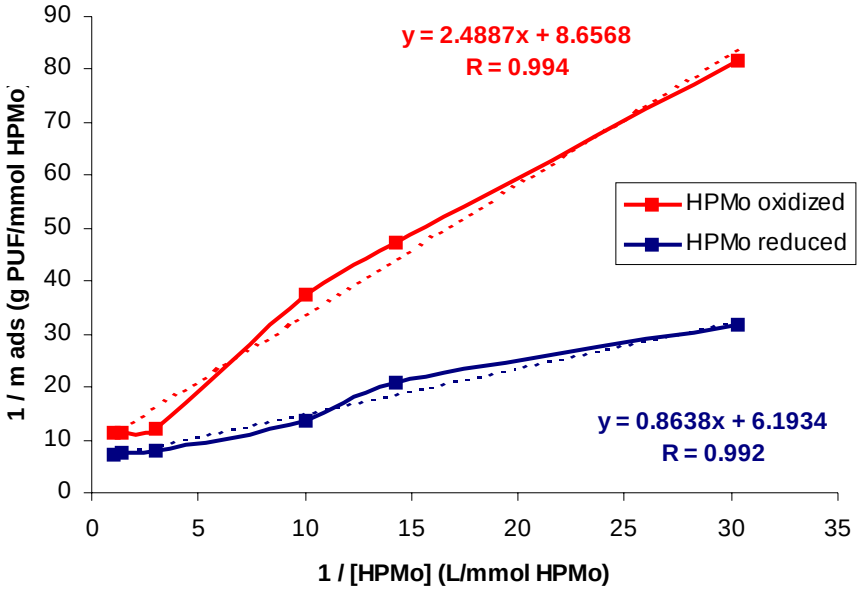


Fig. 5.12 Langmuir isotherm for sorption of phosphomolybdic acid by PUF.

The application of Eq. 5.1 gave as result straight lines for both oxidized and reduced phosphomolybdic acid. Of course, the line equations presented in Fig 5.12 were obtained using linear regression for the experimental data. Nevertheless, the correlation coefficients indicate that very good correlation exists. Therefore, we can assume that the adsorption of phosphomolybdic acid by PUF obeys the Lagmuir theory.

The parameters of the regression equations are used to calculate the limit sorption capacity, the sorption equilibrium constant and the effective area of PUF as follows:

$\frac{1}{m_{ads}} = \frac{1}{K[HPMo]V_m} + \frac{1}{V_m}$	=	$y = ax + b$
Langmuir equation		**Linear regression equation**

- The reciprocal of the limit sorption capacity ($1/V_m$) in the Langmuir equation is equal to the *intercept* (b) in the linear regression equation.
- The sorption equilibrium constant in the Langmuir equation (K) is equal to the ratio *intercept/slop* (b/a) in the linear regression equation.
- The effective area was calculated based on that one molecule of phosphomolybdic acid is contained in a square of 11.7 Å (Kaba et al., 1998). Therefore, the surface occupied by one molecule of phosphomolybdic acid is 136.89 $Å^2$ (1.3689×10^{-18} m^2) and one mmol of phosphomolybdic acid will occupy 1.3689×10^{-18} m^2 * 6.023×10^{23} /1000 = 824.48 m^2/mmol. The effective area occupied by phosphomolybdic acid will be equal to 824.48 m^2/mmol * V_m.

The results are tabulated in Table 5.1.

Table 5.1. Adsorption parameters for phosphomolybdic acid using PUF

HPMo type	Vm = 1/intercept (mmol HPMo / g PUF)	K = intercept / slope (L /mmol)	Effective area occupied by HPMo (m^2 / g)
Oxidized form	0.1155	3.478	95.24
Reduced form	0.1614	7.169	133.12

The results confirm that the reduced form of phosphomolybdic acid is adsorbed by PUF two times better than the oxidized form. This can be inferred from the ratio between the sorption equilibrium constants, which gives the value 2.06. The values for the limit sorption capacity show that the amount of the reduced form of phosphomolybdic acid adsorbed by PUF is 1.4 times higher than the oxidized form. The difference in effective area occupied by the oxidized and the reduced form is significant: the reduced form occupies an area around 40 % more than the oxidized form.

5.4.2 FREUNDLICH ISOTHERM

The adsorption of solutes on solids from liquids often follows the empirical Freundlich isotherm (Atkins, 1986), which states a logarithmic adsorption:

$$w_s = c_1[A]^{1/c_2} \qquad \text{Eq. 5.2}$$

where:

w_s = amount of solute adsorbed per unit mass of adsorbent,

[A] = solute concentration

c_1 and c_2 = adsorption constants

This means that the adsorption process will follow the Freundlich isotherm if the plot ln [A] vs. ln w_s gives a straight line.

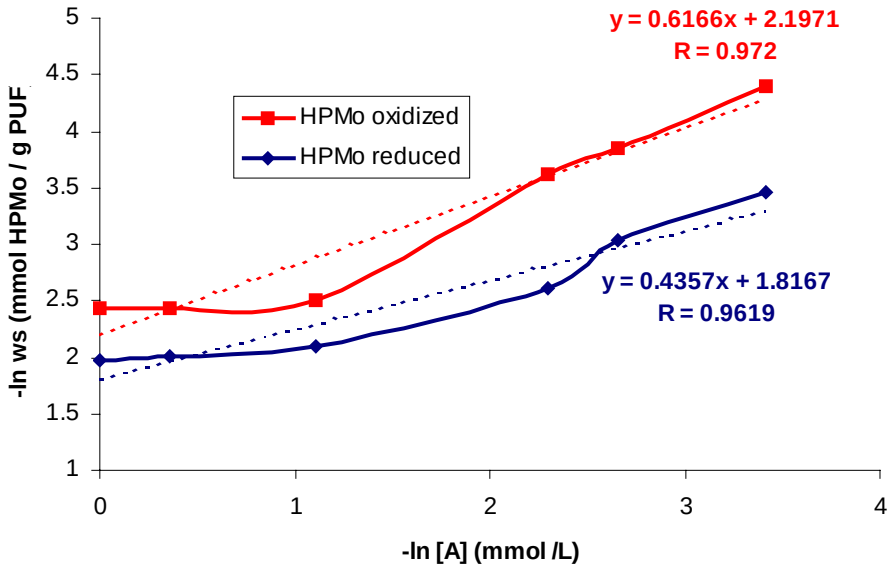


Fig. 5.13 Freundlich isotherm for sorption of phosphomolybdic acid by PUF.

Fig. 5.13 shows no straight lines at all, and therefore, the adsorption of phosphomolybdic acid can be definitely described using the Langmuir

isotherm in which, the main assumption is that the adsorbed material covers the adsorbent as a unique monolayer.

