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HORIZONTAL - ORG

HORIZONTAL STANDARDS ON ORGANIC MICRO-POLLUTANTS FOR IMPLEMENTATION OF EU DIRECTIVES ON SLUDGE, SOIL AND TREATED BIO-WASTE

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Topic 1.5 Environmental assessment

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Organisation name of lead contractor for this deliverable: CAE / ANJOU RECHERCHE
Veolia Water
1 place de Turenne 94417 Saint-Maurice cedex
Tel : +33 1 49 76 52 52 Fax : +33 1 49 76 58 96

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List of Abbreviations

- Aba : Abamectine
- Ampi : Ampicilline
- ASE : Accelerated Solvent Extraction
- Beza : Bezafibrate
- Carba : Carbamazepine
- Chlortetra : Chlortetracycline
- Cipro : Ciprofloxacine
- Diclo : Diclofenac
- EDTA : Ethylene Diamine Tetraacetic Acid
- Enro : Enrofloxacine
- Ery : Erythromycine
- Ery-H_2O : Erythromycine – H_2O
- Feno : Fenofibrate
- Fluox : Fluoxetine
- Gem : Gemfibrozil
- Keto : Ketoprofen
- LOQ : Limit Of Quantification
- Meto : Metoprolol
- Napro : Naproxen
- Norflo : Norfloxacine
- Oflo : Ofloxacine
- Oxy : Oxytetracycline
- Para : Paracetamol
- Para D4 : Paracetamol D4
- Phena : Phenazone
- Primi : Primidone
- Propra : Propranolol
- RSD : Relative Standard Deviation
- Roxy : Roxythromycine
- SA : Acid Salicylic
- Sim : Simetone
- Spira : Spiramycine
- Sulfa : Sulfamethazine phenyl $^{13}C_6$
- Sulfachloro : Sulfachloropyridazine
- Sulfamera : Sulfamerazine
- Sulfametho : Sulfamethoxazole
- Tetra : Tetracycline
- Tylo : Tylosine
- US : Ultra Sonication

CONTENTS

FOREWORD

The European project Horizontal is focussed on the standardization of test procedures in environmental samples. Several studies have been started to elaborate the possibility of horizontal standardization on specific subjects. One of the subjects is the Pharmaceuticals Products. The aim of this project is to build a standard or a report close to a standard but the final step of this particular project ends at the second consultation. The validation and transfer to Task Force 151 will not carry out.

Regarding the technical difficulties and the delay we encountered to work on this project (see document "Horizontal Report" sent to M Van der Sloot the second of december, 2005), we decided to write a report of advancement on ruggedness of our analytical methods applied on sludges in first of all, and some tests will be performed as well on soil and bio-waste. Compost and biological have been tested as well for steroid hormones.

INTRODUCTION

Since their identification in water, pharmaceutical compounds have been targeted as emerging environmental contaminants. Their chemical properties (Log Kow, pKa, polarity….) show tendency towards persistence in solid environmental matrices.

Due to their polarity, persistence and water solubility, some drugs and metabolites are able to pass through the wastewater treatment plants (Sulfonamides, Macrolides, Carbamazepine, Phenazone). Their low adsorption on sludge and soil may cause the contamination of surface and ground water.
The sorption on sludge or soil could let original active substance in hydrophobic links persistent (Fluoroquinolones, Hormones, Avermectin, Tetracyclines). For some of them (Diclofenac, Oxytetracycline, Tylosin, Ibuprofen, Macrolides), partial or total biodegradation may occur possibly producing unknown metabolites more or less active than initial form.

Regarding to this literature review, amongst the different classes, the therapeutic groups for environmental solid matrices most concerned seem to be Steroid Hormones, Fluoroquinolones, Tetracyclines, Analgesics/Anti-Inflammatories and Avermectin.

1. SCOPE

The purpose of this report is to present the development of our analytical methods to analyse steroid hormones and pharmaceutical compounds in solid matrices in term of ruggedness. Hormones and pharmaceuticals analysis have been carried out on two apparatus : respectively a LC/MS-MS Quantum (Thermo Electron) and an ion trap mass spectrometer LCQ (Thermo Electron), older and less performing. Indeed, it occurs some analytical problems in term of repeatability of control point area.
That is why we project to transfer the final part of development of antibiotics and pharmaceutical analytes on the Quantum very soon.

Other difficulty in this project is the lack of sample certified in researched analytes. Even with spiked solid matrices, it is still delicate to verify correctly the impact of extraction step because it does not reproduce a real sample.

What we propose here is a complete analytical method on hormones tested on sludges. The advantage of this part was the fact that hormones concentrations in solid matrices are seldom zero.
For other molecules, the transfer of the method and the application on solid matrices will be performed before june 2006. Nevertheless this document provides a final protocol on extraction and purification tested on spiked sludges with antibiotics and pharmaceutical compounds.

We have already undertaken the developement of the method on a few soil and treated bio-waste samples but not enough to write them on this report.

2. NORMATIVE REFERENCE

Hormones
Concerning pharmaceutical products and steroid hormones normative reference does not exist.
However there are several articles about the determination on hormones in effluent, in waste water and sludge. Some techniques have been developped using fluorescence or diode array detector [1, 2], but analytical method more often carried out is the GC-MS which needs derivatization step before injection [3, 4, 5, 6, 7, 8]. Instead of derivatization which is a very delicate step in the case of complex matrices such as influent and sludge, analysis by HPLC/MS-MS can be undertaken [9, 10, 11, 12, 13, 14]. About sludges an analytical method allows to determine hormones in using size exclusion chromatography, a purification on silica gel before injection on GC-MS [15].

Pharmaceuticals and antibiotics
Concerning antibiotics and pharmaceutical products there are several articles about their analysis in the different environmental matrices (sediments, soils and sludges). The analysis of these compounds is almost always done by liquid chromatography. And most of the analytical methods use the mass spectrometry as detection.
The analysis of sediments is performed by LC-MS [17]. The molecules are extracted by ultra-sonication with 4*45 mL of different organic solvents. Concerning soils [18, 19, 20] the extraction is made by ASE with different solvents as methanol with NH_3, a mixture of methanol/water or a mixture of methanol and citric acid. Then the clean-up of these extracts can be made on different cartridges like Diol or SAX+HLB or MPC.
Concerning sludges the molecules are extracted by ASE with a mixture of acetonitrile and orthophosphoric acid and they are purified on MPC cartridges [21].

3. REAGENTS AND MATERIALS

3.1 Solvents

All the solvent are of analytical grade. The solvents Water, Hexane, Acetone, Ethyl Acetate, Methylene Chloride and Acetonitrile are acquired from J.T. Baker (Atlantic Labo, Eyssines, France), except Methanol obtained from Merk (Fontenay sous Bois, France) and Petroleum Ether obtained from VWR (Saint Quentin Fallavier, France).

3.2 Chemicals and Stocked solutions preparation

Pharmaceutical standards (Annexes 1 to 3) are of analytical grade (>90%)
Pure standards are purchased as powders from :
- Sigma-Aldrich (Saint Quentin, Fallaviers, France) for the native steroid hormones and from C/D/N Isotopes (CIL Cluzeau, Sainte Foy la Grande, France) for their deuterium-labeled steroids
- CIL Cluzeau, (Sainte Foy la Grande, France) for Propranolol, Ampicilline, Carbamazepine, Paracetamol D4, Simetone, Enrofloxacine, Fluoxetine (Sigma-Aldrich, Saint Quentin, Fallaviers, France)
- EURISO-TOP (Saint-Aubin, France) for Sulfamethazine phenyl $^{13}C_6$ (Sigma-Aldrich, Saint Quentin, Fallaviers, France) for all the other ones.

The Surrogate standards are chosen according therapeutic use or practice restricted in France.
A stock solution 1 containing all hormones at 100 mg/L was prepared in Methanol.
Stoked solution 2 containing Ofloxacine, Abamectine, Norfloxacine and Ciprofloxacine at 100 mg/L is prepared in Acetonitrile with 2% of Ammonia.
Stoked solution 3 containing all other antibiotics at 100 mg/L are prepared in Water.
Stoked solution 4 containing all pharmaceuticals at 100 mg/L is prepared in Methanol.

Solutions 2, 3 and 4 are stored in dark glass bottles at –20°C for one month maximum (See part 6) for antibiotics and others pharmaceuticals. The solution of hormones stored in dark glass bottles at –20°C is stable over on year (See part 6) .

All Deuterium-labelled substances are prepared individually in Methanol at a concentration of 100 mg/L and stored in dark glass bottles at –20°C.

3.3 Others chemicals

- Sodium sulfite (Merck, Fontenay sous Bois, France)
- Ethylenediaminetetraacetic acid disodium salt (VWR, Fontenay sous Bois, France)

3.4 Small materials

Cartridges:

Cartouches Oasis HLB, 6 mL, 200 mg, Waters
Cartouches Bond Elut SAX, 3 mL, 500 mg, Varian
Cartouches Bond Elut LMS, 3 mL, 500 mg, Varian
Cartouches Bond Elut PPL, 6 mL, 500 mg, Varian
Cartouches Supelclean LC-Florisil, 6 mL, 1 g, Supelco
Cartouches C_{18}, 6 mL, 1 g, J.T. Baker

Materials :

Soxtec Avanti 2050
Ultrasonication tank VWR

3.5 Sampling

Before the extraction procedure, the sludge is freezed, lyophilised and grinded at 0.2 mm then the dry material is kept at room temperature in amber bottle till pre-treatment analysis.

4. APPARATUS, CHROMATOGRAPHIC AND DETECTION PARAMETERS

In order to tune the detector properly we infuse directly in the mass spectrometer each compound to define the best conditions of detection. So it is possible to sequence the events of definite analytical conditions.

4.1 Analytical method concerning hormones

4.1.1 Chromatographic separation

The HPLC apparatus consists of a Surveyor System, Autosampler, LC pump and a column oven (Thermo Electron, Courtaboeuf, France). The separation is performed on a Hypersil BDS C18 column (125*2.1 mm, 5µm) with a guard column (1*2.1 mm, 5µm) at a flow rate of 0.2 mL/min. The separation is performed under isocratical conditions. The initial conditions for liquid chromatography are Water/MeOH (50/50) to optimize the separation between the alpha and beta Estradiol. Then, the solvent must be higher than 30% of methanol in the extracts to avoid any precipitation.

The table 1 presents the LC gradient conditions.

Eluants : Water (A), Acetonitrile (B), MeOH (C)			
Time	***% A***	***% B***	***% C***
0	50	50	0
10	50	50	0
10.1	0	0	100
40	0	0	100
40.1	50	50	0
47	50	50	0

Table 1 : the LC gradient conditions

4.1.2 Mass spectrometry detection

The detection is carried out using a Quantum (Thermo Electron, Courtaboeuf, France) tandem mass spectrometer. The mass spectrometer is operated in negative mode electrospray ionization (ESI-). The Selected Reaction Monitoring (SRM) mode is chosen for quantification. The spray voltage is fixed at 3500 V and the temperature of the ESI heater was set at 350°C.
For MS-MS analysis the pressure in the collision cell is set at 1.5 mTorr. The optimization of the detection is performed by carrying out infusions of solution at 1 ng/µL directly into the mass spectrometer. The [M-H]- species is observed as the base peak in the mass spectrum for each of the estrogens. This observation of m/z 145 is consistent with the stability of the phenol ring in the system and the observation of this daughter ion for all the estrogens is a good indicator of specificity for these compounds.
The fragmentation parameters (the SRM monitor ions and collision energies optimized) for each compound defined during the analytical development are the following :
The most intensive product ion from each precursor ion is selected and chosen as transition ion for detection and quantification.

Compounds	Collision Energy (en V)	Ion parent m/z	Daughter Ion m/z
Estradiol (E2)	46	271.10	145.20
Estradiol-d5 (E2-d5)	46	276.15	187.20
Ethinylestradiol (EE2)	46	295.10	145.20
Ethinylestradiol-d4 (EE2-d4)	46	299.05	147.20
Estrone (E1)	46	269.10	145.20
Estrone-d4 (E1-d4)	46	273.15	147.20

Table 2 : detection parameters for hormones

Figure of annexe 4 shows a LC-MS/MS chromatogram of solvent-base standard at 1 ng/g for steroid hormones.

4.2 Analytical method concerning antibiotic and pharmaceutical compounds

4.2.1 Chromatographic separation

The analysis is performed with HPLC apparatus consisted of an Agilent 1100 solvent degassing module, autosampler (4°C thermostat), LC pumps and column oven (Agilent, Massy, France). The separation is performed on a Hypersil BDS C18 (125*2.1mm, 5µm) with a guard column BDS Hypersil C18 (10*2.1 mm, 5 µm) at flow-rate of 0.2 mL/min and 25°C thermostat. The injection volume is 35 µL. The tables 3 and 4 present the LC gradient conditions.

Antibiotics

Time	A (%)	B (%)
0	95	5
30	50	50
45	5	95
52	5	95
60	95	5
70	95	5

Table 3 : the LC gradients for antibiotics

Pharmaceutical compounds

Time	A (%)	B (%)
0	95	5
30	0	100
35	0	100
40	95	5
55	95	5

Table 4 : the LC gradients for pharmaceutical compounds

4.2.2 Mass spectrometry detection

The detection is performed using an ion trap mass spectrometer (LCQ-Thermo Electron). The ESI interface is selected for the ionisation mode as provided the best overall sensitivity for compounds of interest. The generated ions are focalized through 2 octapoles before entering the ion trap. The voltage on the ion trap is selected to eliminate all ions except the desired ion(s) in preparation for a MS/MS analysis. The radiofrequency voltages to the ion trap are adjusted to stabilize the desired ion. The radiofrequency voltage is also called Qz value (Activation).

	Pharmaceutical compounds	Parent ion	Isolation	Collision E	Qz activation	Product Ions	ESI +/-
segment 1	paracetamol D4	156.2	1.0	28	0.25	114.1	+
	paracetamol	152.1	1.0	30	0.25	110.0	+
segment 2	phenazone	189.2	1.0	36	0.25	146.1	+
	metoprolol	268.1	1.0	34	0.2	116.0	+
	salicylic acid	137.1	1.0	39	0.3	93.0	-
segment 3	propranolol	260.1	1.0	31	0.25	183.0	+
	carbamazepine	237.1	1.0	29	0.25	194.4	+
	ketoprofene	255.1	1.0	24	0.3	209.0	+
	bezafibrate	361.8	3.0	22	0.3	275.9/315.8	+
segment 4	diclofenac	295.9	2.0	21	0.3	249.9/278.0	+

Table 5 : detection parameters for pharmaceutical compounds

	antibiotic compounds	Parent ion	Isolation	collision E	Qz activation	Product Ions	ESI +/-
segment 1	sulfamerazine	265.1	2.0	29	0.25	155.9/173.9/189.8	+
	sulfamethazine phényl 13C6	285.1	1.0	20	0.25	203.8	+
segment 2	sulfachloropyridazine	284.9	2.0	25	0.25	155.9	+
	tetracycline	445.0	2.0	20	0.3	427.0	+
	sulfamethoxazole	254.1	1.0	29	0.2	155.8/188.0	+
	ofloxacine	362.2	1.0	28	0.3	318.1	+
	oxytetracycline	461.1	3.0	19	0.3	443.0	+
	ciprofloxacine	332.1	2.0	34	0.25	288.1/332.1	+
segment 3	norfloxacine	320.1	1.0	100	0.3	276.3/302.3	+
	ampicilline	349.9	3.0	21	0.25	159.9	+
	simetone	198.1	1.0	38	0.3	114/170.1/128/123.9	+
	spyramicine	438.1	3.0	20	0.25	365.9	+
	chlortetracycline	479.1	2.0	21	0.25	462.0	+
segment 4	tylosine	948.3	2.0	29	0.25	772.0/916.0	+
	erythromycine	734.1	3.0	22	0.3	576.1/716.0	+
	erythro-H20	716.0	4.0	19	0.25	558.0	+
segment 5	roxythromycine	837.1	2.0	25	0.25	679.1	+
	abamectine	889.7	8.0	15	0.25	566.8/710.5	+

Table 6 : detection parameters for antibiotics

The single values corresponded to most abundant ion for each compound is used for the quantification. For some compounds, the fragmentation induces several daughter ions so it is preferable to use sum of ions to guarantee better quantification.

Figures of annexe 8 show the LC-MS/MS chromatograms of solvent-base standard at 100 ng/mL for antibiotics and others pharmaceuticals.

5. CALIBRATION AND LIMITS OF QUANTIFICATION

5.1 Hormones

5.1.1 Working solutions preparation

An intermediate solution at 1 ng/µL is prepared by dilution of the stock solution.
A working solution at 0.1 ng/µL of hormones is prepared by dilution of the intermediate solution at 1 ng/µL in Methanol.
A working solution at 0.1 ng/µL of Deuterium-labelled steroids is also prepared by dilution of the stock solution in Methanol. All solutions were stored at –20 °C prior use.

Calibration standards are prepared with appropriate amounts of the working solution to achieve concentrations between 0.5 and 125 ng/mL.

	0.5ng/mL	1 ng/mL	1.5 ng/mL	2.5 ng/mL	12.5 ng/mL	25 ng/mL	62.5 ng/mL
Volume in µL of the working solution at 0.1 ng/µL	5	10	15	25	125	250	625

All the standards are reduced to dryness under Nitrogen. Then 100 µL of the deuterium-labelled hormones at 0.1 ng/µL and 400 µL of Methanol are added. Standards are gently mixed before adding 500 µL of Water prior to LC/MS-MS analysis.

5.1.2 Standards calibrations curves and repeatabilities of slopes

The figures in annexe 4 present an example of internal calibration curve of in range between 0.5 to 62.5 ng/mL (7 points) for hormones.

	E2	Alpha	EE2	E1
27/10/2004	0.01391	0.02484	0.10150	0.11444
25/10/2004	0.01448	0.02600	0.13618	0.11777
20/10/2004	0.01476	0.02852	0.10875	0.13143
21/10/2004	0.01499	0.02719	0.11635	0.12747
18/10/2004	0.01405	0.02466	0.12057	0.11716
Mean	0.01444	0.02624	0.11667	0.12165
Deviation	0.00046	0.00163	0.01312	0.00736
RSD (%)	3	6	11	6

Table 7 : Statistical tests on slopes of 5 hormone standard calibration curves

Several calibration curves are presented in annexe 7 with recalculated values. Relative standard deviations are under 15 % which is quite acceptable.

5.1.3 Repeatability on Limit Of Quantification (LOQ)

Table 8 illustrates criteria used for acceptable limit of quantification validation.

Target values	**0.5**	**0.5**	**1**	**0.5**
n = 10	beta E2	alpha E2	EE2	E1
1	0.504	0.423	1.223	0.538
2	0.483	0.494	1.067	0.49
3	0.484	0.483	1.068	0.537
4	0.52	0.376	1.018	0.508
5	0.455	0.431	0.981	0.501
6	0.483	0.488	1.003	0.543
7	0.522	0.417	1.029	0.522
8	0.444	0.404	1.031	0.495
9	0.435	0.33	1.097	0.534
10	0.458	0.41	1.21	0.53
Mean	**0.479**	**0.426**	**1.073**	**0.520**
deviation	**0.031**	**0.052**	**0.083**	**0.020**
RSD %	**6%**	**12%**	**8%**	**4%**

Table 8 : Statistical tests on hormones LOQ in ng/mL

According to the RSD and the concentrations obtained during this test of repeatability, the limits of quantification for hormones are the following :

- Beta E2, Alpha E2, E1 : 0,5 ng/mL
- EE2 : 1 ng/mL

5.2 Antibiotics and others pharmaceuticals

5.2.1 Working solutions preparation

All the working solutions are freshly prepared by dilution of stoked solutions.