5.5 ANALYTICAL DETERMINATIONS

5.5.1 RANGE OF LINEARITY

The range of linearity for the determination of all six antibiotics was evaluated preparing standard solutions and the corresponding blank, between the range 0 and 5 µg/ml. After reaction with phosphomolybdic acid and subsequent adsorption on PUF, the color intensity was measured directly on the PUF by means of diffuse reflectance and the data were processed using the Kubelka-Munk function. The mean Kubelka-Munk values from five repetitions plotted against the concentration of the standards is shown in Fig. 5.14.

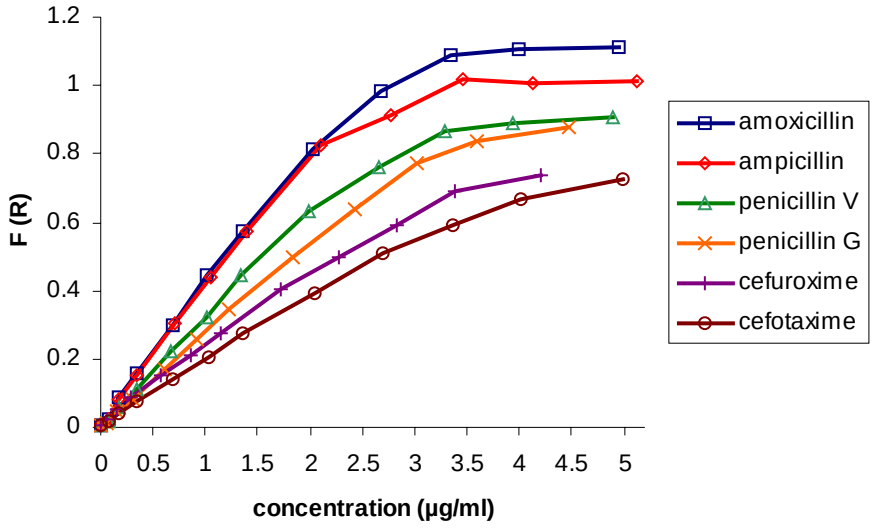


Fig. 5.14 Kubelka-Munk values in function of antibiotic concentration. Mean value of five repetitions. V_{antib} = 40 ml; C_{HPA} = 0.25 mM; T_{react} = 90 °C; $time_{react}$ = 60 min; T_{ads} = 20 °C; $time_{ads}$ = 90 min; m_{PUF} = 0.056 g; $density_{PUF}$ = 35 kg/m³; λ_{DRS} = 720 nm.

The results show that all six antibiotics present a similar behaviour under the same experimental conditions. Visually, the curves show a

linear correlation for all antibiotics up to 2.0 μg/ml. At higher concentrations, the Kubelka-Munk values start to remain constant due to the PUF, under these experimental conditions, is not able to adsorb more amount of reduced phosphomolybdic acid because an equilibrium state of sorption has been reached. In this case, we can evidence that this phenomenon is similar to that one experimented before when the adsorption isotherms were evaluated (Fig. 5.11).

The evaluation of linearity is necessary to be performed in order to verify that the response signal is linearly proportional to the analyte in a defined range of concentration. The range of linearity was evaluated by means of the response factor (RF) method.

The RF method is based on the correlation between response factor and concentration. When plotting the *response factor* versus *concentration*, a slope with zero value indicates that a linear response exists over this concentration range (Christian, 2004).

$$RF = (signal - intercept) / concentration \qquad \text{Eq. 5.3}$$

The response factors for all six antibiotics in the range between 0 and 2 μg/ml are presented in Fig. 5.15, where it is possible to realize that at the lowest concentration (below 0.1 μg/ml), the response factors for all six antibiotics are not fitting in a linear range with the other response factor. Therefore, it is not possible to use the standards at that concentration in the preparation of the calibration curve. The explanation to this observation is that the signals obtained at this low concentration level are near to the signals obtained with the blank, being in this point necessary to evaluate the detection limit, as it will be made in the next sub-chapter.

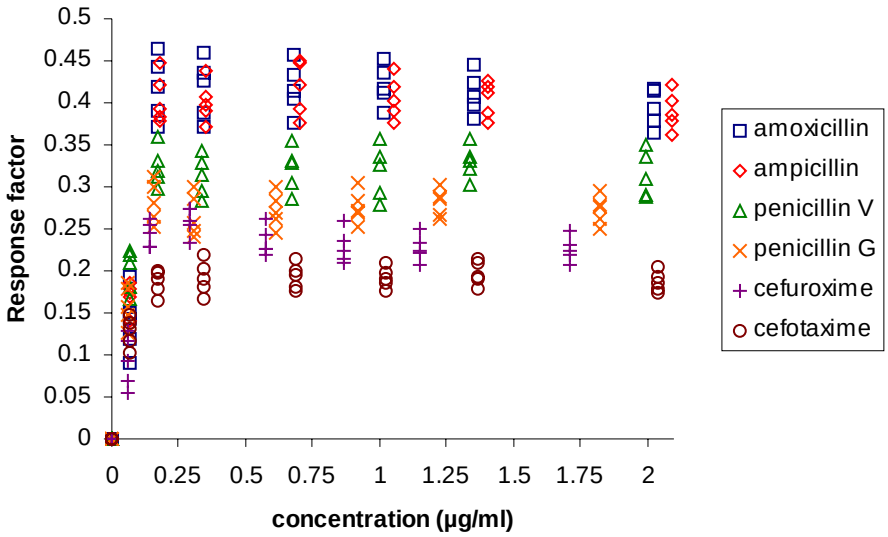


Fig. 5.15 Response factor in the range 0 to 2 µg/ml.

After eliminating the values at the lowest concentration, the regression equations for all response factors present slopes near zero (Fig. 5.16), indicating that the linear range of analysis for all six antibiotics is applicable between 0.1 and 2.0 µg/ml.

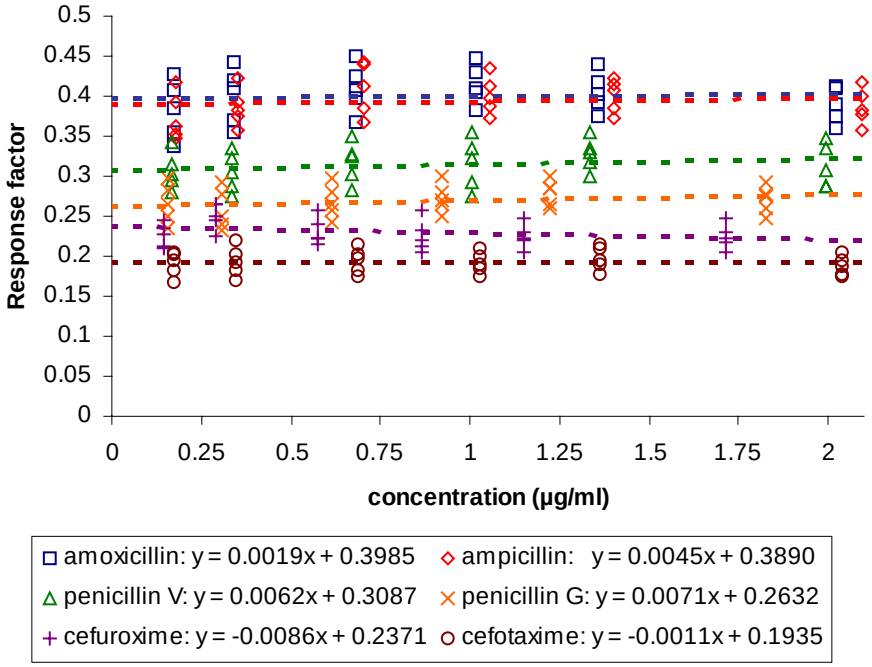


Fig. 5.16 Response factor in the range 0.1 to 2 µg/ml.

5.5.2 CALIBRATION CURVES

Once the linear range has been evaluated, the calibration curves are possible to be constructed in the defined concentration range between 0.1 and 2.0 µg/ml using the respective standard solutions and including the values of the blank for 0.0 µg/ml. The final calibration curves for all six antibiotics are presented in Fig. 5.17.

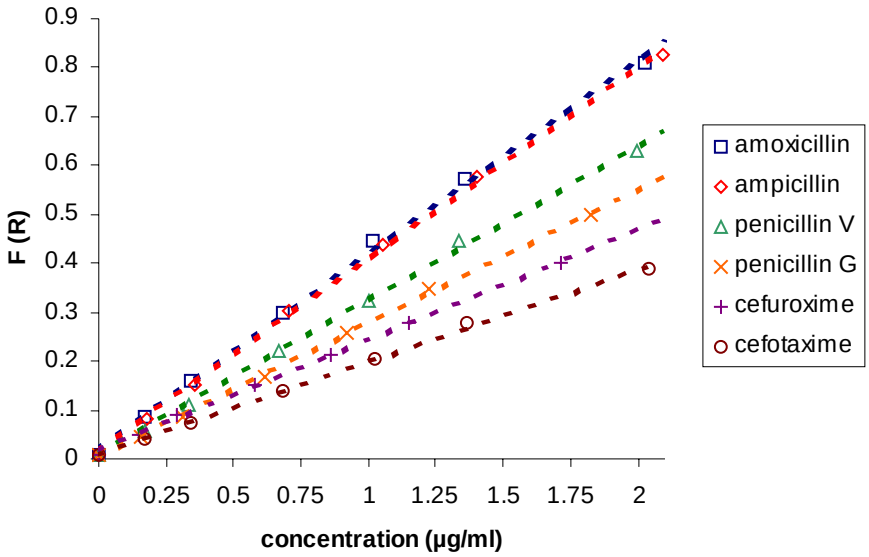


Fig. 5.17 Calibration curves. V_{antib} = 40 ml; C_{HPA} = 0.25 mM; T_{react} = 90 °C; $time_{react}$ = 60 min; T_{ads} = 20 °C; $time_{ads}$ = 90 min; m_{PUF} = 0.056 g; $density_{PUF}$ = 35 kg/m³; λ_{DRS} = 720 nm.

The correlation parameters for all six calibration curves are presented in table 5.2. It is included the limit of detection (LOD), limit of quantitation (LOQ) and relative standard deviation (RSD), calculated using DIN NORM 32645 with a confidence value of 95%.

Table 5.2 Analytical limits calculated by means of the calibration data curve

	Slope	Intercept	Correlation Coefficient	LOD (µg/ml)	LOQ (µg/ml)	RSD (%)
amoxicillin	0.399	0.0220	0.9954	0.038	0.145	4.32
ampicillin	0.3929	0.0157	0.9959	0.039	0.142	3.20
penicillin V	0.3171	0.0069	0.9928	0.035	0.126	2.96

	Slope	Intercept	Correlation Coefficient	LOD (μg/ml)	LOQ (μg/ml)	RSD (%)
penicillin G	0.2726	0.0044	0.9955	0.026	0.096	2.45
cefuroxime	0.2262	0.0172	0.9942	0.037	0.133	3.70
cefotaxime	0.1896	0.0096	0.9946	0.039	0.139	2.28

The limits of quantitation (LOQ) achieved with the present method are at least 10 times lower than other spectrophotometric methods based on the analysis of solutions. Only the determination using pyrocatechol violet (Amin, 2001) presents LOQ near the ones here presented. However, the attempts made by myself to reproduce the mentioned method were unsuccessful, since, not even the colorimetric reaction was possible to be performed under the corresponding procedure.

With the limits of quantitation achieved in the present study it is impossible to reach the detection of β-lactam antibiotics at the concentration levels normally found in surface water, which are in the concentration level of μg/L.

For waste water coming out directly from hospitals and livestock farms, which present concentration levels for β-lactam antibiotics around μg/ml, the method would be able to be applied. The question is: is there necessity to use this method in that application?.

At this point, it is not easy to find a real and practical application according to the objectives proposed at the beginning of this research. Moreover, if it is true that the present method will work perfect for analysing pharmaceutical formulations, the operational procedures make easier the decision to use typical spectrophotometric methods and liquid chromatography for the analysis of that kind of samples.

5.5.3 LIMIT OF DETECTION BY MEANS OF THE BLANK VALUE METHOD.

The International Union of Pure and Applied Chemistry (IUPAC) defines the limit of detection (LOD) as follows: "The limit of detection, expressed as the concentration, c_L, or the quantity, q_L, is derived from the smallest measure, x_L, that can be detected with reasonable certainty for a given analytical procedure. The value of x_L is given by the equation $x_L = x_{bi} + ks_{bi}$ where x_{bi} is the mean of the blank measures, s_{bi} is the standard deviation of the blank measures, and k is a numerical factor chosen according to the confidence level desired, generally 3 for a confidence value of 90% (Thomsen et al. 2003)

In that way, for all six antibiotics, the series of five sequential baseline measurements obtained for the blank and for the standards at concentration around 1 µg/ml were used. The LOD and LOQ for all six antibiotics calculated by means of the method of blank value are shown in table 5.3.

Table 5.3 Analytical limits calculated by means of the blank value method

	Concentration (µg/ml)	Signal F (R)	Standard Deviation	Net Signal	LOD (µg/ml)	LOQ (µg/ml)
blank	0.0000	0.00802	0.00219	0.00802	---	---
amoxicillin	1.0188	0.44473	0.02512	0.43671	0.01533	0.050
ampicillin	1.0542	0.43772	0.02610	0.42970	0.01612	0.053
penicillin V	1.0047	0.32420	0.03186	0.31618	0.02088	0.068
penicillin G	0.9198	0.25580	0.01728	0.24778	0.02439	0.080
cefuroxime	1.1482	0.27651	0.01724	0.26849	0.02810	0.092
cefotaxime	1.0259	0.20508	0.01351	0.19706	0.03421	0.112

The values for the LOD and LOQ obtained by means of the blank value method and the LOD and LOQ obtained by means of the DIN NORM are not comparable at all. The difference arises from the fact that the blank value method is dependent only on the baseline noise and the signal at a unique concentration, and completely independent of any calibration

scheme. Therefore, it is more coherent to calculate the LOD and LOQ based on the data used to calculate the calibration curves, which is the method taken in the DIN NORM.