A working solution at 1 mg/L is prepared by dilution of stoked solution in methanol for the pharmaceuticals and in water/methanol (95/5) for antibiotics.
A mix of the internal standards is prepared by dilution of stocked solution in water/methanol (95/5). The solution at 50 µg/L is prepared by diluting 50 µL of internal standards in 100 mL.

Calibration standards are prepared with appropriate amounts of the working solution to achieve concentrations between 50 and 750 ng/mL.

In 1 mL	0	50 ng/mL	100 ng/mL	250 ng/ mL	500 g/mL	750 g/mL
Pharma. (V in µL)	0	50	100	250	500	750
Antibio (V in µL)	0	50	100	250	500	750

A fixed concentration of internal standards at 50 µg/L is added to each standards and samples.

5.2.2 Standards calibrations curves and repeatabilities of slopes

The figures in annexe 6-a present an example of internal calibration curve in range between 50 to 750 ng/mL for pharmaceuticals compounds.

The following table shows the relative standard deviation on slopes obtained with 4 different calibration curves.

	Gamme 1	Gamme 2	Gamme 3	Gamme 4	**Mean (n=4)**	**Deviation**	**RSD %**
Para	0.03172	0.02364	0.02998	0.01747	0.02570	0.00649	**25%**
Phena	0.01892	0.04146	0.05144	0.02420	0.03401	0.01509	**44%**
Meto	0.02729	0.02463	0.02835	0.01888	0.02479	0.00424	**17%**
SA	0.00295	0.00628	0.00566	0.00198	0.00422	0.00208	**49%**
Propra	0.44604	0.42146	0.41601	0.34750	0.40775	0.04224	**10%**
Carba	1.71388	1.78533	1.68494	1.38213	1.64157	0.17803	**11%**
Keto	0.20072	0.24574	0.23782	0.19192	0.21905	0.02669	**12%**
Beza	0.22183	0.28302	0.24426	0.19129	0.23510	0.03862	**16%**
Diclo	0.09738	0.13424	0.11889	0.08511	0.10891	0.02191	**20%**

Table 9: Statistical tests on slopes of 9 pharmaceuticals standard calibration curves

The whole of these results confirms the good linearity on calibration curves for almost all the compounds. The relative standard deviations are under 25% which is quite acceptable except for phenazone and salicylic acid. But these 2 compounds present not very good results in term of sensibility and repeatability.

Some calibration curves for antibiotics are presented in annexe 6-b. Repeatability test will be undertaken following the transfer on the LC-MS/MS Quantum.

Several calibration curves are presented in annexe 9 with recalculated values for each pharmaceutical compound. The relative standard deviations with the theoretical concentration are correct except for 3 compounds : phenazone, metoprolol and salicylic acid. As we have said before, we don't have a good response for these compounds but we hope that the transfer of the method on the LC/MS-MS Quantum allows to obtain better results in term of sensibility and repeatability.

For antibiotics there are no results presented here concerning the standard calibration curves and the repeatability of slopes. This point will be treated when the transfer of the method on the LC/MS-MS Quantum will be done.

5.2.3 Repeatability on Limit Of Quantification (LOQ)

We have no results concerning the limits of quantification. At the occasion of the transfer this part will be performed.

6. CONSERVATION AND STABILITY OF STOCK SOLUTIONS

Stability has been demonstrated in the intermediate report sent in december 2004. We present under just a summary.

6.1 Stability of Hormone stocked solutions

A solvent standard at 2.5 ng/mL always prepared with the same worked solution demonstrate a good stability of this one during several months.

6.2 Stability of pharmaceutical stocked solutions

For the majority of the substances, with RSD $\leq$ 20%, we can considere a stability of the solution during one month and stored at – 20 °C. In the case of salicylic acid and fluoxetine, the stocked solutions in methanol have to be prepared more frequently, at least every 10 days.

6.3 Stability of antibiotic stocked solution

All stoked solutions are stored at -20 °C. For the moment, regarding to the values obtained for RSD, we have decided to prepare solutions every 10 days for Spyramicine, Ciprofloxacine, Oxytetracycline and Roxythromycine and every month for the other ones.

7. EXTRACTION

This extraction protocol is optimized on sludge matrices in this first step.
Before the extraction procedure, the sludge is freezed, lyophilised and grinded at 0.2 mm then the dry material is kept at room temperature in amber bottle till pre-treatment analysis.

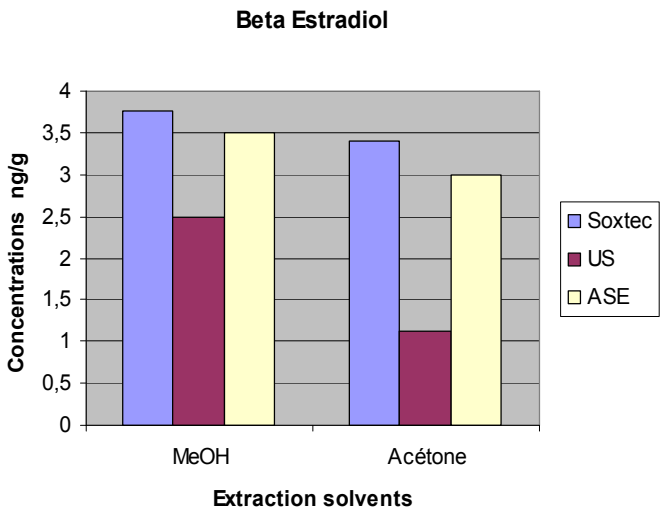
7.1 Hormones

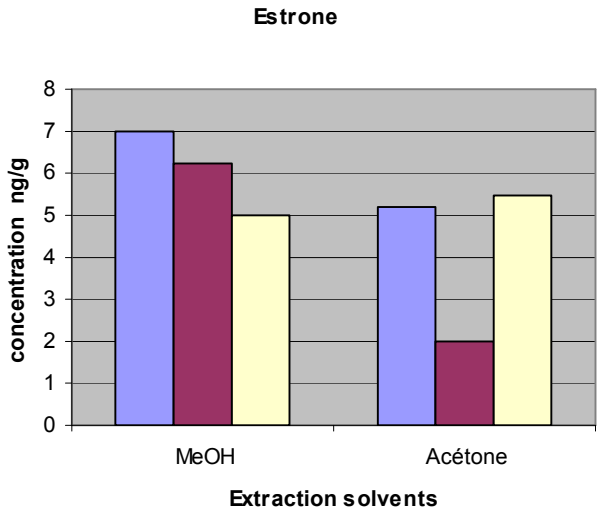
7.1.1 Choice of the technique extraction

We test 3 extraction techniques : Soxtec, ASE and Ultra-Sonication. For each technique the extraction conditions are resumed in the following table :

	Hormones	Pharma & Antibio
Soxtec	100 mL –180°C- 3 hours	100 mL – 100°C – 2 hours
ASE	33 mL – 150°C – 2000 psi – 2 cycles	33 mL - 100 °C – 100 bars – 3 cycles
US	3*20mL – 15 min	3*20 mL – 15 min – room temperature

For hormone extraction 2 solvents, methanol and acetone, are tested.

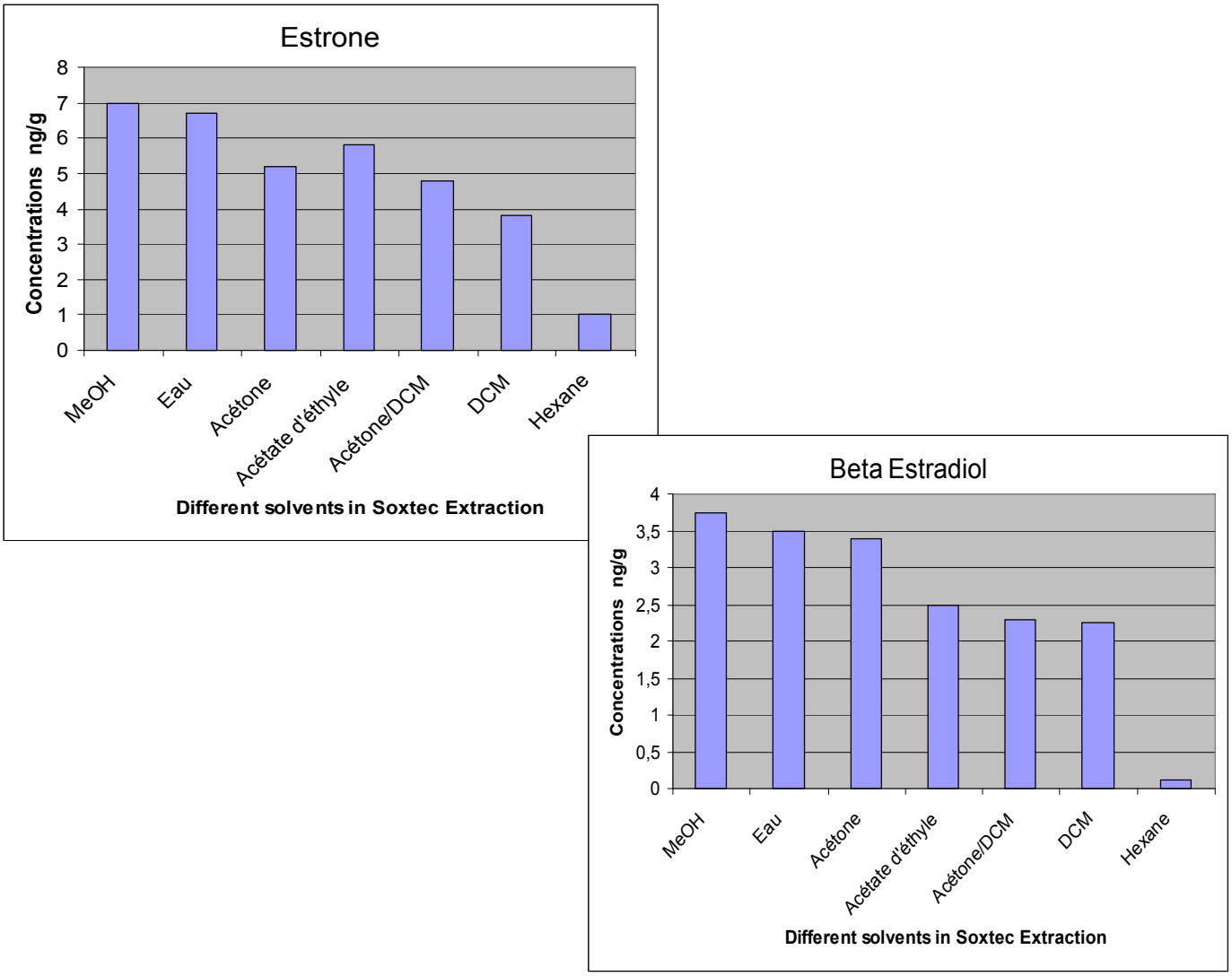




According to the results, Soxtec technique is retained for the hormones extraction.

7.1.2 Choice of solvent extraction

The Soxtec extraction of sludge is performed at 180°C during 3 hours with 100 mL of solvents.
Different solvents, methanol, water, acetone, ethyl acetate, acetone/methylene chloride (1/1), methylene chloride alone and hexane are tested in these conditions of extraction. The results for estrone and beta estradiol are presented in the following graphs.



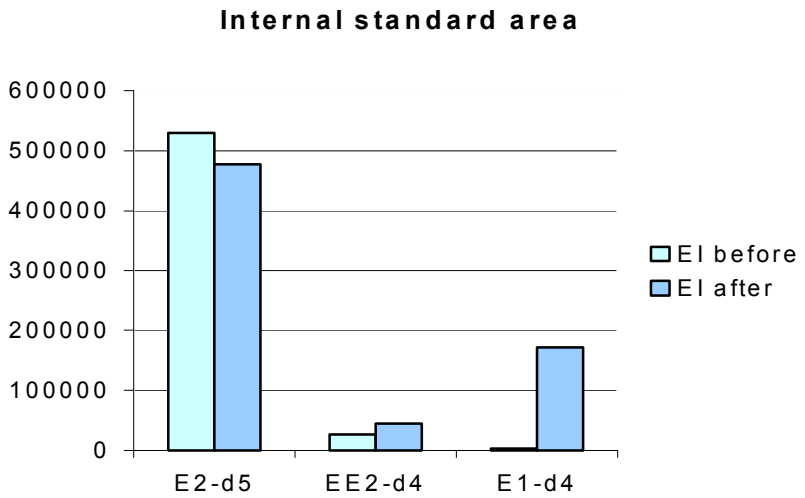
Graph 1 : Hormones soxtec extraction : comparison of different solvents (n = 3)

We can observe that the best extractant power was obtained with the more polar solvents : Methanol, Acetone and Water. Water is preferred to Methanol or Acetone for the extraction of hormones because during the sample preparation we encounter less problems of precipitation, especially at the end of the sample preparation when Methanol and Water are added for the LC-MS-MS analysis. Less matrices effects are also observed by using Water for the extraction.

According to the results obtained during the development of the extraction step, the protocol chosen is :
1 g of lyophilized and ground sludge is Soxtec extracted by 100 mL of Water at 180°C (Foss, Nanterre, France). Sample is immerged in water during 2 hours (boiling mode) and then rinsed for 1 hour (rinsing mode).

7.1.3 Impact of soxtec extraction on steroid hormones

After testing soxtec extraction on sludge, sand is spiked with 10 ng/g hormones in order to verify the impact of heating. Internal standards are Deuterium-labelled steroids. They are added at the beginning of extraction or after the soxtec extraction.
The following graph presents area of internal standards :



Graph 2 : Internal standard added before or after soxtec extraction

This table 10 illustrates hormones concentrations for a target of 10 ng/g :

	E2	Alpha	EE2	E1
Internal standard before extraction	9.6	10.4	9.9	5.90
Internal standard after extraction	7.9	9.2	7.0	9.9

Table 10 : Concentrations in ng/g of hormones with internal standard added before and after soxtec extraction

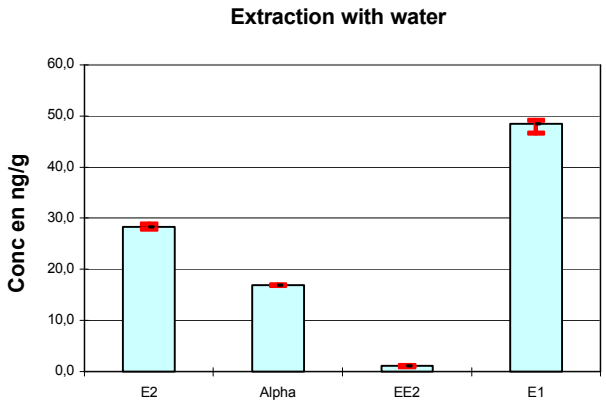
The graph 2 shows an important variation of Deuterium-labelled estrone E1-d4. Indeed, this compound is affected by the heat. We decide to spike after extraction, and the table 10 presents good results for hormones with internal standard spiked in these conditions.

The table 11 demonstrates the efficiency of the method on sand sample :

	E2	*Alpha E2*	*EE2*	*E1*
Spiked	12.1	10.2	12.8	10.1
Spiked	11.6	9.5	9.9	9.7
Mean	11.8	9.9	11.4	9.9
Recovery	**118%**	**99%**	**114%**	**99%**

Table 11 : recoveries for synthetic sample spiked at 10 ng/g

Other tests are performed with a sludge extracted six times in these conditions (graph 3).



Graph 3 : a sludge soxtec extracted 6 times with HPLC Water

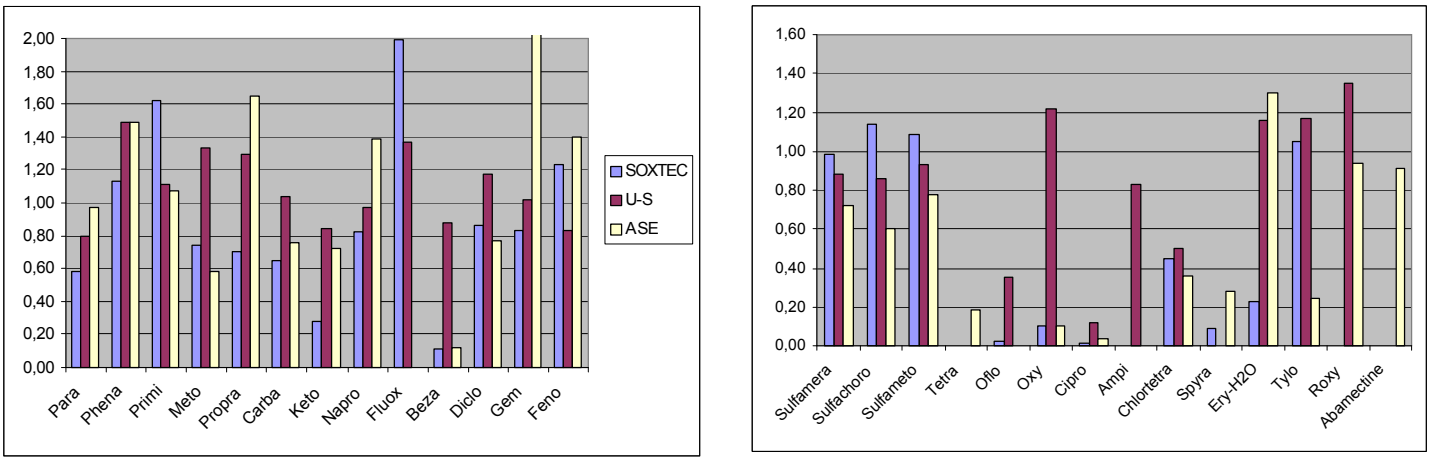
We obtain a very good repetability as soon as the internal standard is spiked after extraction when the extract is at room temperature.

7.2 Pharmaceutical and antibiotics compounds

7.2.1 Choice of the technique extraction

Tetracyclines are known to form chelate complexes with metal ions and bind to proteins and silanol groups.
Except for tetracycline substances, it seems to be not necessary to proceed at cleaning glass before antibiotic extraction as the tests in the intermediate report in december 2004 demonstrated it.

For the pharmaceutical compounds, the same techniques are carried out in the same conditions. Methylene Chloride/Acetone (40/60) mixture is used. The target value is 1. It is the ratio between the found area and the area of control point.



Graph 4 : ratio for 3 different techniques

US and ASE could be preferable to the Soxtec technique.

7.2.2 Choice of solvent extraction

Pharmaceutical analytes

Tests concerning the choice of solvent extraction were not probant in the intermediate report. So several tests are carried out using different solvents : acetone, methanol, ethyl acetate, water/methanol (50/50), acetonitrile with 0.1 % of NH_3 and methanol with citric acid 0,2 M (50:50).

A mixture made up with 500 ng of each analyte is used to spike sludge. Ultra-sonication is carried out with conditions described above. The same sludge is extracted as well without spiking. All operation is repeated 3 times. The result treated is the area of spiked sludge minus the area of no-spiked sludge. The annexe 10-a gives the mean values.

The best results are obtained with acetonitrile with 0.1 % of NH_3 and methanol with citric acid 0.2 M. The same tests are done in the same conditions. The annexe 11-a presents data.

Acetonitrile with 0.1 % of NH_3 is the best solvent extraction in particular for paracetamol, carbazepine and propanolol, which are the drugs more cited in the environment. For the salicylic acid it is not the best extraction solvent but we must find a compromise.