5.6 INTERFERENCES

5.6.1 EVALUATION OF INTERFERENCES

Due to phosphomolybdic acid can be reduced by several chemical agents, an evaluation of possible interferences was carried out on a total of fifty substances from the following groups: reducing agents, pharmaceuticals, amino acids, sugars, active metabolites and phenols. These compounds have been selected by reason of they are either related with metabolic pathways, medicine, natural products and industrial process, and therefore, they cover a wide range of chemical substances with high probability to be found in waste water.

The evaluation was performed preparing individual solutions at 100 μg/ml for all possible interferences and using 0.25 mM phosphomolybdic acid. All solutions were heated simultaneously at 90 °C for 60 minutes and finally, the absorbance of each solution was measured at 705 nm. The absorbance values are shown in Fig. 5.18, including amoxicillin and penicillin G for comparison effects.

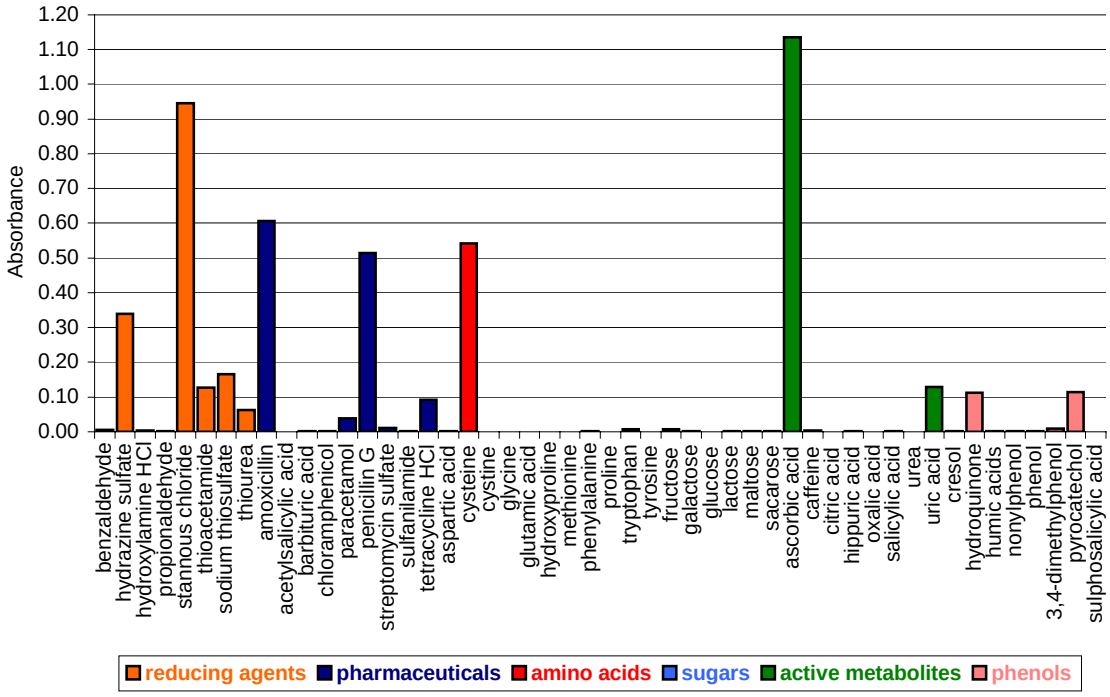


Fig. 5.18 Response signals (absorbance) of interferences. C = 100 µg/ml; C_{HPA} = 0.25 mM ; T_{react} = 90 °C; $time_{react}$ = 60 min.

The results indicate that the strongest interferences are produced by ascorbic acid, stannous chloride and cysteine because they present the similar and even much higher absorbance values than those obtained with amoxicillin and penicillin G, the antibiotics used as reference. Other interferences are produced by hydrazine sulfate, thioacetamide, sodium thiosulfate, tetracycline hydrochloride, uric acid, hydroquinone and pyrocatechol. Sugars and aminoacids (except cysteine) do not interfere at all. Even aldehydes (belzaldehyde and propionaldehyde), chemical species with reducing properties, did not present positive reaction with phosphomolybdic acid. This similar result was previously reported for the reaction between formaldehyde and phosphomolybdic acid (Lo and Chu, 1944).

Once the interferences have been evaluated, it would be interesting to know if those compounds can normally take part of the matrix in a sample under analysis. For natural products as ascorbic acid, cysteine and uric

acid, there is a high probability to be found in waste water because they are excreted after the metabolism of humans and animals. Pharmaceuticals, in general, can be present as the result of medical practice, specially in untreated waste waters from hospitals. The rest of evaluated interferences would be present according to human-industrial-commercial activity. For that reason, the elimination of strong interferences is essential for the application of the method since the signals obtained at the same concentrations of β-lactam antibiotics are comparable and due the high probability to be found in waste water.

5.6.2 PRELIMINARY STUDY BY MEANS OF TLC.

Due to the sample is present in an aqueous medium and the analytes and the interferences are polar compounds, reversed phase elution was used. A preliminary test using reversed phase thin layer chromatography (C18) was performed, in order to visualize the behaviour of the antibiotics and some interferences under neutral medium and acid medium. The TLC retention factors are shown in table 5.4.

Table 5.4 Retention factors by means of TLC

name	Neutral medium Developing solvent: water	Acid medium Developing solvent: 0.1 M HCl
Amoxicillin	0.523	0.341
Ampicillin	0.184	0.130
Penicillin V	0.423	0.058
Penicillin G	0.436	0.058
Cefuroxime	0.723	0.035
Cefotaxime	0.656	0.024
Ascorbic acid	0.983	0.980
Cysteine	0.972	0.970
Phenol	0.277	0.333

The results indicate that in acid medium all antibiotics present low Rf values and can be separated from ascorbic acid and cysteine because these are eluting with the solvent front. Under neutral and acid medium, phenol presents similar Rf values like the antibiotics and therefore, is not possible to be separated.

5.6.3 SOLID PHASE EXTRACTION

The elimination of interferences was evaluated using solid phase extraction technique (SPE) by means of commercial available SPE cartridges.

5.6.3.1 Determination of breakthrough volume

Based on the previous results obtained with TLC, amoxicillin was used to evaluate the *breakthrough* volume, since it presented the highest Rf value under acid medium, indicating that it was less strong retained on the sorbent and it will be the first compound to elute from a column. In the other side, cefotaxime was used to evaluate the *elution* volume, since it presented the lowest Rf value under acid medium, and therefore, it was strongly retained on the sorbent.

The breakthrough volume for amoxicillin using five different commercial SPE cartridges is shown in Fig. 5.19.

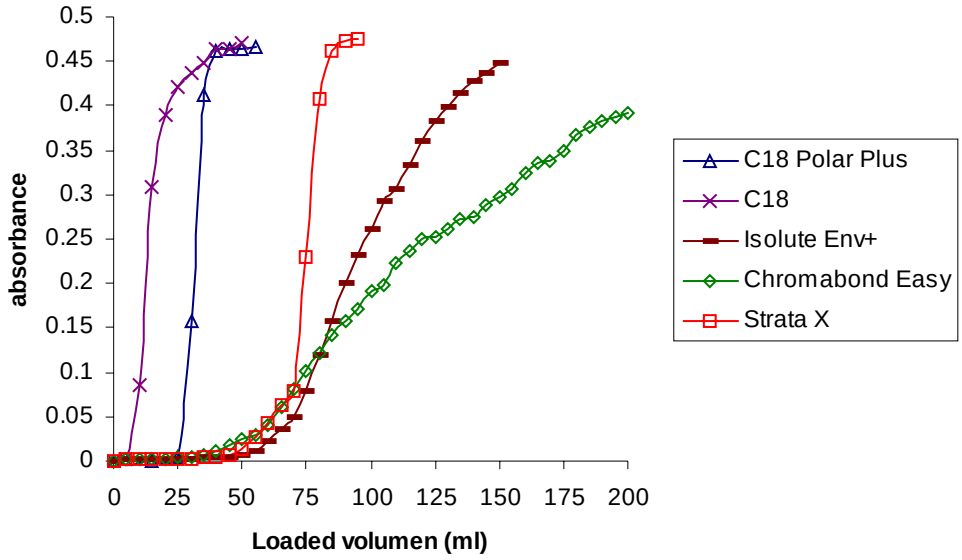


Fig. 5.19 Breakthrough for amoxicillin. C_{amox} = 25 µg/ml; pH = 1.5 in HCl medium; Flow = 3 ml/min.

The highest load capacity was achieved using cartridges packed with polystyrene-divinylbenzene, with a breakthrough volume of 50 ml.

In a similar way, the breakthrough volume for ascorbic acid and cysteine was evaluated. No more than 2 ml of sample for C18 Polar Plus and 3 ml of sample for Chromabond Easy and Strata X were necessary to load on the cartridge, indicating that these two interfering compounds are not retained at all on the sorbents.

5.6.3.2 Removal of ascorbic acid from SPE cartridges

Once the cartridges were loaded with ascorbic acid, an elution test was performed to evaluate the behaviour of the cartridges for removing the interferences. Hydrochloric acid 0.1 M was used as cleaning solvent. The results are shown in Fig. 5.20.

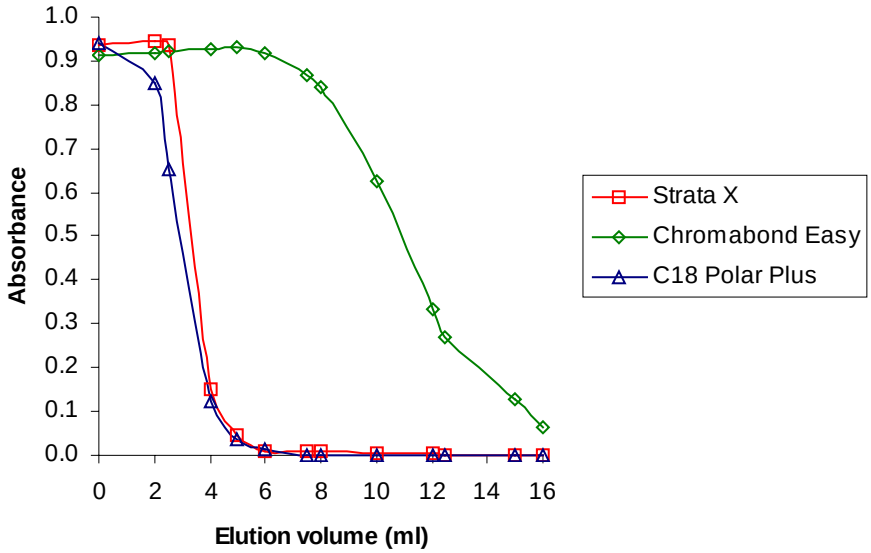


Fig. 5.20 Elution of ascorbic acid from SPE cartridges. Eluent = 0.1 M HCl; Flow = 3 ml/min.

The removal of ascorbic acid is achieved in a similar way using C18 Polar Plus and Strata X, with minimun 7.5 ml of 0.1 M HCl. In the case of Chromabond Easy, not even 16 ml of 0.1 M HCl are able to remove from the cartridge all the ascorbic acid.

Therefore, among all SPE cartridges, Strata X must be finally selected for cleaning up the sample, since it presents a sharp curve for the breakthrough of amoxicillin after 50 ml of loaded sample, and also a sharp curve for removal of ascorbic acid after 3.0 ml of 0.1 HCl, with minimum cleaning volume of 7.5 ml, although 10.0 ml are better for practical purposes.

5.6.3.3 Effect of pH on the breakthrough volume

The effect of the pH over the breakthrough was evaluated using Strata X and amoxicillin. As is possible to visualize in Fig. 5.21, the pH exerts an important effect on the breakthrough point.

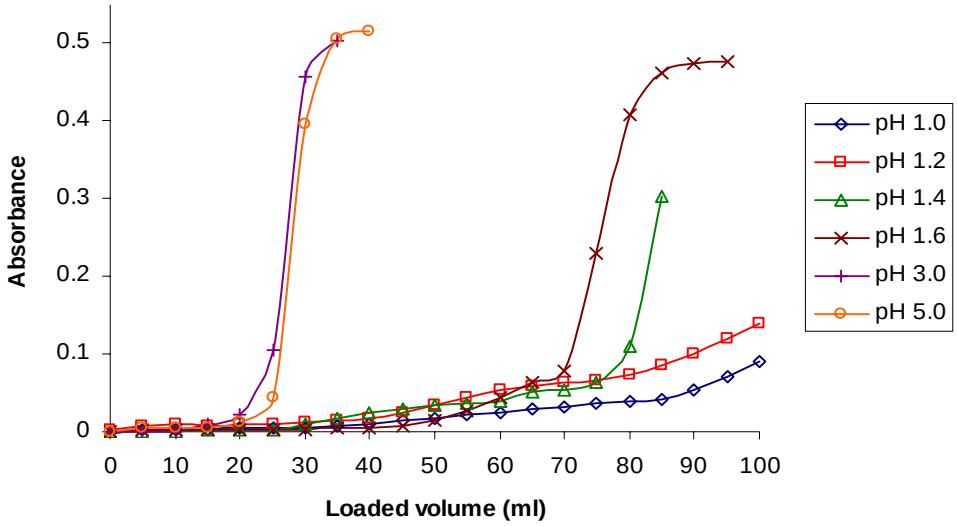


Fig. 5.21 Breakthrough for amoxicillin in function of pH. C_{amox} = 25 µg/ml; Flow = 3 ml/min.