Antibiotics

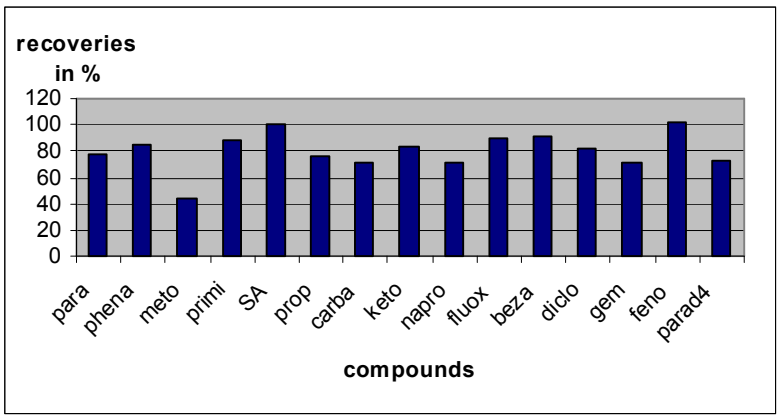
For antibiotics the same tests are carried out with identical solvents. A mixture made up with 500 ng of each analyte is used to spike sludge. Ultra-sonication is carried out with conditions described above. The same sludge is extracted as well without spiking. All operation is repeated 3 times. The result treated is the area of spiked sludge minus the area of no-spiked sludge. The annexe 10-b presents data.
Acetonitrile with 0.1 % of NH_3 and methanol with citric acid 0.2 M provide the best results.

Several mixtures of solvents are performed for the extraction with acetonitrile with 0.1 % of NH_3 and methanol with citric acid 0.2 M. Good areas are obtained with following mixtures. Finally we choose to extract :
- 2 × 20 ml of acetonitrile at 0.1 % of NH_3 following by 2 × 20 ml with methanol with citric acid 0.2 M.
- And the second test is to combine the extraction with 3 × 20 ml of acetonitrile/methanol (50/50).
Areas are compared in the annexe 11-b.

The second combination acetonitrile/methanol (50/50) is chosen because it offers good results for 2 groups of antibiotics which are sulfonamides and macrolides. And according to bibliography we note that dehydro-erythromycin, roxithromycin and sulfamethoxazole are the most present molecules in the environment. However according to our results, the best solvent of extraction for these three molecules is acetonitrile/methanol (50/50).

7.2.3 Impact of ultra-sonic extraction on pharmaceutical compounds

In order to control the impact of this step, we perform an ultra-sonic extraction on a standard solution of pharmaceutical compounds. Areas obtained are compared to a standard solution at the same concentration. The results are demonstrated on this graph with the recoveries :



Graph 5 : Impact of ultra-sonic extraction on standard solution

Excepted for the metoprolol we do not observe any big variation. The fact that 100 % is not reached is explained by the variation given by the detector. That is why we expect best results with the LC-MS MS quantum Thermo Electron on which pharmaceutical and antibiotic methods will be transfered in march 2006.

We can notice that the extraction step does not affect the internal standard : Deuterium-labelled paracetamol. It will spike at the beginning of the protocol.

Nevertheless impact of ultrasonic step has to be verified on a standard solution of antibiotics. This test is not carried out yet. We will execute it the next month.

8. CLEAN-UP

8.1 Hormones

After the extraction step, a purification step was developped before the analysis of the compounds using LC-MS/MS. The purification step was previously developped by our laboratory and described [16]. 1% of Acetone is added to the water extract prior the purification on C18 cartridge. The C18 cartridge is conditioned with 10 mL of ethyl acetate, 10 mL of methanol and 10 mL of Water prior use. After loading the water extract the C18 cartridge is rinsed with 10 mL of water/methanol (50/50, v/v), 10 mL water/acetone (65/35, v/v) and 10 mL hexane. Then the cartridge is dried under nitrogen during approximately 1 hour. The steroids are desorbed with 10 mL of a mixture of ethyl acetate/methanol (5/1, v/v). The extracts are reduced to dryness under nitrogen and reconstituted in 0.2 mL of hexane/methylene chloride (50/50, v/v) prior the purification step on Florisil cartridge. The extract is placed on the surface of the cartridge previously conditioned with 10 mL of hexane/methylene chloride (50/50, v/v). Then the cartridge is rinsed with 10 mL of Petroleum ether, 10 mL of hexane/methylene chloride (50/50, v/v) and 5 mL of methylene chloride. The elution is performed using 10 mL of methylene chloride/acetone (90/10, v/v). The extract is reduced to dryness under nitrogen and reconstituted in 1 mL of water/methanol prior to LC-MS/MS analysis.

The recoveries are verified in order to be sure that no compounds are lost during the rinsing steps on the C18 and the Florisil cartridges.

8.2 Pharmaceutical compounds

Pharmaceuticals compounds

Protocol has been studied previously on water samples. Netherless, cartridges tested on water samples are used for sludge extracts obtained with ultrasonic.
After the extraction step, the sludge extract (which the final volume is approximately 60 mL) is evaporated until 3-4 mL. Then this volume is mixed with 100 mL of water and this “aqueous” extract is then purified. 500 ng of sludge are extracted 8 times. Four extracts are purified with HLB cartridge and the others are purified on SAX and HLB.

The graph in annexe 12-a presents the comparison between the two protocols.
The purification on SAX following HLB gives satisfaction. So it is this protocol which is kept now.

The next table demonstrates the repeatability on the four repetitions on SAX-HLB.

	RSD (%) on the 4 extracts
Para	11.2
Phena	25.8
Meto	20.8
SA	14.1
Propra	15.3
Carba	10.0
Keto	12.0
Beza	6.8
Diclo	9.9

Table 12 : Relative standard deviations for the 4 extracts purified with SAX+HLB

The values of RSD show a good repeatability for the purification on the SAX+HLB cartridges.

So this protocol of clean-up with the 2 cartridges SAX+HLB is described below.
The SAX and HLB cartridges are conditioned with 5 mL of methanol and 5 mL of buffer.
After loading the extract, the SAX cartridge is removed and the HLB cartridge is rinsed with 5 mL of buffer, 2.5 mL of 0.1 M NaOAc, 5 mL of water HPLC and finally 2 mL of methanol 20%. Then the HLB cartridge is dried under nitrogen during approximately 30 minutes. The pharmaceuticals compounds are desorbed with 8 mL of methanol. The extract is reduced to dryness under nitrogen and reconstituted in 1 mL of water/methanol (95:5) prior to LC-MS/MS analysis.

Antibiotics

The same tests are performed on antibiotics. As well as pharmaceutical compounds, protocol has been studied previously on water samples. PPL following by LMS cartridge is compared to SAX following by HLB on sludge extracts, on graph in annexe 12-b.

Purification with SAX and HLB is much better. The next table demonstrates the repeatability on the four repetitions on SAX-HLB.

	RSD (%) on the 4 extracts
Sulfamera	8.8
Sulfachloro	12.7
Sulfametho	3.2
Tetra	11.3
Oflo	10.7
Oxy	15.6
Norflo	25.2
Cipro	14.8
Ampi	
Chlortetra	
Spira	4.7
Tylo	11.3
Ery	16.4
Ery-H2O	8.7
Roxi	
Aba	14.1

Table 13 : Relative standard deviations for the 4 extracts purified with SAX+HLB

The values of RSD show a good repeatability for the purification on the SAX+HLB cartridges.

9. HORMONES IN SLUDGES AND COMPOSTS : RESULTS

In first, this part of the document is written to demonstrate the efficiency of the method. We will begin by a last test. Then we will communicate some values obtained on different types of sludge with different treatments.

9.1 A sludge spiked with 2.5 ng/g of steroid hormones

The table 14 allows to appreciate the repeatability of a slight spike repeated three times. In first are presented the sludges without spiking. A recovery is calculated and values are between 94 % and 101 %.

	E2	EE2	E1
Sludge 1	9.8	0.6	4.6
Sludge 2	10.3	0.7	4.5
Sludge 3	10.3	0.8	4.6
Mean	10.1	0.7	4.5
Spiked sludge1	12.5	3.4	6.9
Spiked sludge2	12.5	3.1	7.2
Spiked sludge3	12.5	3.3	7.2
Mean	12.5	3.3	7.1
Recovery	**94%**	**101%**	**101%**

Table 14 : sludge spiked with 2.5 ng/g of hormones

9.2 Results on different types of sludge and compost

The sludge at different step of treatment is extracted three times. Mean and relative standard deviations are illustrated in the table 15.

		concentration in ng/g					
		test 1	test 2	test 3	**Mean**	Standard deviation	% RSD
Primary sludge	17Beta-Estradiol	1.25	1.25	1.25	**1.25**	**0.00**	**0%**
	17 alpha-Estradiol	0.75	0.5	0.5	**0.58**	**0.14**	**25%**
	Ethinylestradiol	1.25	0.8	0.75	**0.92**	**0.29**	**31%**
	Estrone	2.25	1.75	1.75	**1.92**	**0.29**	**15%**
Treated sludge	17Beta-Estradiol	1	1	0.75	**0.92**	**0.14**	**16%**
	17 alpha-Estradiol	0.5	0.5	0.5	**0.50**	**0.00**	**0%**
	Ethinylestradiol	7.75	7.0	7	**7.25**	**0.43**	**6%**
	Estrone	85.25	88.75	82.25	**85.42**	**3.25**	**4%**

Table 15 : statistical values obtained with sludges at two steps of process

In table 16 different types of sludge and compost are analysed. For each sample, the protocol is carried out three times. The relative standard deviation for the compost shows for low values a less better precision. Some more analysis have been undertaken on this matrix in the next table.
Otherwise, we can see some differences : Estrone is more present in biological sludge and in compost whereas 17 β estradiol is in much more quantities in primary sludge.

		Concentration in ng/g					
		test 1	test 2	test 3	**Mean**	Standard deviation	% RSD
Biological sludge	**17Beta-Estradiol**	2.8	2.5	2.3	**2.5**	0.25	10%
	17 alpha-Estradiol	3.5	2.8	2.5	**2.9**	0.52	18%
	Ethinylestradiol	<LQ (=0.25)	<LQ (=0.25)	<LQ (=0.25)	**<LQ**		
	Estrone	14.3	14	13.3	**13.8**	0.52	4%
Primary sludge	**17Beta-Estradiol**	19.8	17.3	14.8	**17.3**	2.50	14%
	17 alpha-Estradiol	2.8	2.8	2.3	**2.6**	0.29	11%
	Ethinylestradiol	<LQ (=0.5)	<LQ (=0.25)	<LQ (=0.25)	**<LQ**		
	Estrone	10.25	10.25	10.5	**10.33**	0.14	1%
Compost	**17Beta-Estradiol**	0.8	1.3	1.0	**1.0**	0.25	25%
	17 alpha-Estradiol	1.0	1.0	1.0	**1.0**	0.00	0%
	Ethinylestradiol	1.5	2.5	2.3	**2.1**	0.52	25%
	Estrone	47.5	53.3	53.8	**51.5**	3.47	7%

Table 16 : Hormone value on different types of sludge

	Estradiol	**alpha Estradiol**	**Ethinylestradiol**	**Estrone**
Compost A	0.9	1.2	1.6	18.2
Compost A	0.9	1.2	1.4	17.5
Compost B	1.4	< LQ	0.9	15.0
Compost B	1.4	< LQ	1.0	16.0
Compost C	< LQ	1.0	< LQ	3.5
Compost C	< LQ	0.7	< LQ	3.2
Manure	0.6	1.6	< LQ	107.8
Manure	0.6	1.7	< LQ	107.5
Compost D	< LQ	0.9	< LQ	0.9
Compost D	< LQ	1.5	< LQ	0.8
Compost E	1.1	1.2	2.8	3.1
Compost E	1.2	1.2	2.6	3.2

Table 17 : some values on different composts and manure

The last table emphasizes the application of the analytical method on a such matrix. Each sample has been extracted twice. We can not see a big deviation, however these analysis have been carried out on different types of compost.
Compost B comes from vegetable origin and has been mixed with sludge. Compost D is only made up vegetable origin.

10. PERSPECTIVES

The analytical method developed on steroid hormones in sludges is an efficient technique. The results illustrated in the part above are only a few examples but some more extractions have been undertaken and the method appears to be rugged.
In the next step we have to adapt the analytical method on hormones to different types of solid matrices such soil and treated bio-waste.
For antibiotic and pharmaceutical compounds we need to transfer the method on the LC/MS-MS (Quantum, Thermo-electron). Then we will verify the ruggedness of antibiotics in term of repeatability on linearity and LOQ. The impact of extraction on antibiotics will be treated as well.

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ANNEXES

Annexe1

Steroid Hormones	**CAS number**	**Purity %**	**Temperature preservation**	**Formula**	**molar Mass $g.mol^{-1}$**	**Abbreviation**
Estrone	53-16-7	>99	25°C	$C_{18}H_{22}O_2$	270.4	E1
17β-Estradiol	50-28-2	>98	25°C	$C_{18}H_{24}O_2$	272.4	E2
17α-Estradiol	57-91-0	99	25°C	$C_{18}H_{24}O_2$	272.4	α-E2
Ethinylestradiol	57-63-6	>98	25°C	$C_{20}H_{24}O_2$	296.4	EE2
Estrone d4 Estrone-2,4,16,16-d_4	53866-34-5	>95	25°C	$C_{18}H_{18}O_2D_4$	274.4	E1 d4
Estradiol d5 17β-Estradiol-2,4,16,16,17-d_5	221093-45-4	>98	25°C	$C_{18}H_{19}O_2D_5$	277.4	E2 d5
Ethinylestradiol d4 17α-Ethinylestradiol-2,4,16,16-d4	350820-06-3	>98	25°C	$C_{20}H_{20}O_2D_4$	300.4	EE2 d4

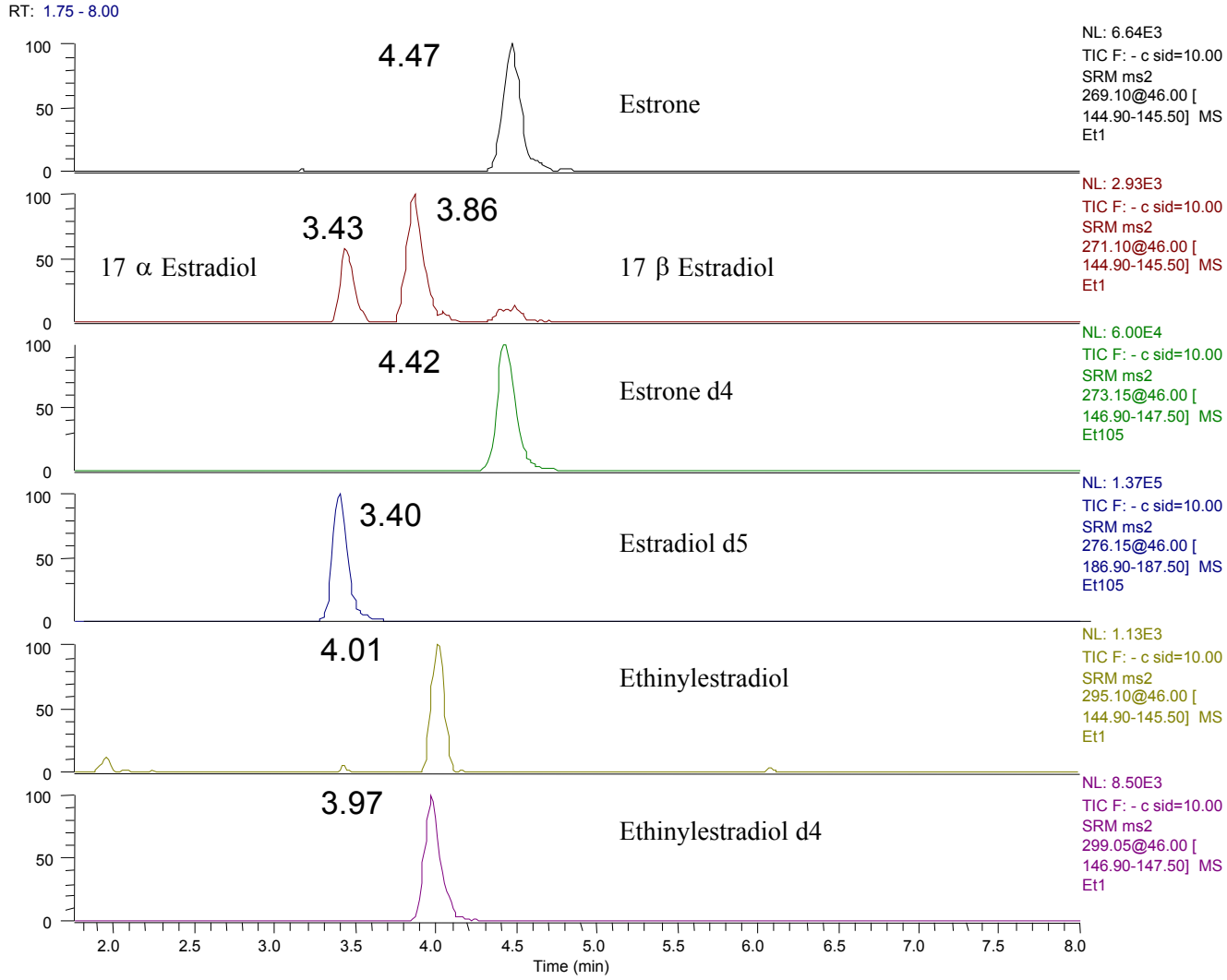
Annexe 2

Antibiotic Substances	CAS number	Purity	Temperature	Dessicator	Light sensitivity	Formula	Molar mass $g.mol^{-1}$	Abbreviation
Ampicilline trihydrate	7177-48-2	86,7	2 to 8°C		Yes	$C_{16}H_{19}N_3O_4S.3H_2O$	403,4	Ampi
Chlortetracycline hydrochloride	64-72-2	97	4° C		Yes	$C_{22}H_{23}ClN_2O_8.HCl$	515,35	Chlortetra
Erythromycine	114-07-8	95,7	20° C		Yes	$C_{37}H_{67}NO_{13}$	733,9	Ery
Oxytetracycline dihydrate	6153-64-6	93,5	20° C		Yes	$C_{22}H_{24}N_2O_9.2H_2O$	496,5	Oxy
Roxithromycine	80214-83-1	90	-20°C	Yes	Yes	$C_{41}H_{75}N_2O_{15}$	837,1	Roxi
Sulfachloropyridazine	80-32-0	100	20° C		Yes	$C_{10}H_9ClN_4O_2S$	284,7	Sulfachloro
Sulfamethoxazole	723-46-6	100	20° C		Yes	$C_{10}H_{11}N_3O_3S$	253,28	Sulfametho
Sulfamerazine	127-79-7	99	20° C		Yes	$C_{11}H_{12}N_4O_2S$	264,3	Sulfamera
Spiramycine	8025-81-8	100	2 to 8°C	Yes	Yes	$C_{43}H_{74}N_2O_4$	815/857/871	Spira
Tetracycline	60-54-8	98	4 °C	Yes	Yes	$C_{22}H_{24}N_2O_8$ aq	444,44	Tetra
Tylosine Tartrate	74610-55-2	95	4 °C	Yes	Yes	$C_{45}H_{77}NO_{17}.C_4H_6O_6$	1066,2	Tylo
Ofloxacine	82419-36-1	100	2 to 8°C		Yes	$C_{18}H_{20}FN_3O_4$	361,4	Oflo
Abamectine	71751-41-2	97,9	20° C		Yes	B1A $C_{48}H_{72}O_{14}$ B1B $C_{47}H_{70}O_{14}$	872 858	Aba
Ciprofloxacine	85721-33-1	98	20° C		Yes	$C_{17}H_{18}FN_3O_3$	331,35	Cipro
Norfloxacine	70458-96-7	100,00	2 to 8°C	Yes	Yes	$C_{16}H_{18}FN_3O_3$	319,3	Norflo
Internal Standards								
Simetone	673-04-1	99%	20° C		Yes	$C_8H_{15}N_5O$	197,24	Sim
Sulfamethazine phenyl $^{13}C_6$	57-68-1	90%	20° C	Yes	Yes	$C_{12}H_{14}N_4O_2S$	284,34	Sulfa