At low pH (between 1.0 to 1.6), the breakthrough point is constant at this pH range with around 50 ml of the loaded sample. At higher pH, the breakthrough point is reduced around 20 ml of the loaded sample.

The different breakthrough points, added to the different shapes of the curves, suggest that the observed differences are related with the ionic species of amoxicillin at different pH. In that way, firstly, let's take a look of the ionisation species for amoxicillin at the corresponding pKa values (Fig. 5.22). In addition, the same effect that the pH produced on the breakthrough and curve shapes using Strata X was observed also with the other cartridges in corresponding experiments.

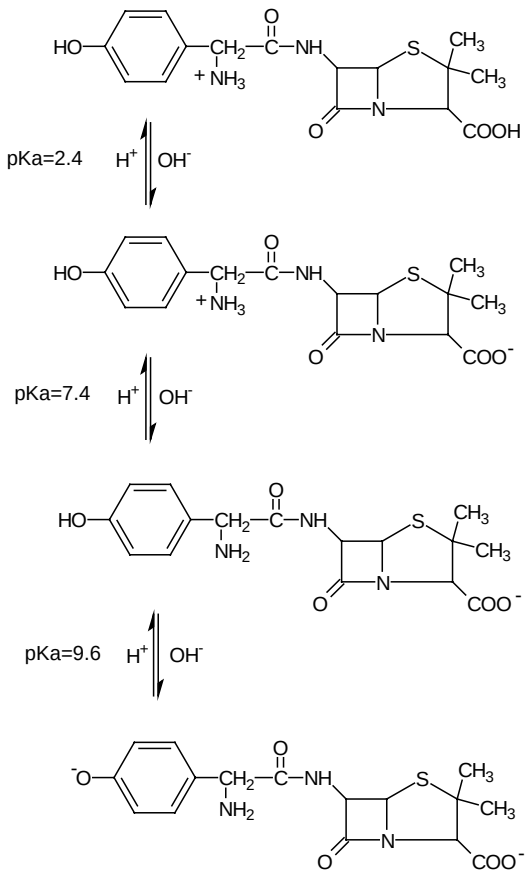


Fig. 5.22 Ionic species of amoxicillin.

To explain the differences in the breakthrough point and the shapes of the curves, a plot of the fraction of ionic species against the pH is useful, as shown in Fig. 5.23 for amoxicillin.

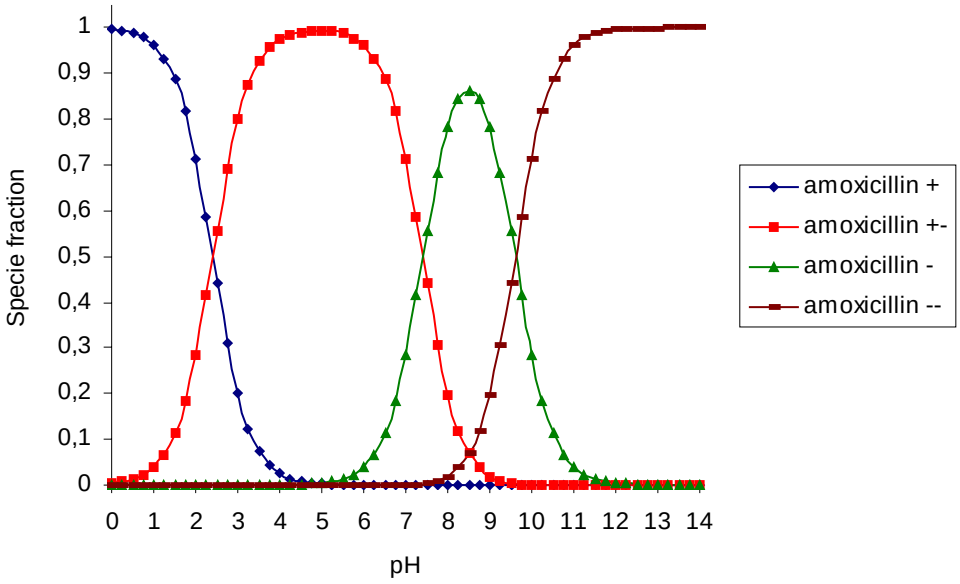


Fig. 5.23 Fraction of ionic species of amoxicillin in function of pH.

The fractions of the different ionic species for the pH range between 0 and 6 are shown in Table 5.5. While the pH increases, the ionic amoxicillin species with positive and negative internal charges start to be formed. At pH 3.0, this species is already 80% and at pH 5.0 presents the maximal value with more than 99%. The lower breakthrough point indicates that this species is not strongly retained on the sorbent as the mono-protonated one, being the reason that at pH 1.4, the absorbance (Fig 5.21), arises abrupt, changing the shape of the curve.

Table 5.5 Fraction of ionic species of amoxicillin at different pH

pH	amoxicillin +	amoxicillin +-	amoxicillin -	amoxicillin --
0	0.99603471	0.00396529	1.57861E-10	3.96529E-20
0.5	0.98756726	0.01243274	1.56519E-09	1.24327E-18
1	0.96171348	0.0382865	1.52421E-08	3.82865E-17
1.2	0.94064902	0.05935094	3.74479E-08	1.49083E-16
1.4	0.90909083	0.09090908	9.09091E-08	5.73598E-16
1.6	0.86319292	0.13680686	2.16824E-07	2.16824E-15
2	0.71525194	0.28474693	1.1336E-06	2.84747E-14

pH	amoxicillin +	amoxicillin +-	amoxicillin -	amoxicillin --
2.5	0.44268526	0.55730772	7.01609E-06	5.57308E-13
3	0.20075362	0.79921456	3.18173E-05	7.99215E-12
4	0.02449386	0.97511794	0.000388201	9.75118E-10
5	0.00249568	0.99354883	0.003955389	9.93549E-08
6	0.00024151	0.96147199	0.038276889	9.61472E-06

5.6.3.4 Determination of elution volume

The elution of retained β-lactam antibiotics on each cartridge was evaluated using cefotaxime. In that way, each cartridge was loaded with the 50 ml of sample containing cefotaxime at 25 µg/ml, then, each cartridge was washed with 8.0 ml of 0.1 M HCl and finally, the elution of cefotaxime was performed using methanol - 0.1 M HCl [1:1]. The results are shown in Fig. 5.24.