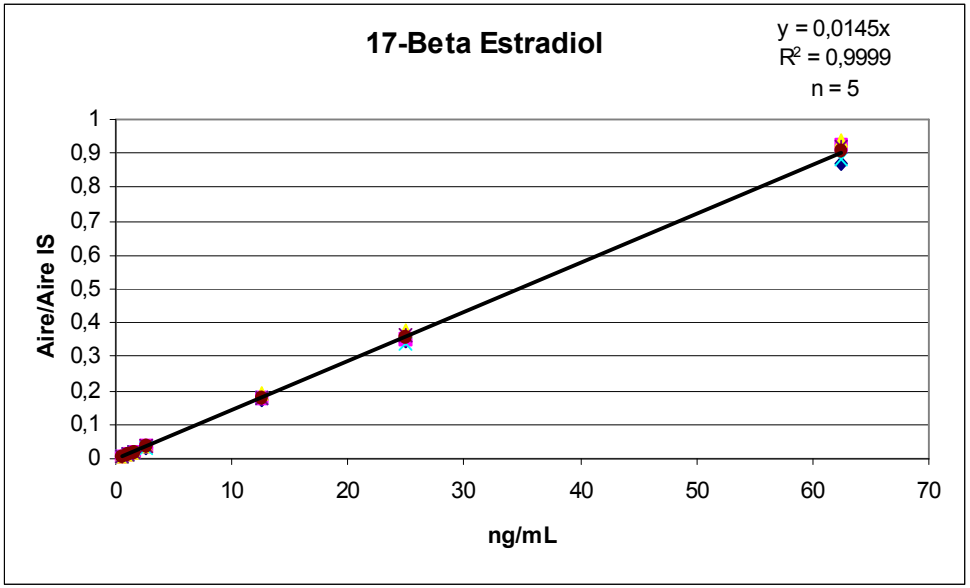
Annexe 3

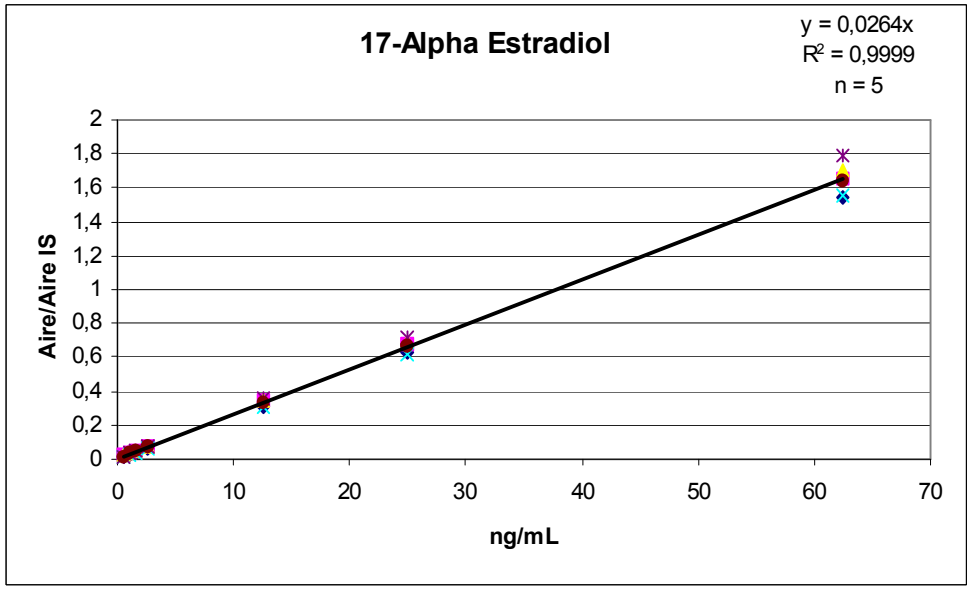
Pharmaceuticals	CAS number	Purity %	Temperature preservation	Dessicator	Light sensitivity	Formula	Molar mass $g.mol^{-1}$	Abbreviation
4 –Acetominophenol (Paracetamol)	103-90-2	99,00	20°C		Yes	$C_8H_9NO_2$	151,17	Para
Acide salicylique	69-72-7	99,00	20°C		Yes	$C_7H_6O_3$	138,12	SA
Antipyrine (Phenazone)	60-80-0	100,00	20°C		Yes	$C_{11}H_{12}N_2O$	188,23	Phena
Bezafibrate	41859-67-0	100,00	20°C		Yes	$C_{19}H_{20}ClNO_4$	361,8	Beza
Carbamazepine	298-46-4	100,00	2 to 8 °C	Yes	Yes	$C_{15}H_{12}N_2O$	236,3	Carba
Diclofenac	15307-79-6	100,00	20°C		Yes	$C_{14}H_{10}Cl_2NO_2Na$	318,1	Diclo
Ketoprofen	22071-15-4	100,00	20°C		Yes	$C_{16}H_{14}O_3$	254,3	Keto
(±) Metoprolol	56392-17-7	99,00	20°C		Yes	$(C_{15}H_{25}NO_3)_2C_4H_6O_6$	684,8	Meto
Propranolol hydrochloride	3506-09-0	99,50	2 to 8 °C		Yes	$C_{16}H_{21}NO_2HCl$	295,8	Propra
Internal Standards								
Paracetamol d4	64315-36-2	100,00	20°C		Yes	$C_8H_5D_4NO_2$	155,17	ParaD4

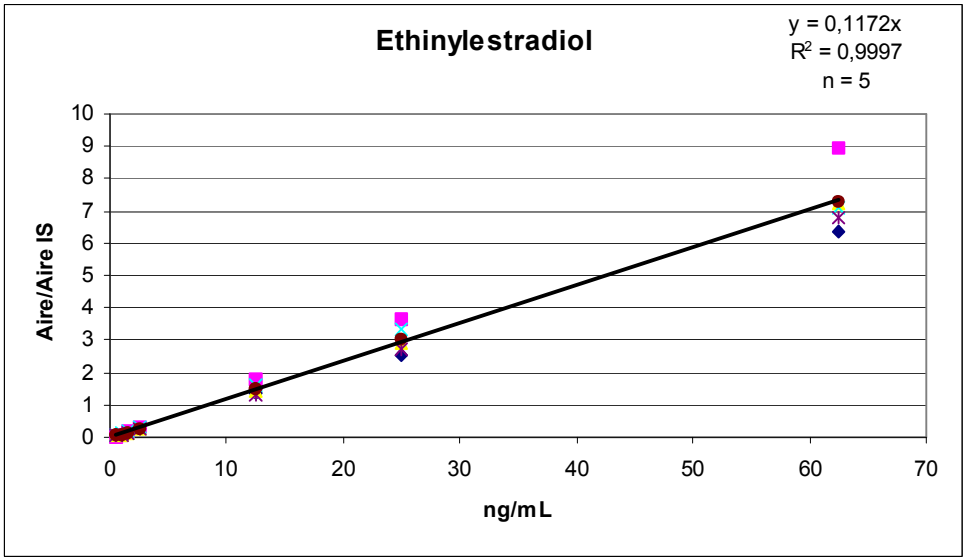
Annexe 4 : LC-MS/MS chromatogram of solvent-base standard at 1 ng/mL for Steroid hormones

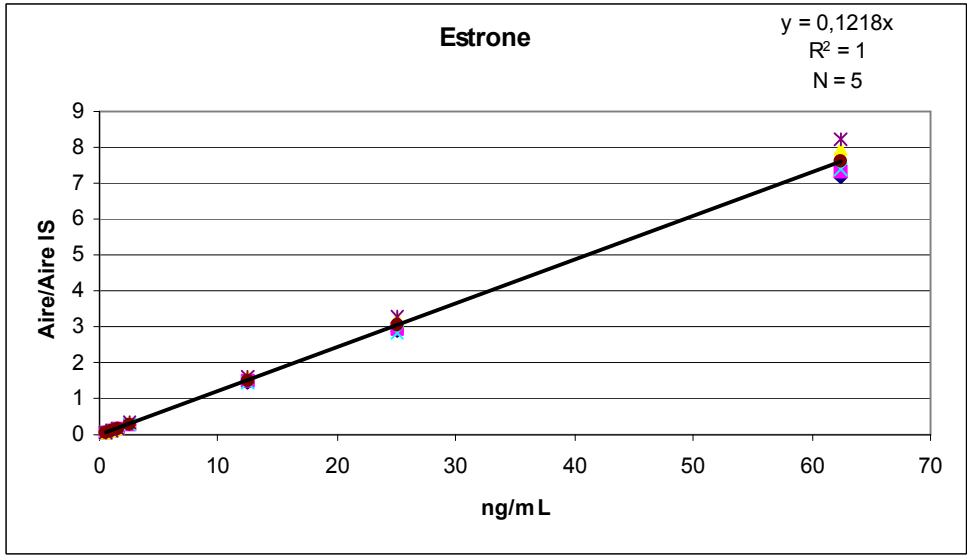


Annexe 5 : internal calibration curve of in range 0.5 to 62.5 ng/mL (7 points) for hormones.



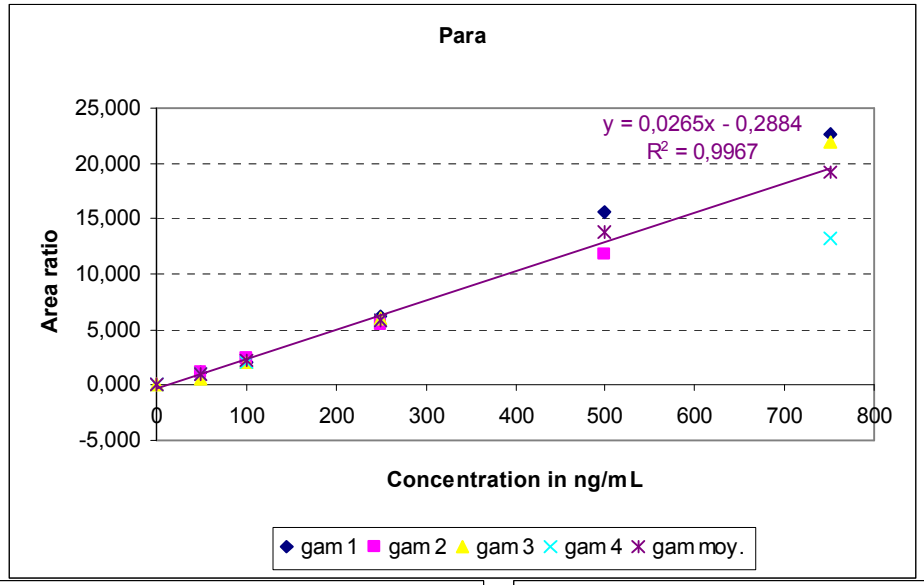


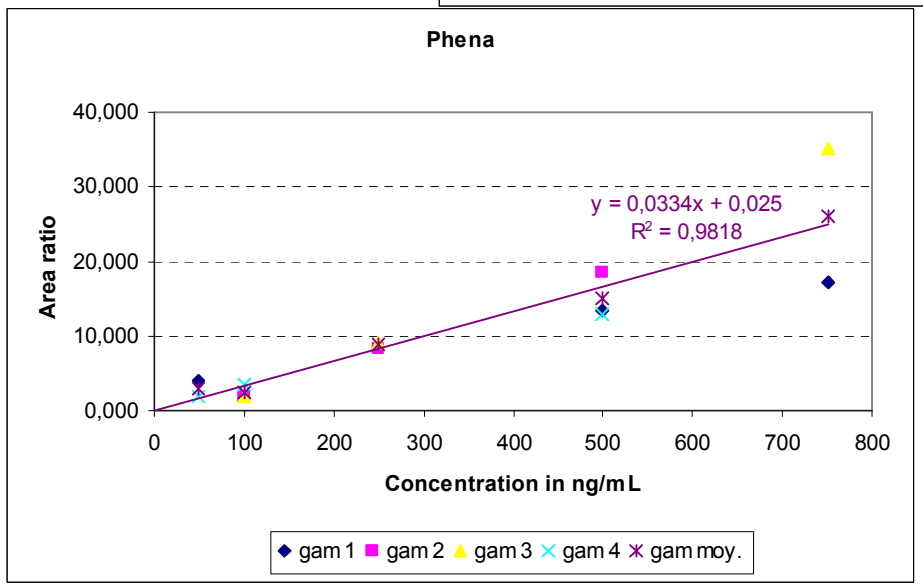


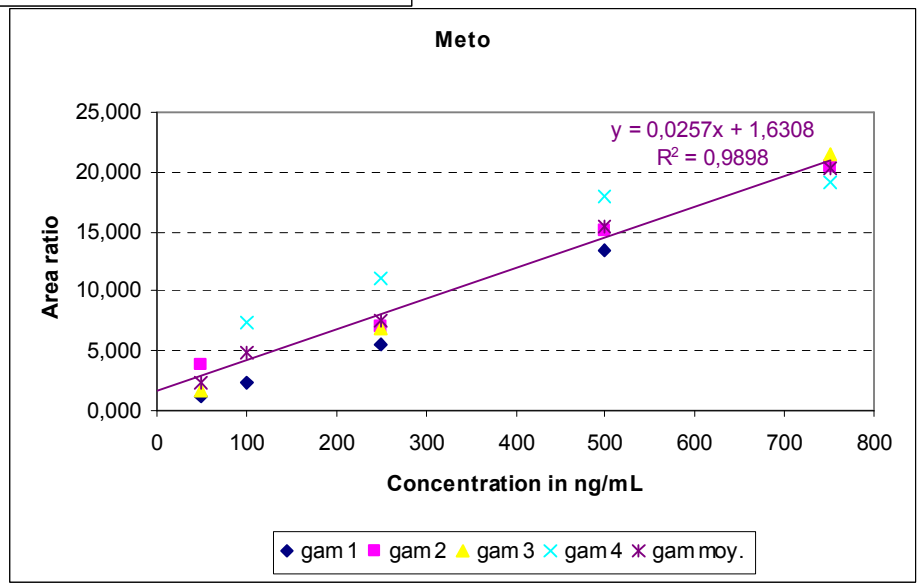


Annexe 6-a : internal calibration curves of in range 50 to 750 ng/mL for pharmaceutical compounds.

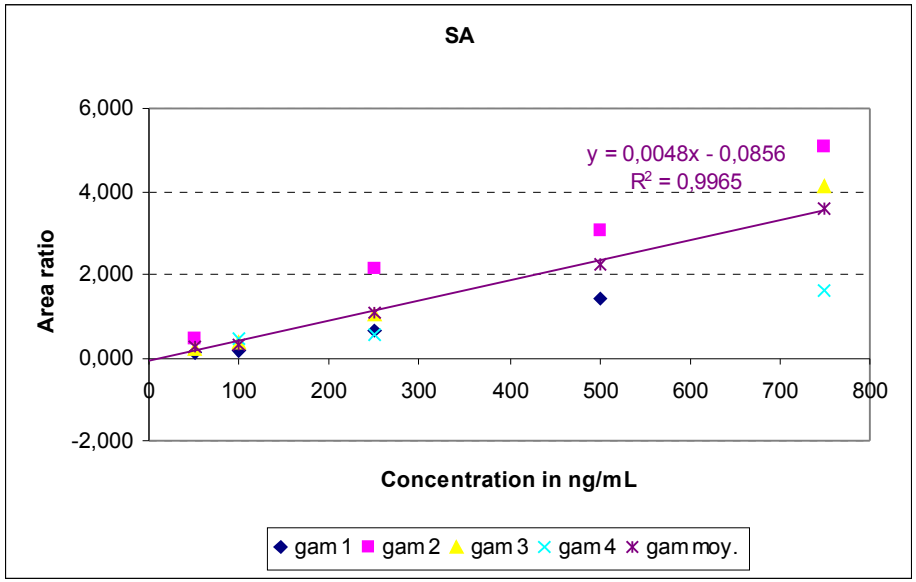
Pharmaceuticals (1)

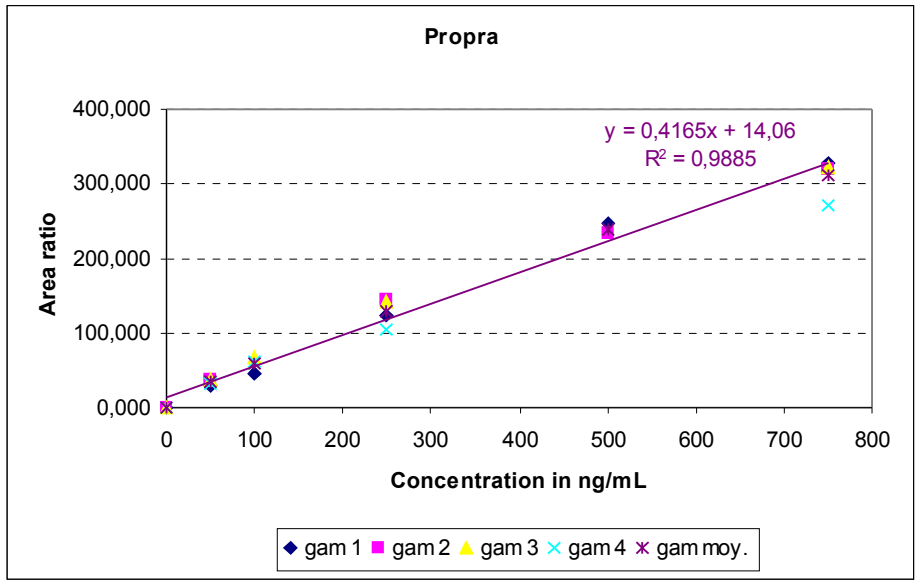


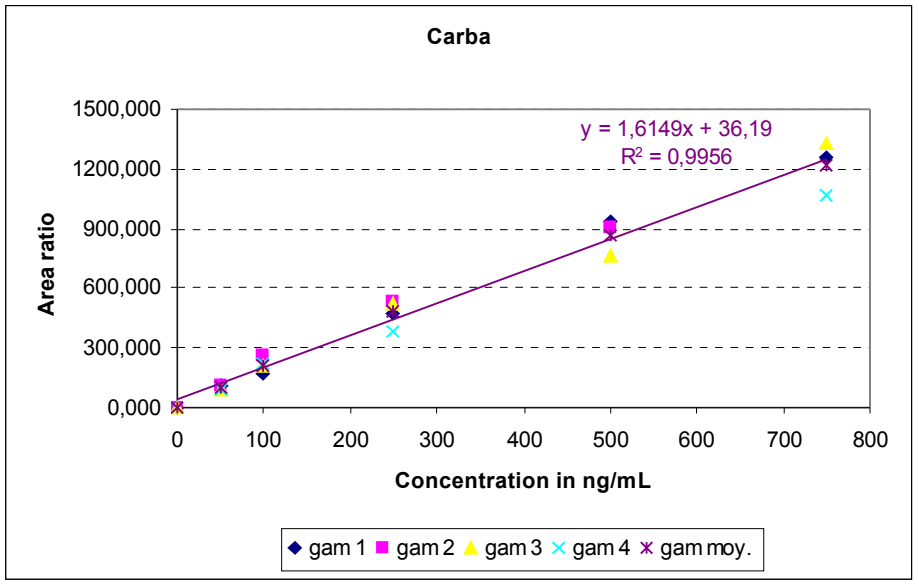




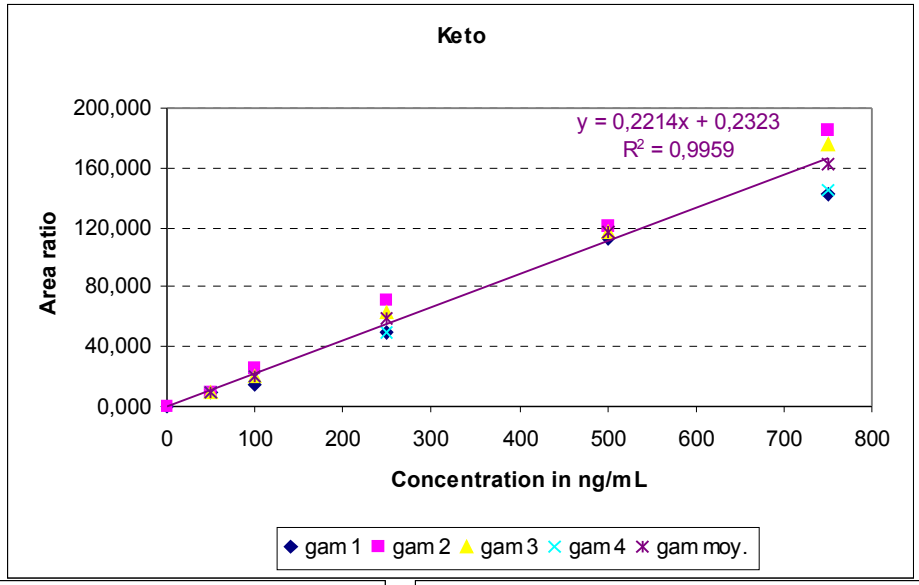
Pharmaceuticals (2)

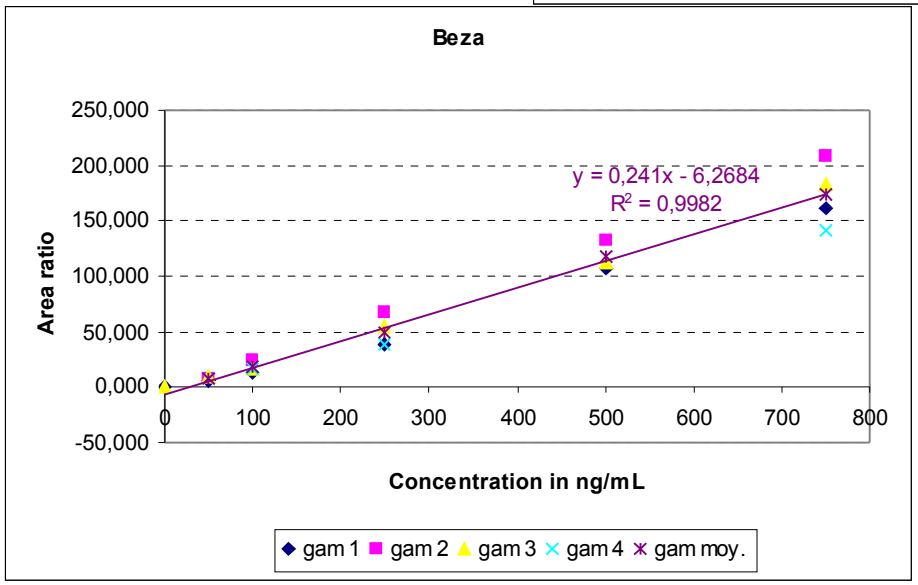


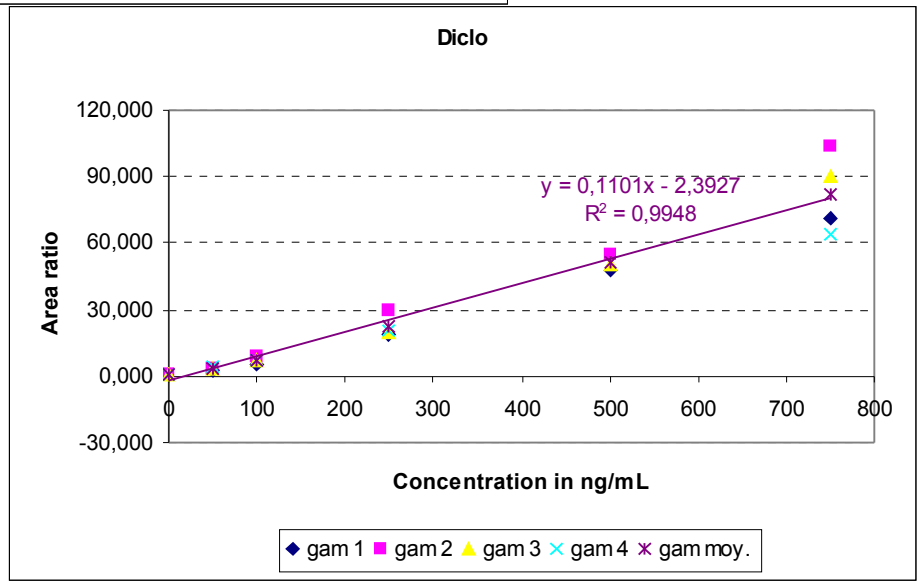




Pharmaceuticals (3)

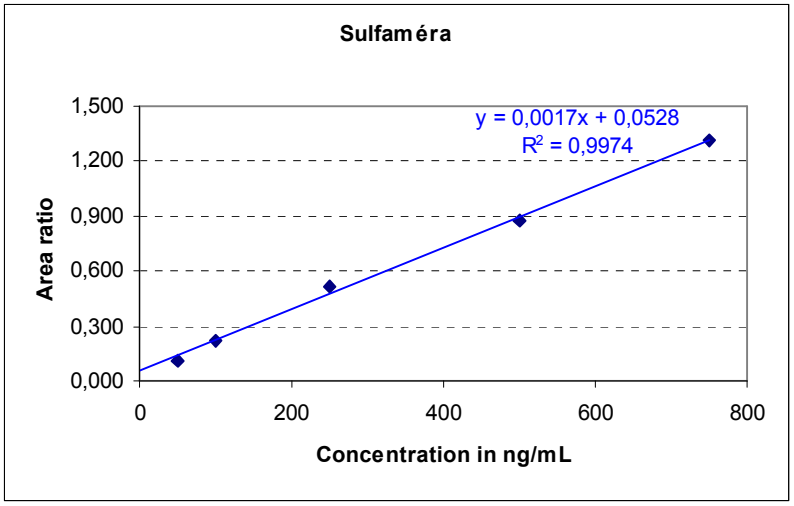


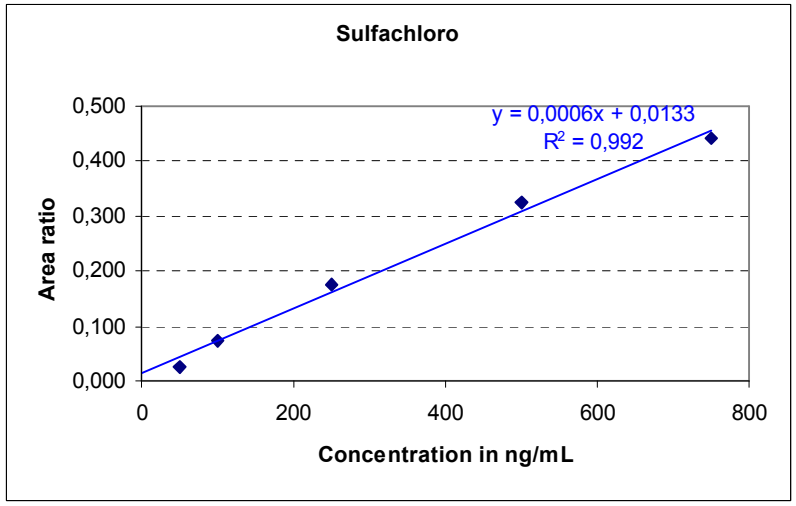


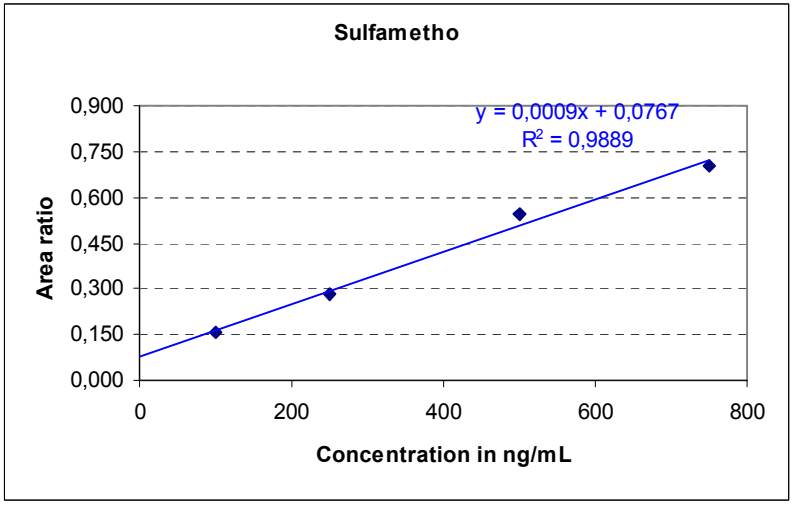


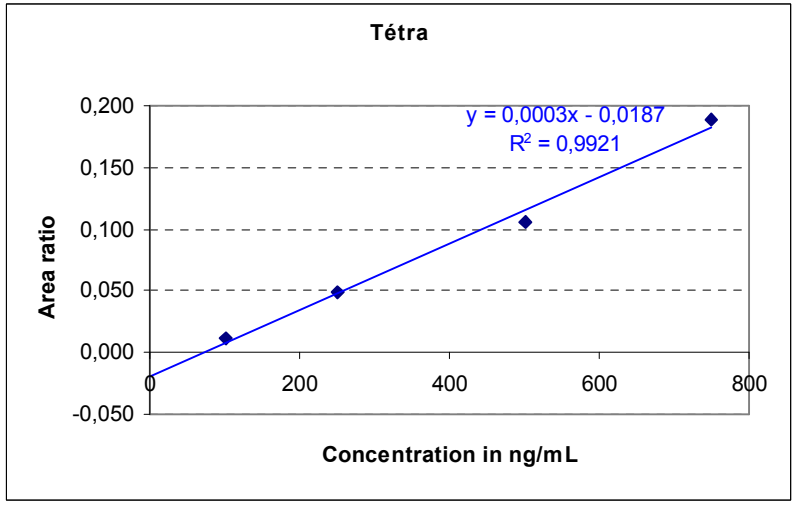
Annexe 6-b : internal calibration curves of in range 50 to 750 ng/mL for antibiotics.

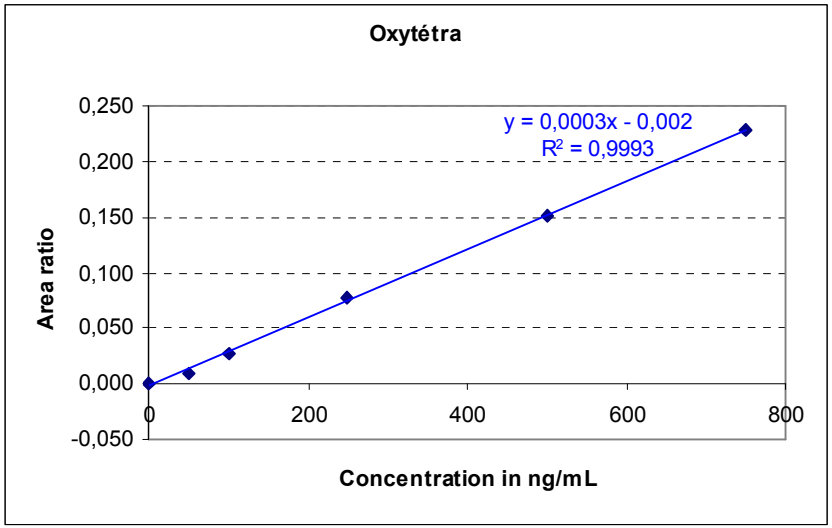
Antibiotics (1)

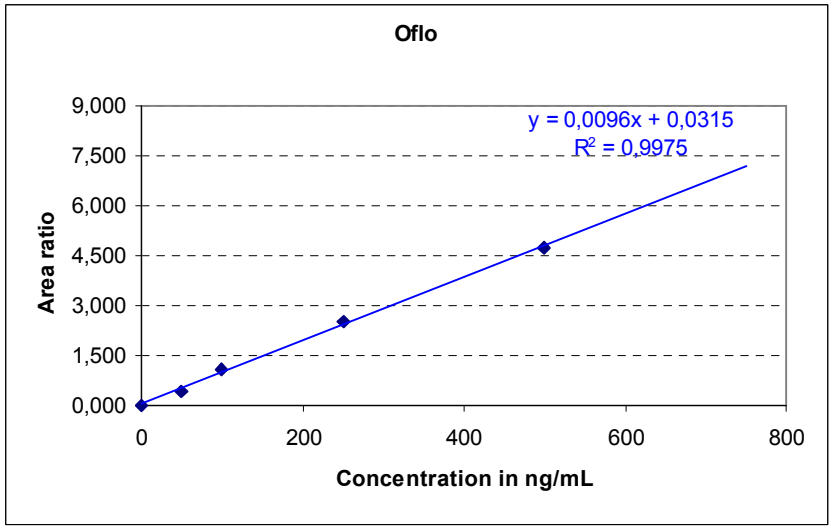


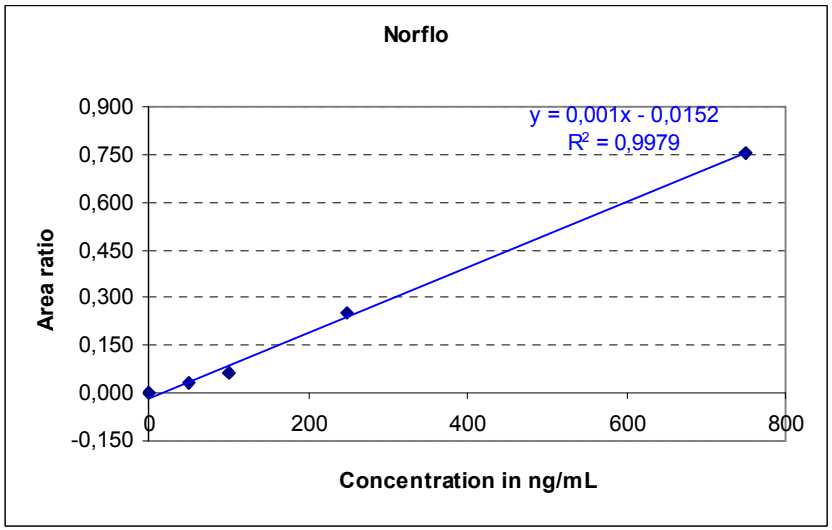


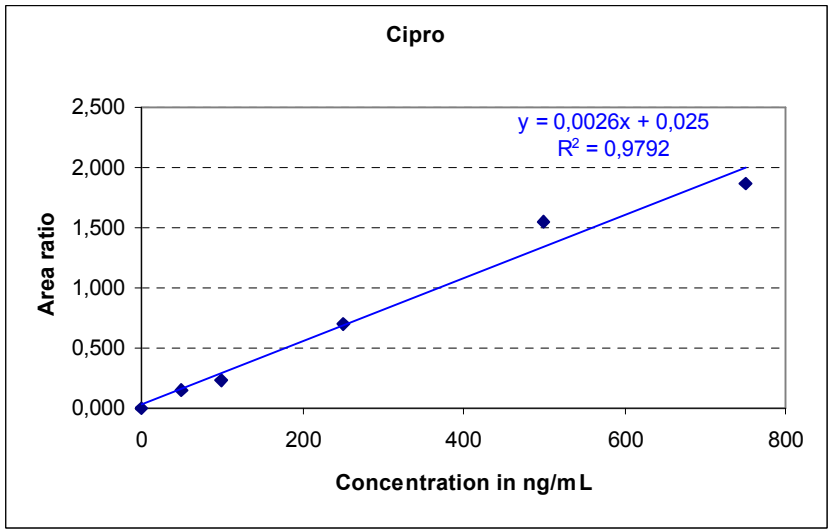


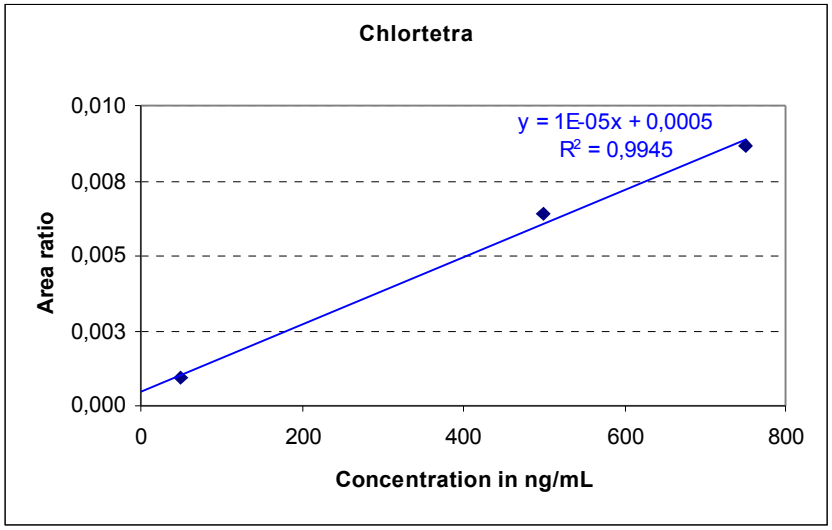


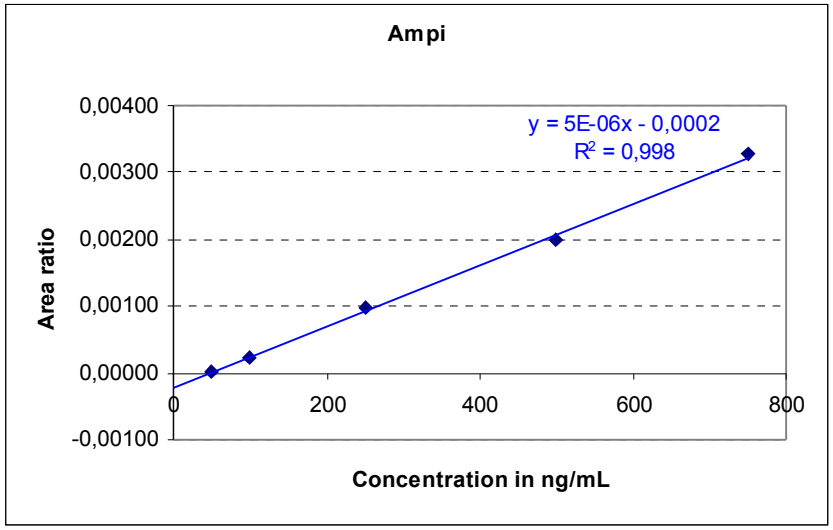

Oxytétra
y = 0,0003x - 0,002
R² = 0,9993
Area ratio
Concentration in ng/mL
0,250
0,200
0,150
0,100
0,050
0,000
-0,050
0
200
400
600
800

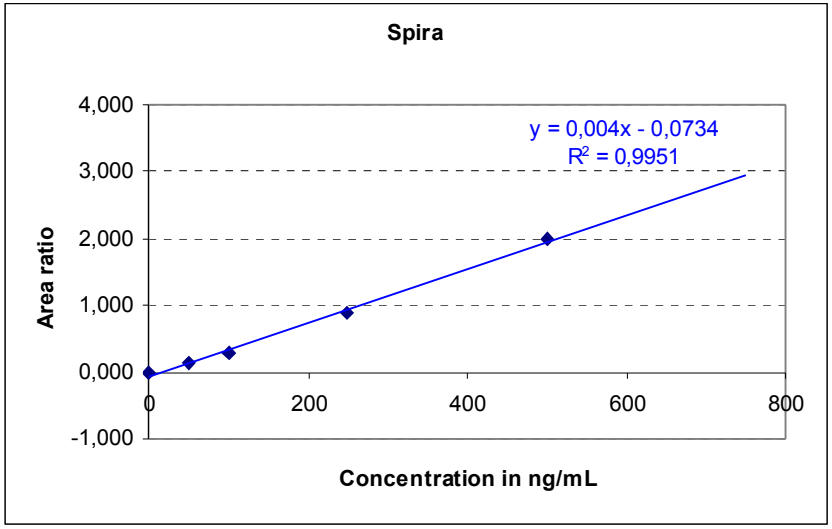

Oflo
y = 0,0096x + 0,0315
R² = 0,9975
Area ratio
Concentration in ng/mL
9,000
7,500
6,000
4,500
3,000
1,500
0,000
0
200
400
600
800