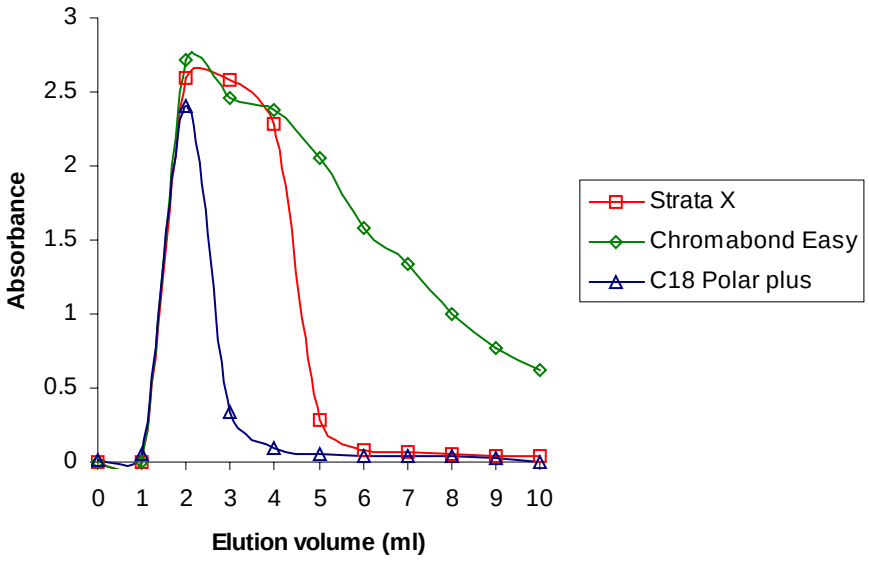


Fig. 5.24 Elution of cefotaxime from SPE cartridges. Eluent = methanol - 0.1 M HCl in proportion [1:1].

For C18 Polar Plus, 5.0 ml of the elution solvent are necessary to remove all cefotaxime. This was expected based on the previous results because this sorbent is not able to adsorb strongly the analyte. For

Chromabond Easy, not even 10 ml are enough to remove all cefotaxime, showing a similar effect as that one presented for removal of ascorbic acid. Finally, Strata X, the sorbent selected for the elimination of interferences needs 7.0 ml of the elution solvent to remove all cefotaxime, but using 10 ml would be better for practical reasons.

5.6.3.5 Evaluation of colorimetric reaction in presence of methanol

Once the retained antibiotics were eluted from the cartridge, it was necessary to evaluate if the presence of methanol in the eluting solvent affects the reaction between the antibiotics and phosphomolybdic acid. In that way, solutions of amoxicillin at 2.0 µg/ml containing methanol at different proportions were tested against 0.25 mM phosphomolybdic acid. The samples were heated at 90 °C for 60 minutes and the absorbance of the blue color obtained was measured at 705 nm. The results are shown in Fig. 5.25.

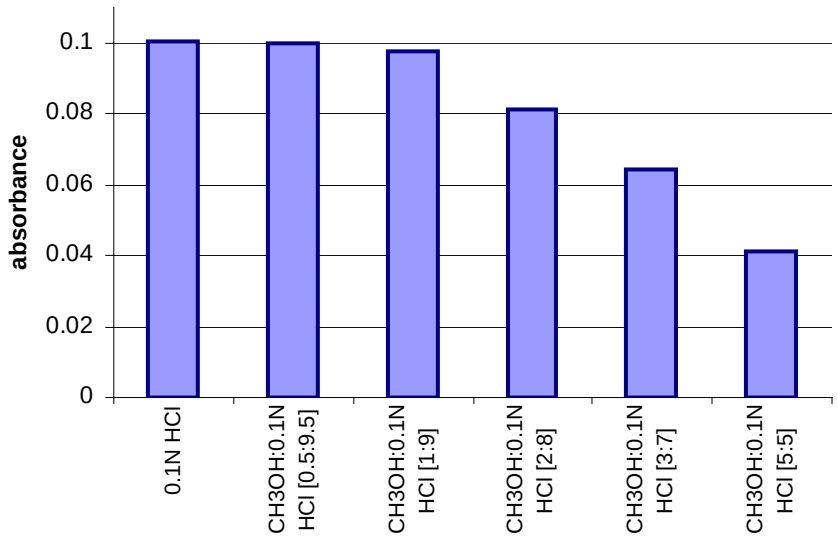


Fig. 5.25 Effect of methanol in the colorimetric reaction between amoxicillin and phosphomolybdic acid.

It is evident that the presence of methanol generates a negative effect in the reaction between amoxicillin and phosphomolybdic acid. Therefore, it

is necessary to remove the methanol from the eluted material before the reaction with phosphomolybdic acid.

5.6.3.6 Recovery evaluation

The recovery evaluation for five Strata X cartridges was performed using amoxicillin, and based on the selected SPE parameters, as follows: volume of sample loaded on the cartridge = 50 ml (amoxicillin, 20 μg/ml at pH 1.0); removal of interferences = 10 ml of 0.1 M HCl (4 fold cartridge volume) and elution step = 10 ml of methanol - 0.1 M HCl [1:1]. The results are shown in Table 5.6.

Table 5.6 Recovery of amoxicillin from Strata X

	Concentration (μg/ml)	**Recovery (%)**
Loaded sample	22.65	---
Cartridge 1	20.94	92.45
Cartridge 2	21.05	92.93
Cartridge 3	20.83	91.96
Cartridge 4	21.23	93.73
Cartridge 5	21.17	93.46
Mean	**21.04**	**92.90**
RSD (%)	**0.778**	**0.778**

5.6.3.7 Confirmatory evaluation for removing interferences

A confirmatory evaluation for the removal of interferences was developed applying the SPE process followed by the reaction with phosphomolybdic acid and further adsorption on PUF. In that manner, individual solutions containing only one interference at 5 μg/ml were used. The interfering compounds evaluated were: ascorbic acid, cysteine, paracetamol, stannous chloride, tetracycline, uric acid and sodium thiosulfate. 50 ml of each solution were loaded on a corresponding Strata X cartridge, followed by 10 ml of 0.1 M HCl used for the washing step and by 10 ml of methanol - 0.1 M HCl [1:1] used for the elution step. The

eluted material was recuperated directly on a reactor tube and then, the methanol was evaporated by means of nitrogen stream under vacuum, until remaining approximately 0.5 ml of the eluted material. On each reactor tube, 10 ml of 0.25 mM phosphomolybdic acid were added and then heated at 90°C for 60 minutes. After cooling at room temperature, a PUF disk was added at each reactor and shaken mechanically for 90 minutes. From all the interferences tested, only paracetamol (para-acetyl-amino-phenol) gave a positive signal. This shows that by means of SPE, phenols are very difficult to be separated from β-lactam antibiotics, situation that was previously faced in the preliminary study using thin layer chromatography.

CHAPTER SIX – CONCLUSIONS AND RECOMMENDATIONS

Indians and animals know better
how to live than white man;
nobody can be in good health
if he does not have all the time
fresh air, sunshine and good water.

Flying Hawk

The present work involves the development and optimization of a simple, cheap and sensitive method for the determination of β-lactam antibiotics in aqueous solutions, having as main application the analysis of environmental samples directly in the field. The method does not include the use of contaminating reagents, nor expensive and sophisticated instrumentation, nor microorganisms and enzymes.

The method is based on the reduction of phosphomolybdic acid by means of the thiols obtained after the acid hydrolysis of β-lactam antibiotics. The reduced form of phosphomolybdic acid - known as heteropoly blues - is formed proportionally to the amount of β-lactam antibiotic present in the sample. The heteropoly blues are adsorbed on disks of polyurethane foam, the intensity of the color is measured directly on the solid material using Diffuse Reflectance Spectroscopy and correlated with the concentration using the Kubelka-Munk Function.

The method presents the advantages to have more sensitivity compared with spectrophotometric methods using analysis of solutions. In general, a limit of detection of 0.03 μg/ml, a limit of quantitation of 0.1 μg/ml and a good reproducibility with RSD of 4.0% was achieved for all six evaluated β-lactam antibiotics.

The method presents the limitation of being not selective, due to phosphomolybdic acid can be reduced by several agents. Nevertheless, strong possible interferences like ascorbic acid, stannous chloride and cysteine were completely eliminated using commercial solid phase extraction sorbent "Strata X". Paracetamol, a wide spread used pharmaceutical product, which gives positive response with phosphomoybdic acid but not at the same intensity level as β-lactam antibiotics, could not be eliminated and therefore, is a limitant of the method, specially for real samples where the possibility to find paracetamol is high, e.g. hospital waste water.

The method makes no difference between the β-lactam antibiotics present in the sample, therefore, the results should be expressed based on the β-lactam antibiotic which is more often administrated and also, more often found in waste water: that is, amoxicillin. In that manner, it is suggested to state the results of analysis as "β-lactam antibiotics expressed as amoxicillin". This particularity must be considered in the way that a possible over-estimation of the real amount of β-lactam antibiotics can result from this suggestion, because the sensitivity for amoxicillin, compared with the rest five β-lactam antibiotics, is the highest one achieved in the present method. Nevertheless, an over-estimation would be preferred instead of under-estimation.

The option to change the 45° illumination geometry – used in the present method for measuring the diffuse reflectance – by an integration sphere would be attractive to be evaluated. It is expected better sensitivity and reproducibility due to more light will reach the detector and due to the reflectance measurements will be not affected from variations on the physical surface material.

Another option for evaluating better sensibility would be to use polyurethane foam disks with smaller diameter than 20 mm, but keeping the tickness in 5 mm. The heteropoly blues will present higher intensity of color when adsorbed in a smaller volume of sorbent. To reduce the thickness to less than 5 mm is not recommended due to there will be not enough material to interact with the light beam and so, it can reach the surface of the sample holder.

Due to tetracyclines present also positive reaction with phosphomolybdic acid, it would be probable to extend the method to analyze this important kind of antibiotics. The different behaviour of tetracyclines and β-lactam antibiotics faced with SPE might be possible to evaluate their presence individually and as a whole.

Based on a recently study, the present method shows a practical relevance for direct application in the fields due to it can be applied employing only a computer and without the use of diffuse reflectance spectroscopy. In that way, a desktop scanner can be used to measure the colored polyurethane foam disks and the image obtained can be processed using Adobe Photoshop as a graphic editor. The calibration plot of the luminosity as a function of concentration can be built up using Origin software (Shishkin, et al. 2004).

CHAPTER SEVEN – SUMMARY

The things that will destroy us are: politics without principle;
pleasure without conscience; wealth without work;
knowledge without character; business without morality;
science without humanity; and worship without sacrifice.

Mahatma Gandhi

The present study was focused to develop an analytical method to analyse β-lactam antibiotics present in aqueous solutions and able to be applied directly in the fields. The method here presented is based on the chemical reaction between β-lactam antibiotics with phosphomolybdic acid, a heteropolyacid able to be reduced by the thiols obtained after the acid hydrolysis of the β-lactam antibiotics. The blue product obtained, which is proportional to the antibiotic amount, is adsorbed on polyurethane foam, and the intensity of color is directly evaluated on the solid material by means of diffuse reflectance spectroscopy.

For all six evaluated β-lactam antibiotics (amoxicillin, ampicillin, penicillin V, penicillin G, cefotaxime, cefuroxime), the analytical parameters achieved were: LOD = 0.03 μg/ml, LOQ = 0.1 μg/ml, RSD = 4.0 %. The study of possible interferences showed that using solid phase extraction is possible to eliminate even strong interferences as ascorbic acid, cysteine and reducing salts like stannous chloride.

The present method has avoided, as much as possible, the use of expensive and contaminating solvents and chemical reagents. The method presents the possibility to be applied using only a desktop scanner and a personal PC for measuring the color of the adsorbed material on the PUF.

CHAPTER EIGHT – BIBLIOGRAPHY

Without the meditative background that is criticism, works become isolated gestures, historical accidents, soon forgotten.

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2002 – 2005	Faculty of Natural Sciences, Department of Analytical Chemistry, University Duisburg - Essen, Duisburg - Germany. **Doctor in Natural Sciences** Thesis: "New method for determination of β-lactam antibiotics by means of diffuse reflectance spectroscopy using polyurethane foam as sorbent"

PROFESSIONAL EXPERIENCE

1997 – 2000	Quality Control Analyst: *Physical and Chemical analysis of raw material and elaborated products*, Laboratorios Industriales Farmaceuticos del Ecuador, Quito – Ecuador. Quality Control Analyst: *Physical, Chemical and Instrumental analysis of raw material and elaborated products*, Schering Plough, Quito – Ecuador. Technical Assistant: *Execution of developmental projects and improvement of living conditions in Rural Andean Areas,* Latin-American Network for an Ecological and Economic Habitat (ECOSUR), Riobamba – Ecuador

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ACADEMIC EXPERIENCE

1994 – 2005	Lecturer's assistant of General Chemistry, ESPOCH, Riobamba - Ecuador. Area's assistant of General and Inorganic Chemistry, ESPOCH, Riobamba – Ecuador. Lecturer of Elaboration of pharmaceutical products, Institute of Natural Studies "Misael Acosta Solis", Riobamba – Ecuador. Scientific assistant, Department of Analytical Chemisty, Duisburg-Essen University, Duisburg - Germany.

RESEARCH – DEVELOPMENT EXPERIENCE

1997 – 2001	Analysis and characterization of natural water springs for human consumption, Community of Montalvo, Pastaza – Ecuador. Associated researcher for development of methodology for the measurement and estimation of poverty lines and indicators of nutrition and feeding, SIISE, Quito – Ecuador. Research on isolation and biological activities of natural products from plants, Laboratory of Pharmaceutical Chemistry and Natural Products, ESPOCH, Riobamba – Ecuador.

PUBLICATIONS

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2004	**Rodriguez, R and Kuss, H-M.** Determination of β-lactam antibiotics by diffuse reflectance spectroscopy using heteropoly acids adsorbed on polyurethane foam. *Transactions of the University of Kosice.* 2, 30-37

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