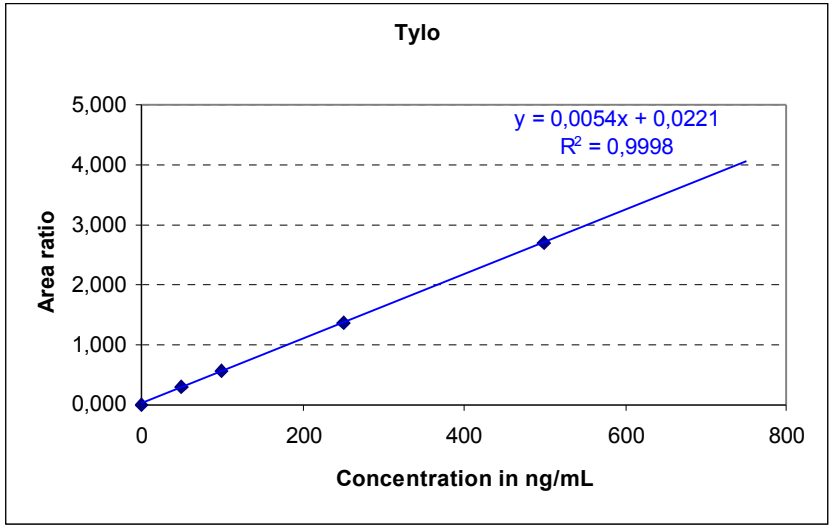

Norflo
y = 0,001x - 0,0152
R² = 0,9979
Area ratio
Concentration in ng/mL
0,900
0,750
0,600
0,450
0,300
0,150
0,000
-0,150
0
200
400
600
800

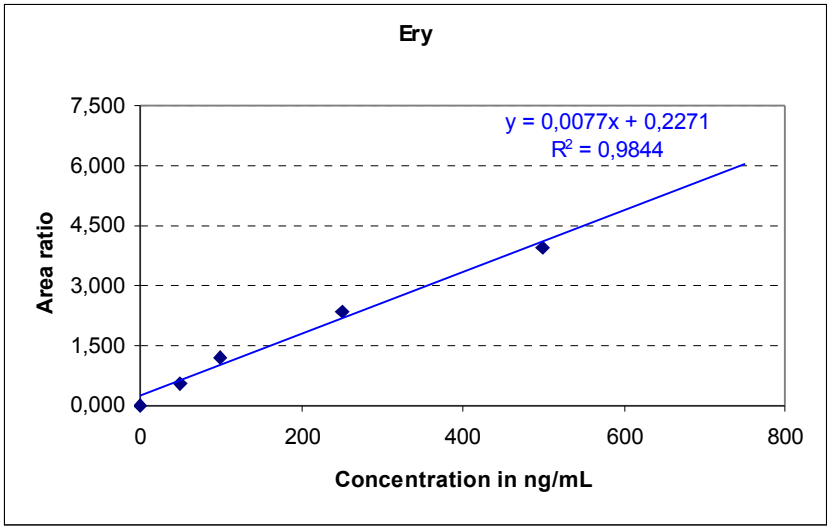

Cipro
y = 0,0026x + 0,025
R² = 0,9792
Area ratio
Concentration in ng/mL
2,500
2,000
1,500
1,000
0,500
0,000
0
200
400
600
800

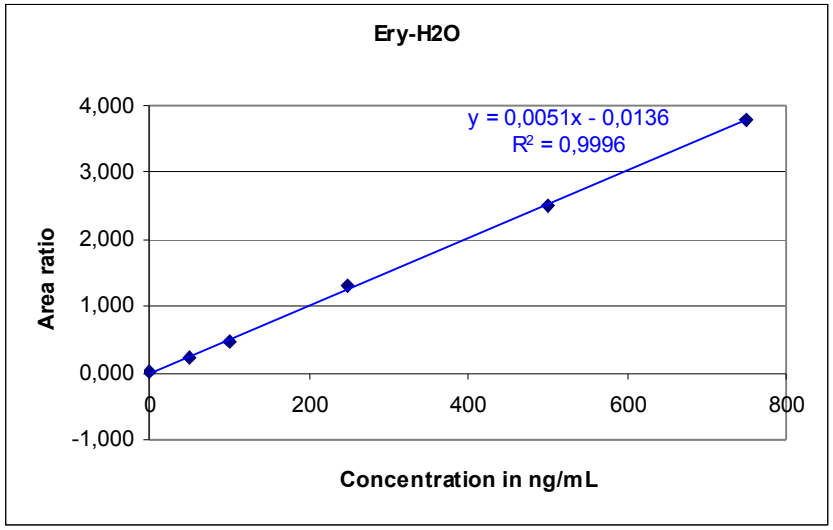

Chlortetra
y = 1E-05x + 0,0005
R² = 0,9945
Area ratio
0,010
0,008
0,005
0,003
0,000
0
200
400
600
800
Concentration in ng/mL

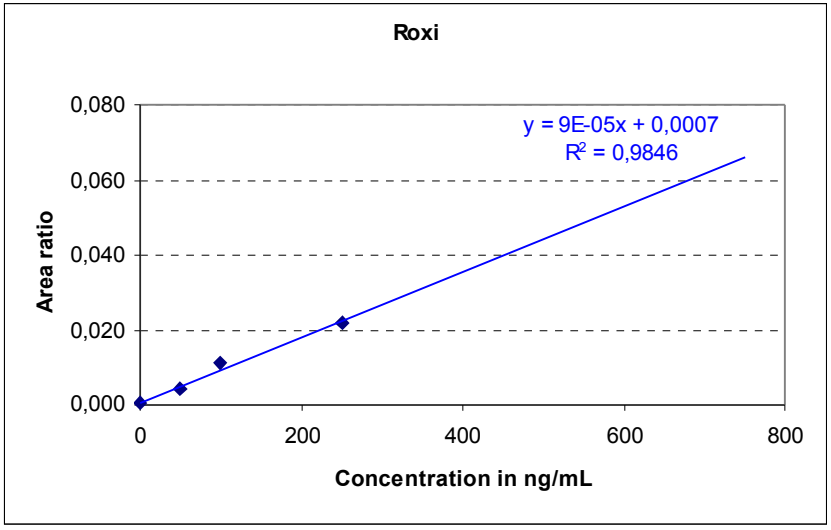

Ampi
y = 5E-06x - 0,0002
R² = 0,998
Area ratio
0,00400
0,00300
0,00200
0,00100
0,00000
-0,00100
0
200
400
600
800
Concentration in ng/mL

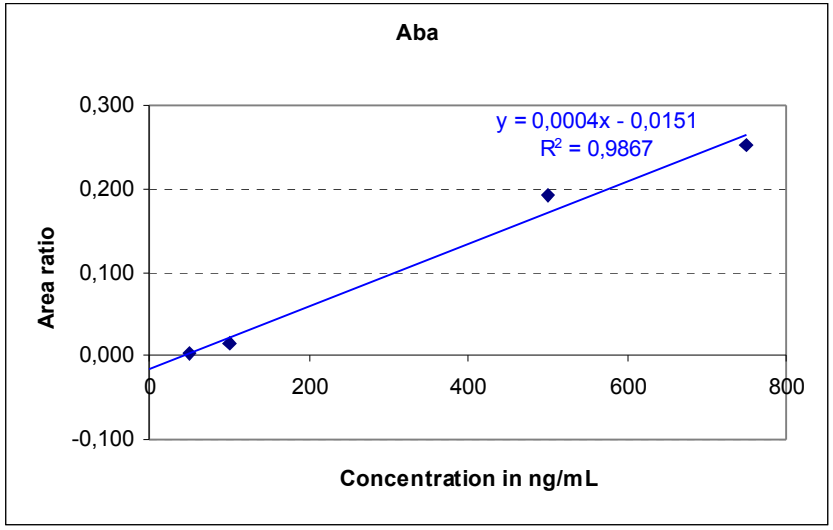

Spira
y = 0,004x - 0,0734
R² = 0,9951
Area ratio
4,000
3,000
2,000
1,000
0,000
-1,000
0
200
400
600
800
Concentration in ng/mL


Tylo
y = 0,0054x + 0,0221
R² = 0,9998
Area ratio
5,000
4,000
3,000
2,000
1,000
0,000
0
200
400
600
800
Concentration in ng/mL


Ery
y = 0,0077x + 0,2271
R² = 0,9844
Area ratio
Concentration in ng/mL
7,500
6,000
4,500
3,000
1,500
0,000
0
200
400
600
800


Ery-H2O
y = 0,0051x - 0,0136
R² = 0,9996
Area ratio
Concentration in ng/mL
4,000
3,000
2,000
1,000
0,000
-1,000
0
200
400
600
800


Roxi
y = 9E-05x + 0,0007
R² = 0,9846
Area ratio
Concentration in ng/mL
0,080
0,060
0,040
0,020
0,000
0
200
400
600
800


Aba
y = 0,0004x - 0,0151
R² = 0,9867
Area ratio
Concentration in ng/mL
0,300
0,200
0,100
0,000
-0,100
0
200
400
600
800

Annexe 7 : Hormones calibration curves with recalculated values

17-Beta Estradiol

ng/g	26/03/2004	01/04/2004	09/07/2004	22/09/2004	18/10/2004	20/10/2004	21/10/2004	25/10/2004	27/10/2004	Mean	Standard deviation	RSD
0,5	0,487	0,4375	0,436	0,495	0,494	0,53	0,556	0,495	0,495	0,492	0,038	**8%**
1	0,983	1,025	0,809	1,046	1,048	0,873	1,033	1,036	1,035	0,988	0,087	**9%**
1,5	1,475	1,615	1,439	1,449	1,545	1,474	1,315	1,407	1,497	1,468	0,084	**6%**
2,5	2,555	2,59	2,912	2,387	2,344	2,728	2,408	2,575	2,434	2,548	0,183	**7%**
12,5	12,79	11,837	13,767	13,112	12,667	12,443	12,675	12,642	12,508	12,716	0,520	**4%**
25	24,975	25,052	27,183	25,124	24,374	24,923	25,035	24,545	25,085	25,144	0,807	**3%**
62,5	63,397	65,3125	64,721	61,244	63,028	62,53	62,487	63,989	62,551	63,251	1,257	**2%**
125	127,43	127,657	125,257	127,106						126,863	1,094	**1%**
187,5	183,905	182,4725	181,476	186,038						183,473	1,979	**1%**

17-alpha Estradiol

ng/g	26/03/2004	01/04/2004	09/07/2004	22/09/2004	18/10/2004	20/10/2004	21/10/2004	25/10/2004	27/10/2004	Mean	Standard deviation	RSD
0,5	0,495	0,512	0,435	0,482	0,476	0,435	0,492	0,453	0,482	0,474	0,027	**6%**
1	0,885	0,965	0,979	1,111	1,017	1,136	1,004	0,938	1,009	1,005	0,079	**8%**
1,5	1,572	1,48	1,559	1,428	1,57	1,517	1,49	1,578	1,5	1,522	0,052	**3%**
2,5	2,39	2,362	2,389	2,43	2,496	2,476	2,552	2,607	2,517	2,469	0,083	**3%**
12,5	13,457	12,085	13,179	12,377	12,317	12,413	12,421	12,84	12,703	12,644	0,445	**4%**
25	26,587	27,137	27,062	25,881	24,945	24,96	25,182	25,954	25,236	25,883	0,874	**3%**
62,5	63,4	66,8375	66,539	60,249	62,678	62,564	62,359	63,252	62,183	63,340	2,103	**3%**
125	118,65	120,305	121,26	125,818						121,508	3,069	**3%**
187,5	190,562	186,315	184,599	188,222						187,425	2,562	**1%**

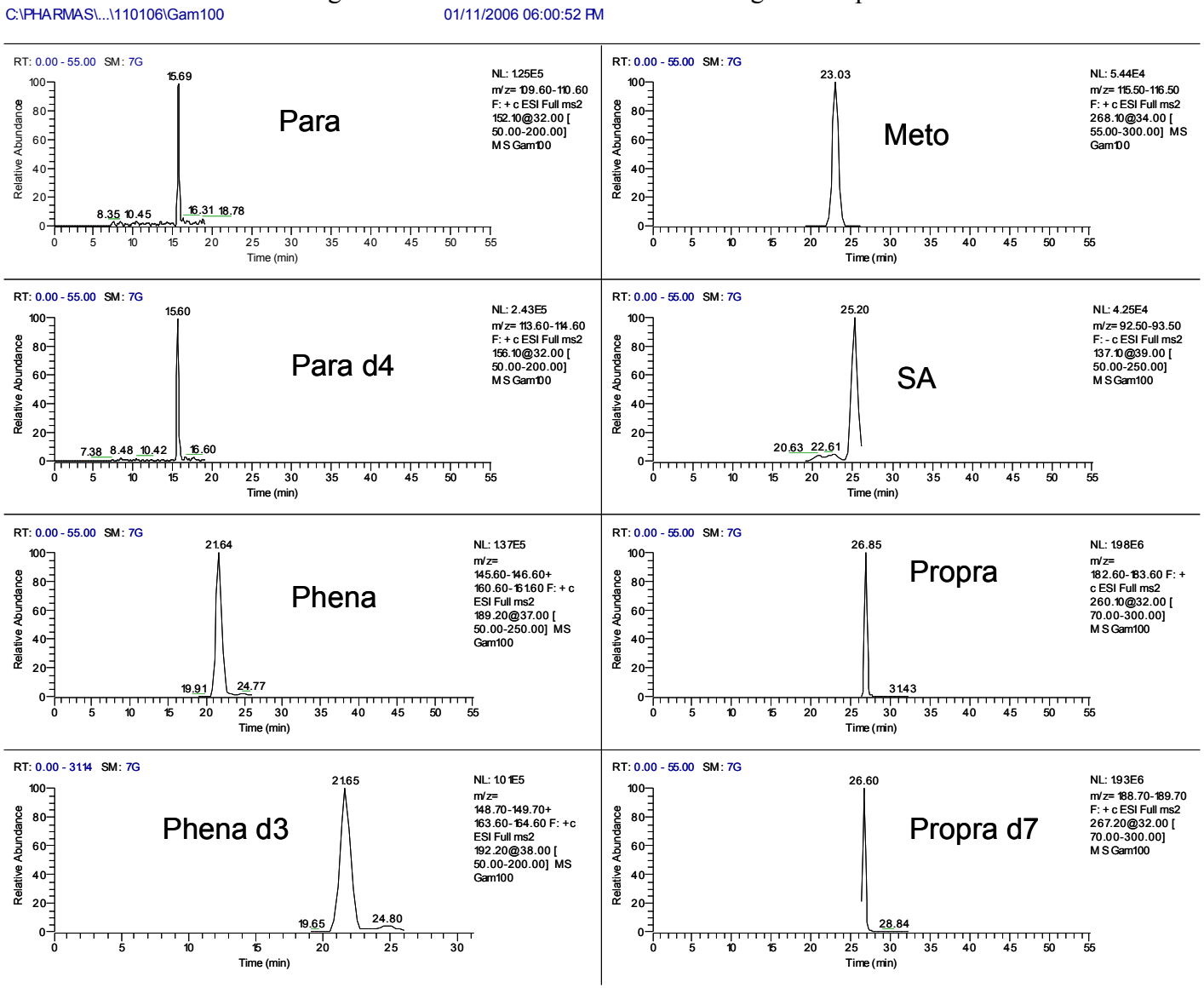
Ethinylestradiol

ng/g	26/03/2004	01/04/2004	09/07/2004	22/09/2004	18/10/2004	20/10/2004	21/10/2004	25/10/2004	27/10/2004	Mean	Standard deviation	RSD
0,5	0,423	0,41	0,495	0,543	0,4	0,579	0,522	0,466	0,492	0,481	0,062	**13%**
1	0,877	0,77	0,924	0,987	1,017	0,948	0,967	0,84	0,989	0,924	0,081	**9%**
1,5	1,562	1,52	1,636	1,293	1,501	1,42	1,567	1,541	1,419	1,495	0,103	**7%**
2,5	2,717	2,202	2,231	2,529	2,661	2,371	2,364	2,418	2,541	2,448	0,178	**7%**
12,5	14,167	16,317	14,66	12,624	13,359	12,363	12,422	13,375	13,28	13,619	1,270	**9%**
25	25,267	29,3	24,026	25,193	27,656	25,195	25,149	26,969	25,214	25,997	1,645	**6%**
62,5	62,6725	69,645	60,616	65,799	58,905	62,624	62,51	66,054	62,7	63,503	3,203	**5%**
125	123,775	126,217	122,789	125,569						124,588	1,583	**1%**
187,5	186,53	171,62	190,623	183,513						183,072	8,171	**4%**

Estrone

ng/g	26/03/2004	01/04/2004	09/07/2004	22/09/2004	18/10/2004	20/10/2004	21/10/2004	25/10/2004	27/10/2004	Mean	Standard deviation	RSD
0,5	0,405	0,525	0,479	0,529	0,494	0,517	0,493	0,497	0,501	0,493	0,037	**7%**
1	1,0325	1,02	0,942	1,02	1,002	1	1,007	0,98	1,003	1,001	0,027	**3%**
1,5	1,532	1,395	1,5	1,38	1,625	1,456	1,494	1,505	1,49	1,486	0,073	**5%**
2,5	2,545	2,577	2,441	2,515	2,357	2,524	2,505	2,505	2,495	2,496	0,064	**3%**
12,5	13,027	12,407	13,267	12,473	12,472	12,228	12,607	12,633	12,46	12,619	0,326	**3%**
25	26,17	24,742	26,937	24,806	24,399	25,101	25,083	25,214	25,249	25,300	0,783	**3%**
62,5	65,805	61,142	62,29	62,482	63,15	62,672	62,311	62,543	62,516	62,768	1,258	**2%**
125	126,467	125,337	125,31	126,685						125,950	0,729	**1%**
187,5	181,015	188,852	184,834	186,11						185,203	3,256	**2%**

Annexe 8-a : LC-MS/MS chromatogram of solvent-base standard at 100 ng/mL for pharmaceuticals. ***Pharmaceuticals(1)***



Pharmaceuticals (2)

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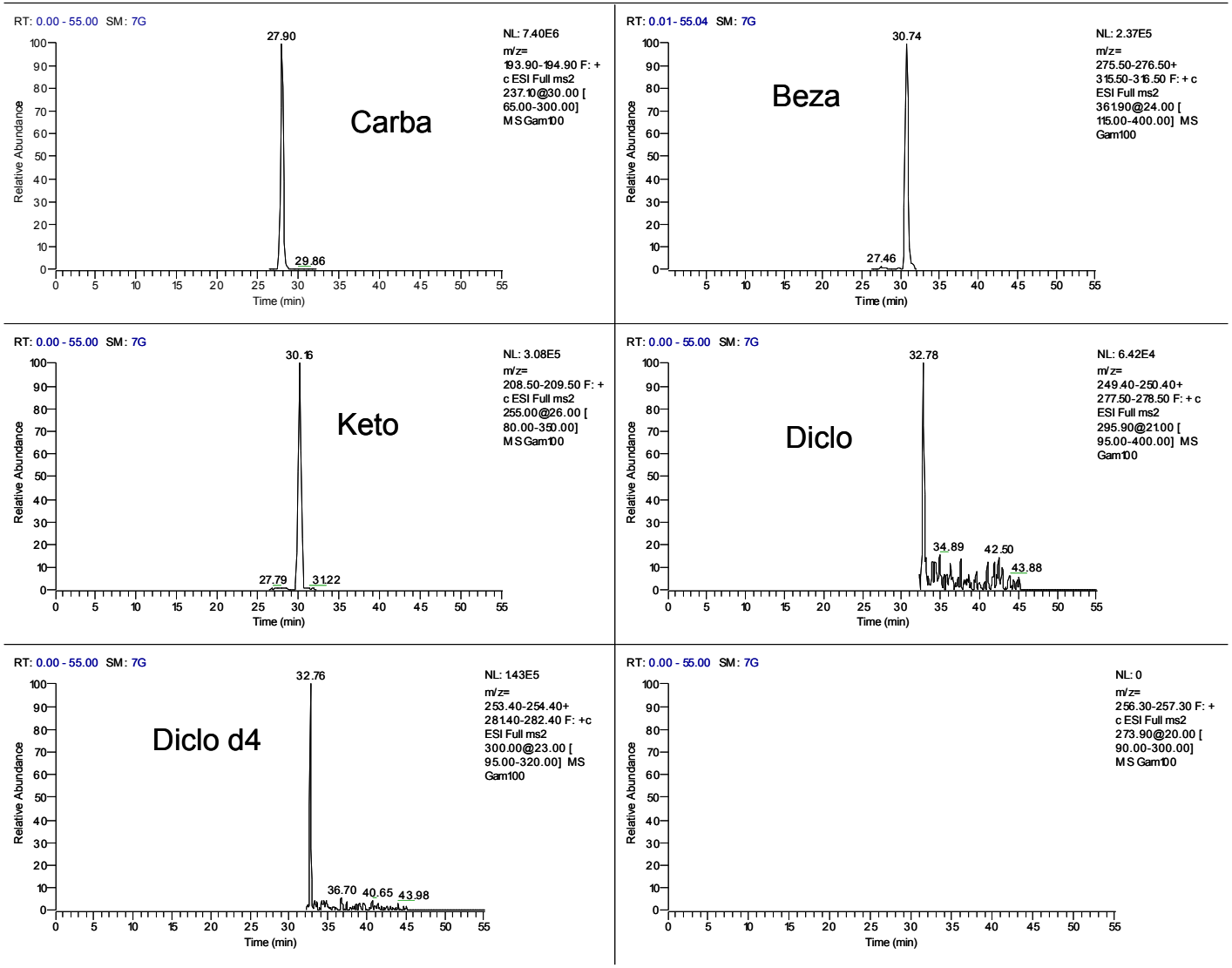
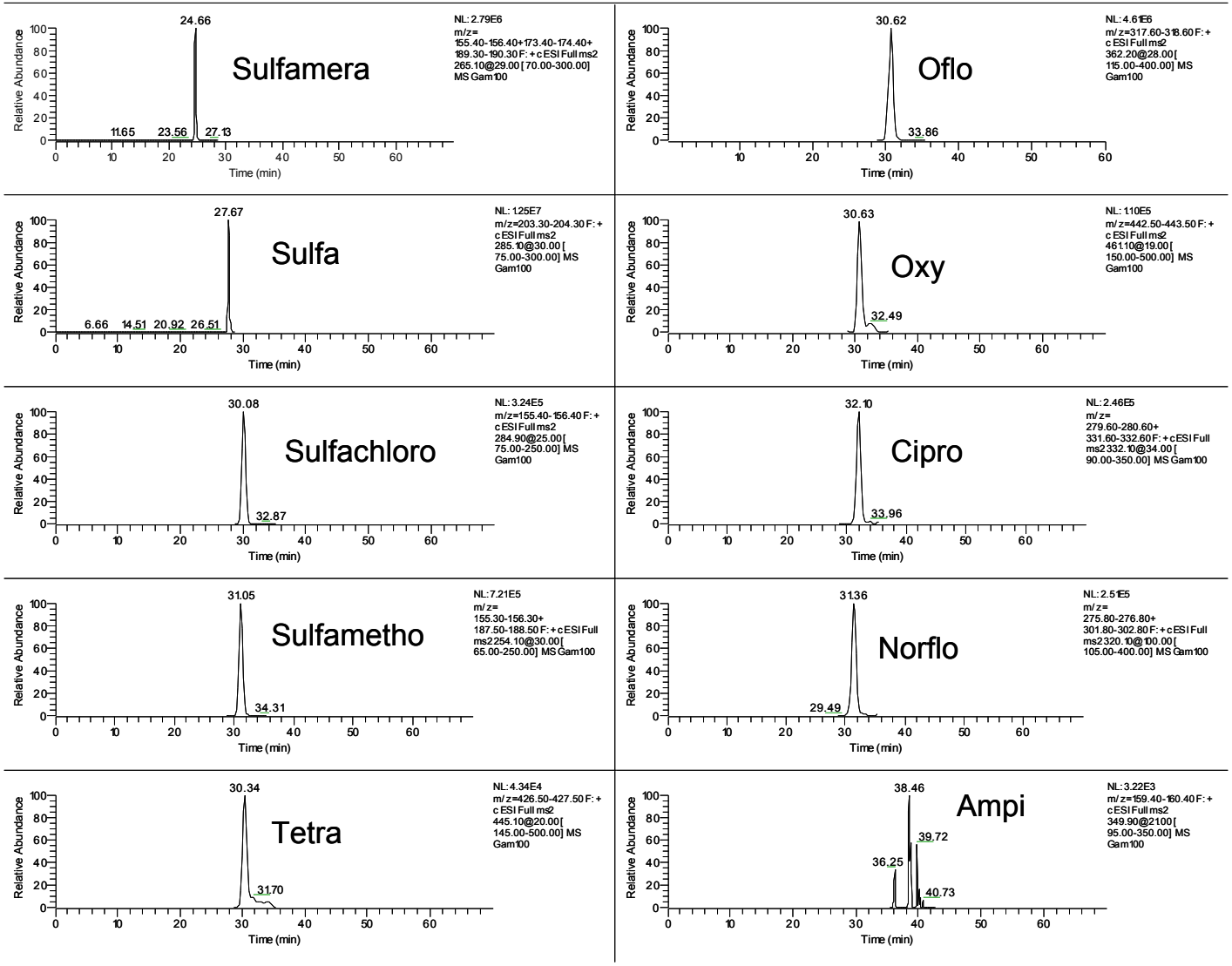


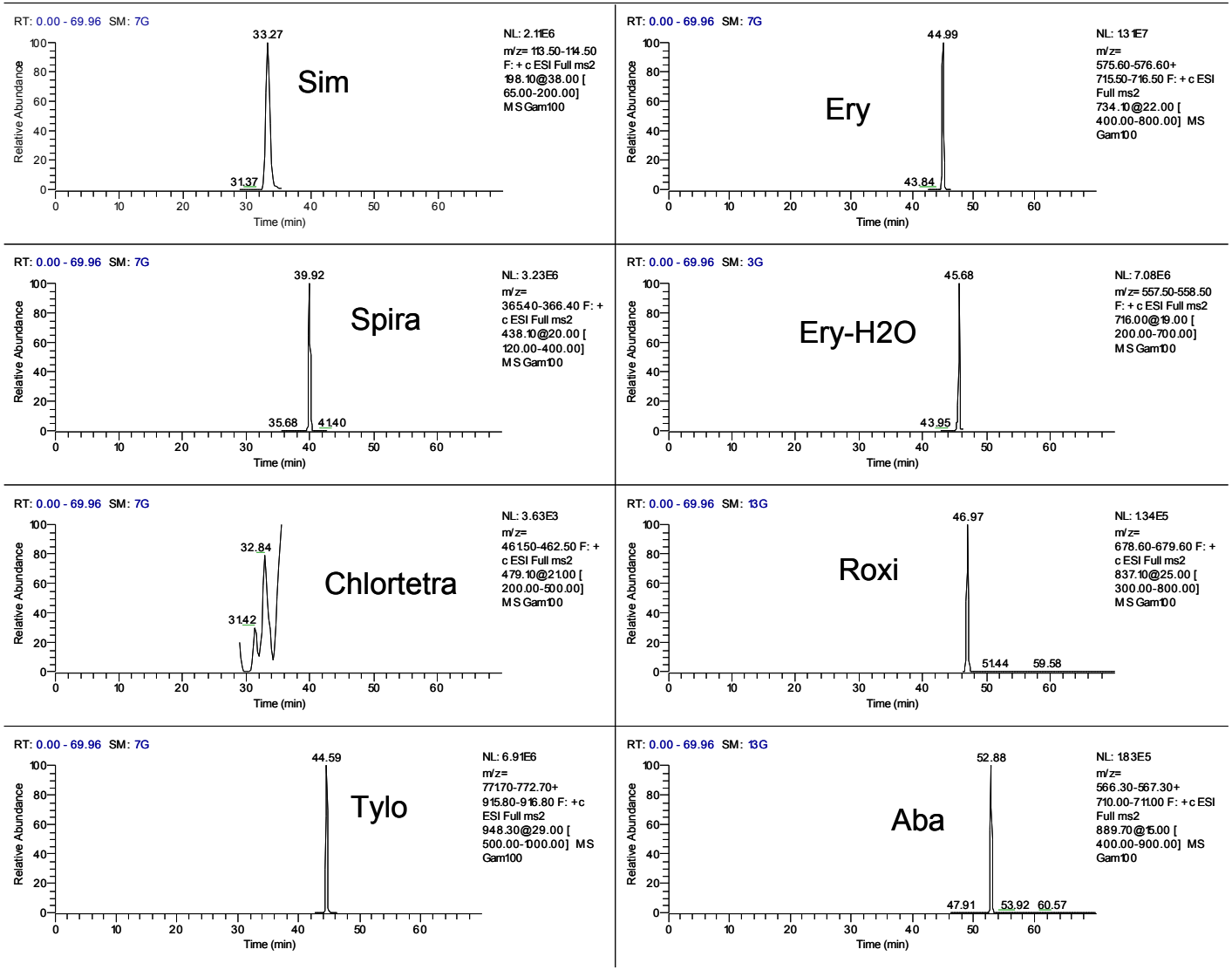
Annexe 8-b : LC-MS/MS chromatogram of solvent-base standard at 100 ng/mL for antibiotics ***Antibiotics (1)***

C:\ANTIBIOS\...\170106\Gam100 01/17/2006 05:00:49 PM



Antibiotics (2)

C:\ANTIBIOS\...\170106\Gam100 01/17/2006 05:00:49 PM



Annexe 9 : Pharmaceuticals calibration curves with recalculated values

Para

ng/mL	Gamme 1	Gamme 2	Gamme 3	Gamme 4
50	64,9	50,2	47,4	35,1
100	94,0	109,5	92,2	93,3
250	226,9	234,5	225,3	308,9
500	520,1	505,8	375,4	736,1
750	744,1	596,4	759,5	732,3

Mean	Standard deviation	RSD	Deviation with the theorical concentration
49,4	12,2	24,8%	**1,2%**
97,2	8,2	8,4%	**2,8%**
248,9	40,2	16,1%	**0,4%**
534,4	149,4	28,0%	**6,9%**
708,1	75,3	10,6%	**5,6%**

Phena

ng/mL	Gamme 1	Gamme 2	Gamme 3	Gamme 4
50	39,0	93,9	133,7	6,1
100	11,4	97,2	104,0	70,2
250	577,6	254,4	244,8	376,6
500	530,8	498,3	285,6	447,0
750	730,2	375,9	751,2	268,7

Mean	Standard deviation	RSD	Deviation with the theorical concentration
68,2	56,7	83,2%	**36,3%**
70,7	42,1	59,6%	**29,3%**
363,4	154,9	42,6%	**45,3%**
440,4	108,8	24,7%	**11,9%**
531,5	245,6	46,2%	**29,1%**

Meto

ng/mL	Gamme 1	Gamme 2	Gamme 3	Gamme 4
50	62,5	73,6	59,1	
100	102,9	253,9	246,4	56,0
250	222,9	203,6	237,2	248,9
500	511,7	526,8	281,5	616,6
750	1 220,4	746,1	753,7	678,5

Mean	Standard deviation	RSD	Deviation with the theorical concentration
65,1	7,6	11,7%	**30,1%**
164,8	100,4	61,0%	**64,8%**
228,2	19,5	8,6%	**8,7%**
484,2	142,8	29,5%	**3,2%**
849,7	249,5	29,4%	**13,3%**

SA

ng/mL	Gamme 1	Gamme 2	Gamme 3	Gamme 4
50	62,5	31,7	75,1	
100	91,9	223,2	98,5	119,2
250	240,5	301,6	216,7	168,7
500	505,1	447,9	325,6	612,6
750	904,7	768,7	759,6	699,5

Mean	Standard deviation	RSD	Deviation with the theorical concentration
56,4	22,3	39,6%	**12,9%**
133,2	61,1	45,9%	**33,2%**
231,9	55,3	23,8%	**7,3%**
472,8	119,5	25,3%	**5,4%**
783,1	86,7	11,1%	**4,4%**

Propra

ng/mL	Gamme 1	Gamme 2	Gamme 3	Gamme 4
50	50,6	47,4	47,0	52,7
100	91,3	156,7	116,9	135,2
250	262,0	304,9	297,0	259,7
500	537,5	516,4	409,7	687,8
750	722,1	720,9	732,3	741,9

Mean	Standard deviation	RSD	Deviation with the theorical concentration
49,4	2,7	5,5%	**1,1%**
125,0	27,8	22,2%	**25,0%**
280,9	23,4	8,3%	**12,4%**
537,8	114,5	21,3%	**7,6%**
729,3	9,8	1,3%	**2,8%**

Carba

ng/mL	Gamme 1	Gamme 2	Gamme 3	Gamme 4
50	50,7	41,0	40,3	43,4
100	90,0	122,7	110,8	132,2
250	261,8	276,7	299,2	254,2
500	534,6	483,0	440,8	695,4
750	724,3	559,9	772,3	744,7

Mean	Standard deviation	RSD	Deviation with the theorical concentration
43,9	4,8	10,8%	**12,3%**
113,9	18,2	16,0%	**13,9%**
273,0	19,8	7,3%	**9,2%**
538,5	111,5	20,7%	**7,7%**
700,3	95,6	13,7%	**6,6%**

Keto

ng/mL	Gamme 1	Gamme 2	Gamme 3	Gamme 4
50	46,3	33,8	44,4	43,1
100	77,2	99,2	90,2	103,6
250	247,4	286,6	273,1	255,0
500	565,2	487,0	494,9	666,6
750	710,7	747,6	747,4	748,3

Mean	Standard deviation	RSD	Deviation with the theorical concentration
41,9	5,6	13,3%	**16,2%**
92,6	11,7	12,6%	**7,4%**
265,5	17,7	6,7%	**6,2%**
553,4	83,2	15,0%	**10,7%**
738,5	18,5	2,5%	**1,5%**

Beza

ng/mL	Gamme 1	Gamme 2	Gamme 3	Gamme 4
50	58,1	46,6	54,2	60,2
100	90,6	103,7	90,4	111,1
250	201,4	255,7	239,2	221,3
500	510,2	488,8	479,9	706,3
750	760,1	755,4	768,0	757,4

Mean	Standard deviation	RSD	Deviation with the theorical concentration
54,8	6,0	10,9%	**9,6%**
98,9	10,2	10,3%	**1,1%**
229,4	23,4	10,2%	**8,2%**
546,3	107,4	19,7%	**9,3%**
760,2	5,5	0,7%	**1,4%**

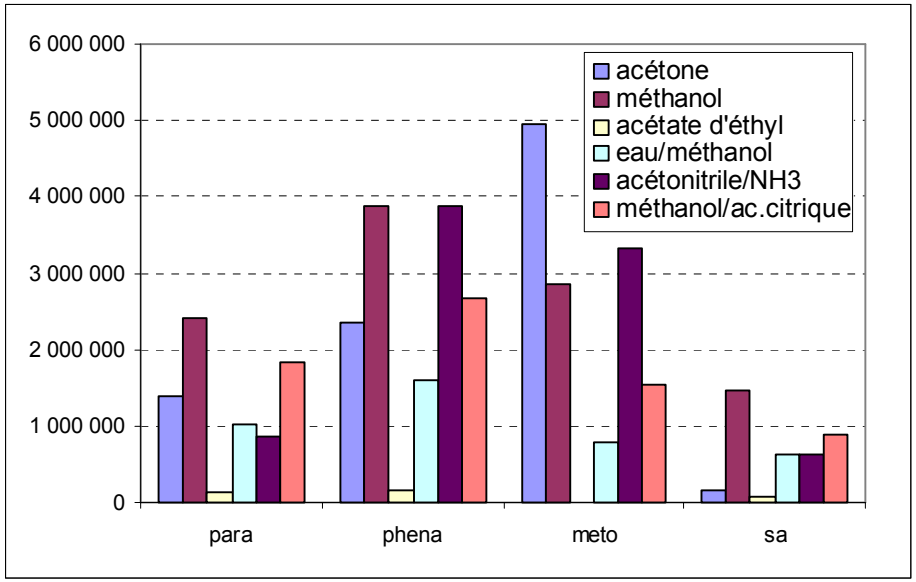
Diclo

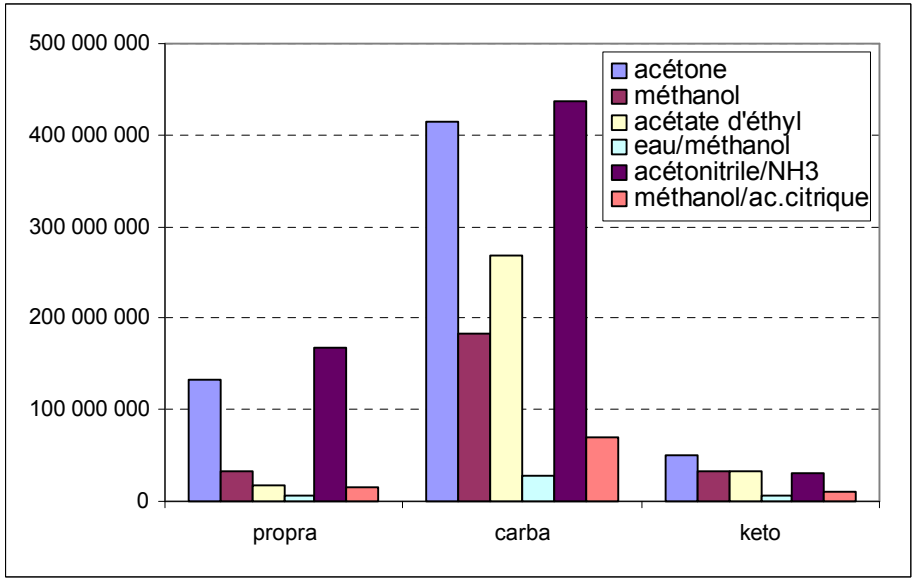
ng/mL	Gamme 1	Gamme 2	Gamme 3	Gamme 4
50	52,1	53,4	62,4	51,1
100	79,5	90,0	95,7	86,9
250	216,8	248,9	195,1	247,2
500	517,0	431,6	459,9	652,2
750	752,3	797,1	794,8	752,6

Mean	Standard deviation	RSD	Deviation with the theorical concentration
54,7	5,2	9,5%	**9,5%**
88,0	6,7	7,7%	**12,0%**
227,0	25,9	11,4%	**9,2%**
515,2	98,0	19,0%	**3,0%**
774,2	25,1	3,2%	**3,2%**

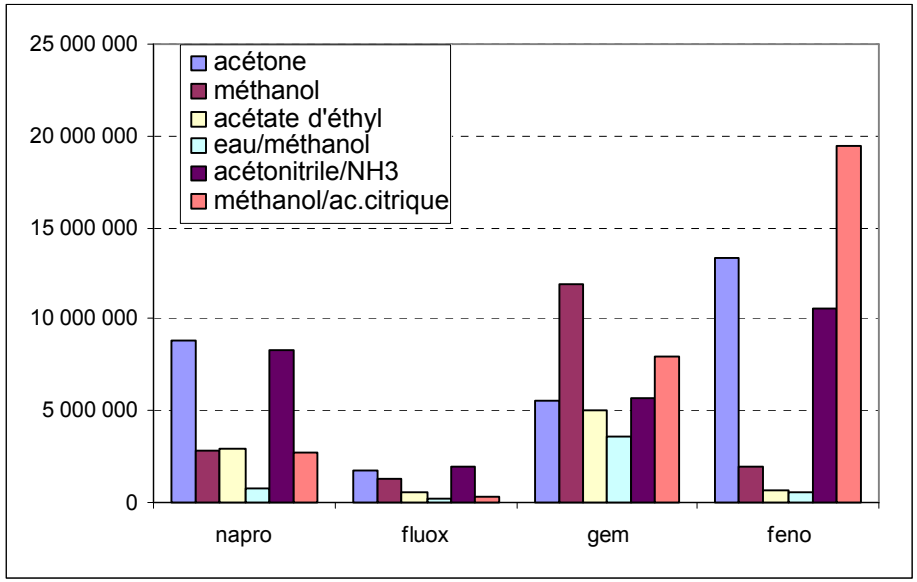
Annexe 10-a : Pharmaceuticals US extraction : comparison of six different solvents

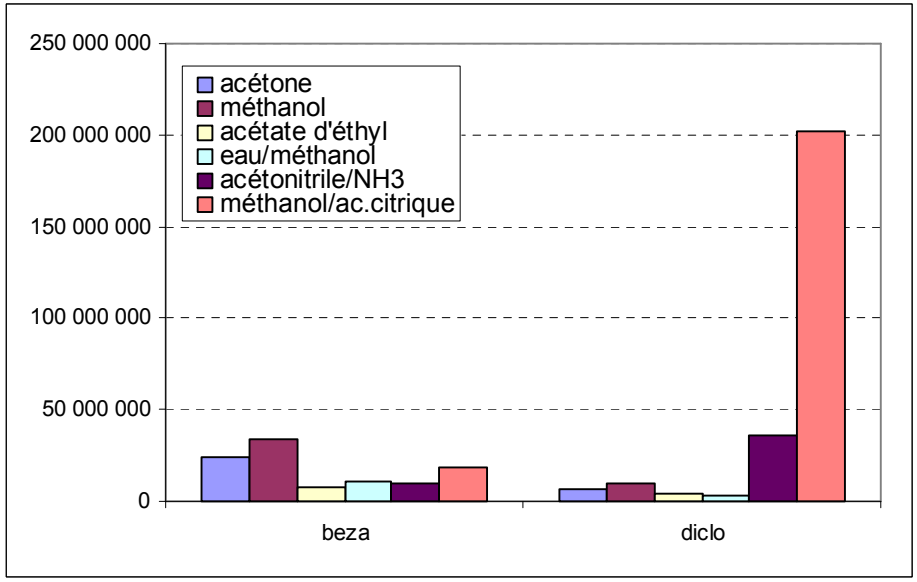
Pharmaceuticals (1)



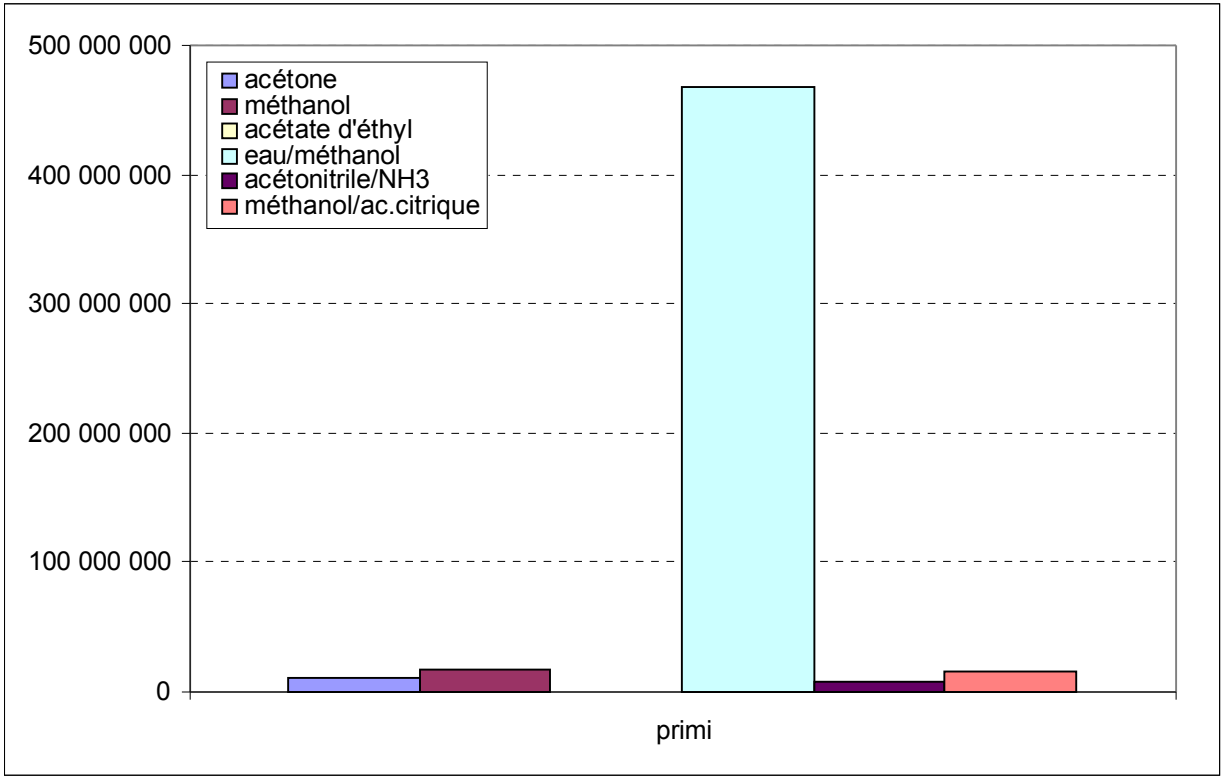


Pharmaceuticals (2)



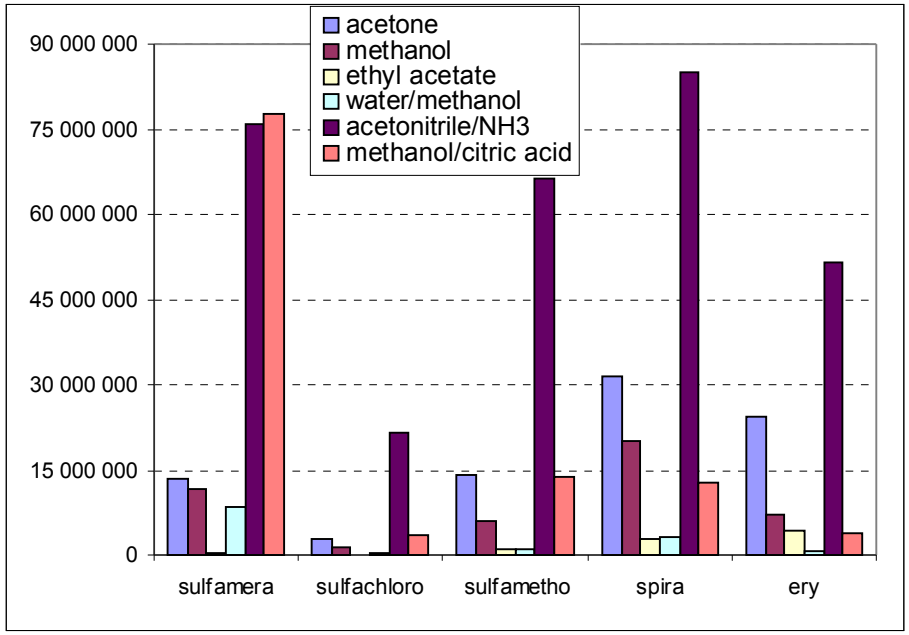


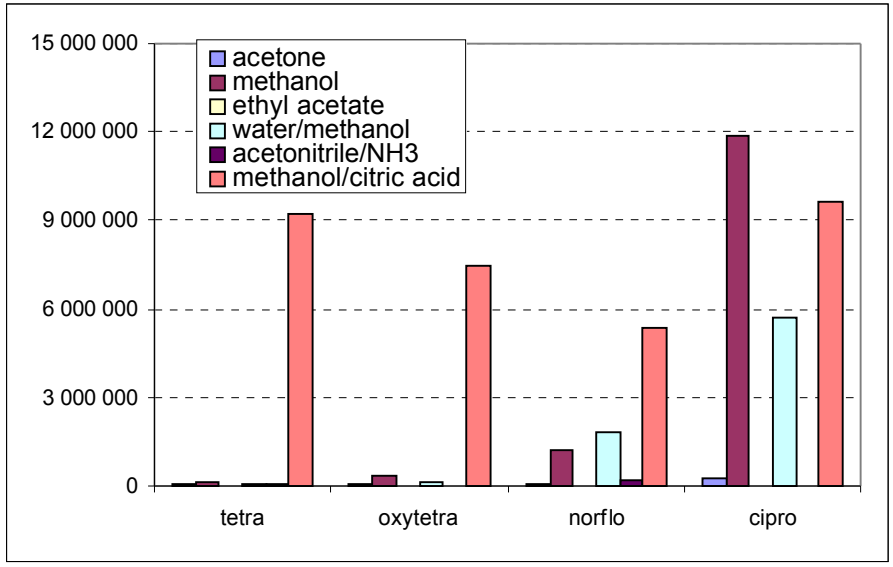
Pharmaceuticals (3)



Annexe 10-b : Antibiotics US extraction : comparison of six different solvents

Antibiotics (1)





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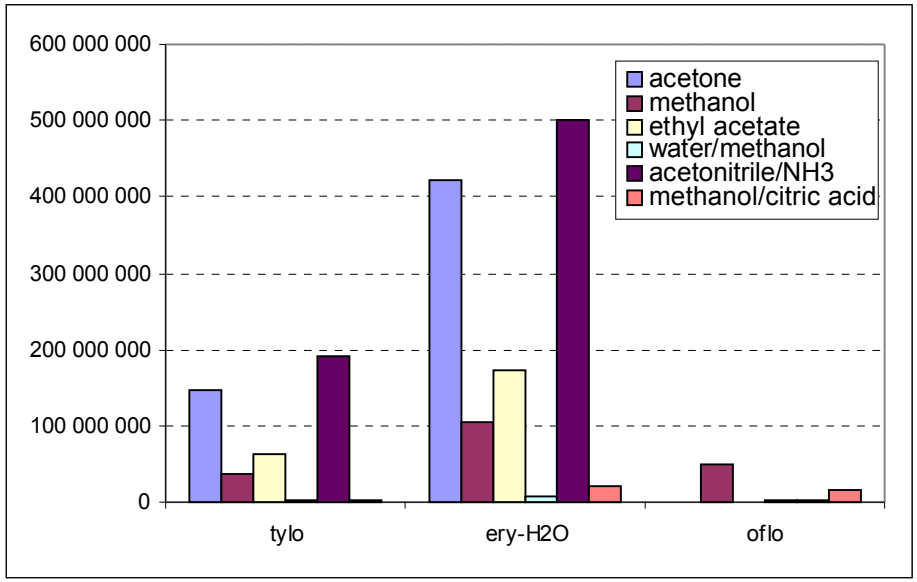
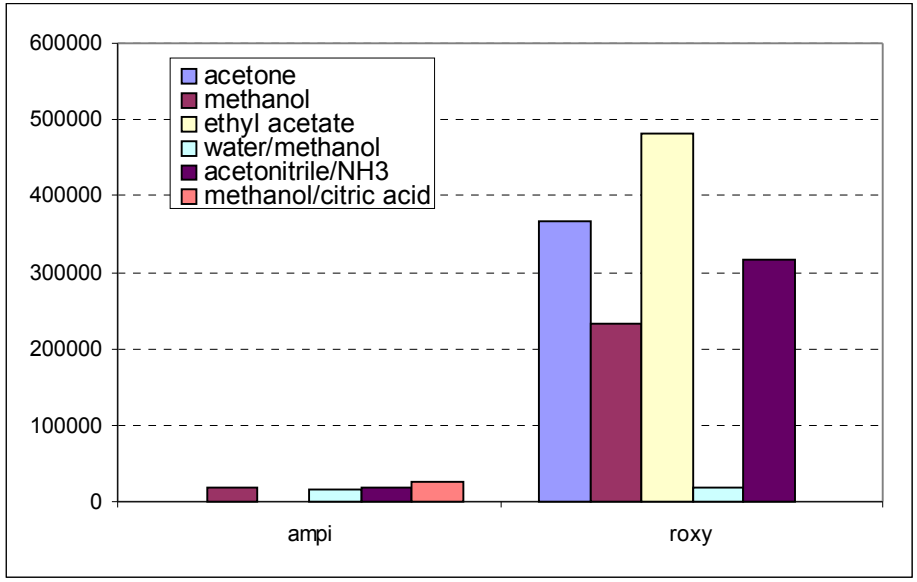
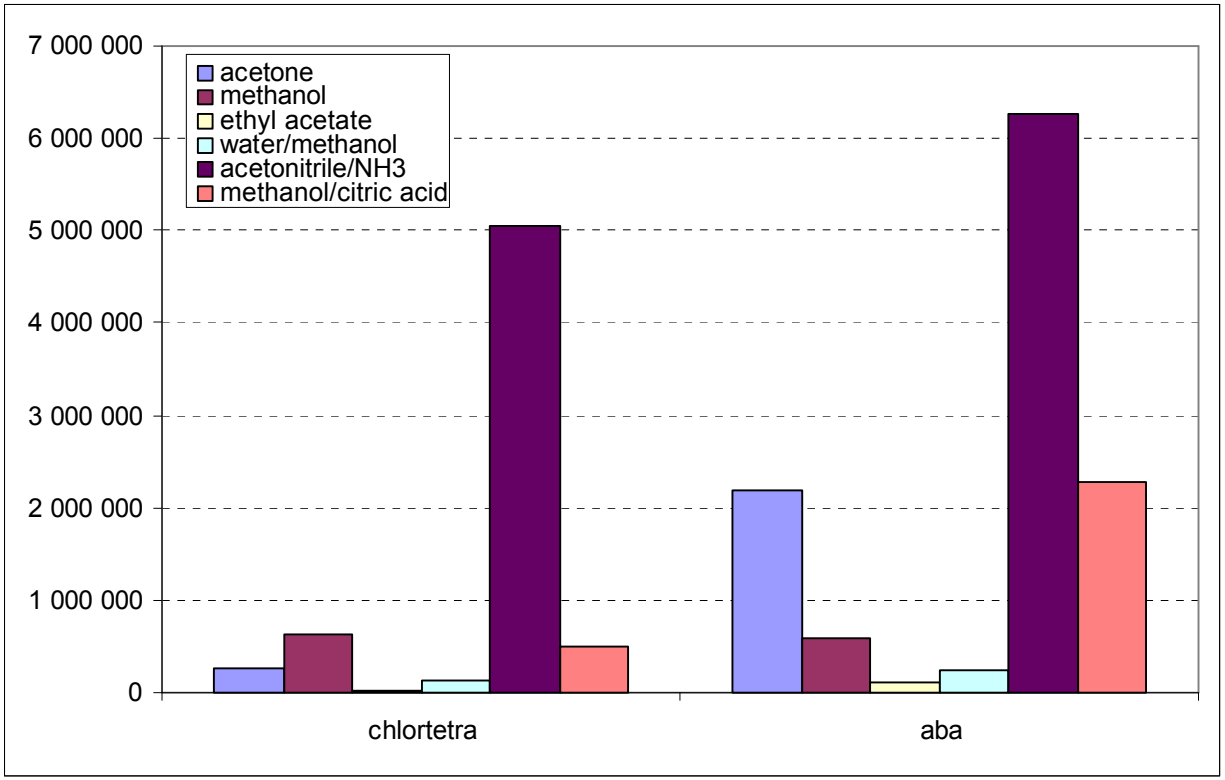
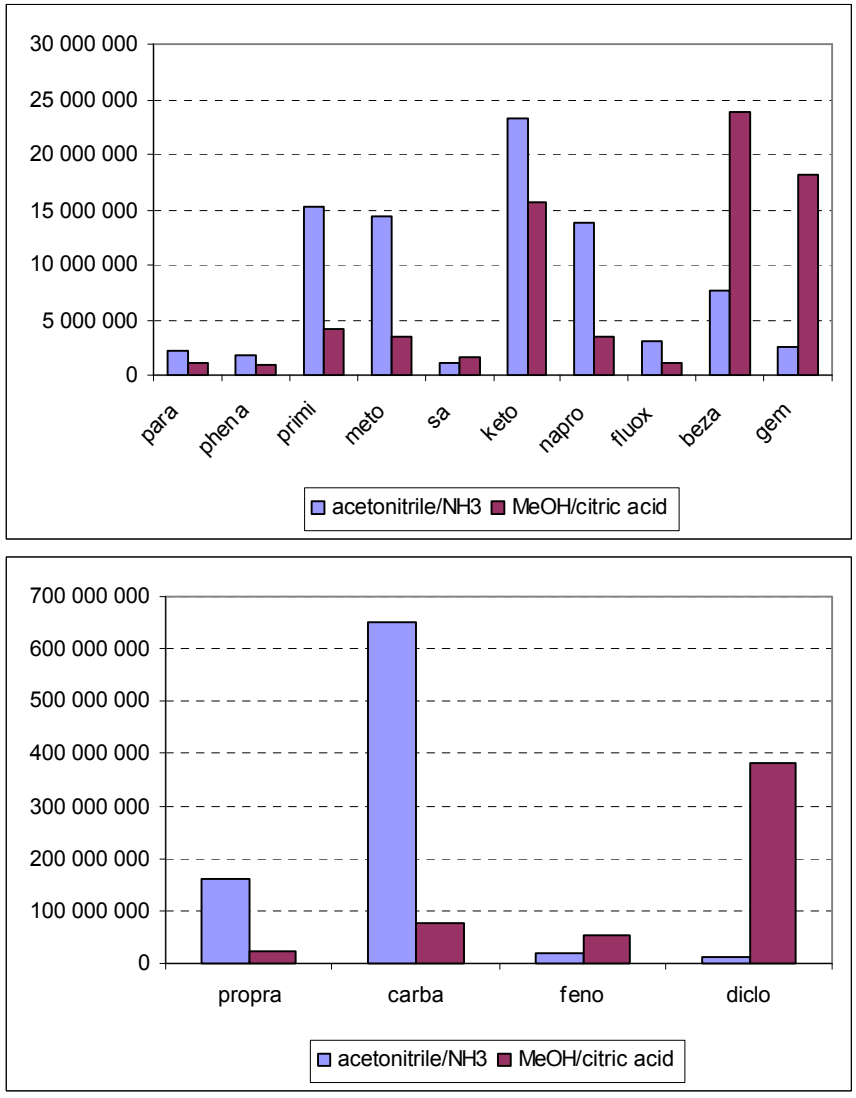




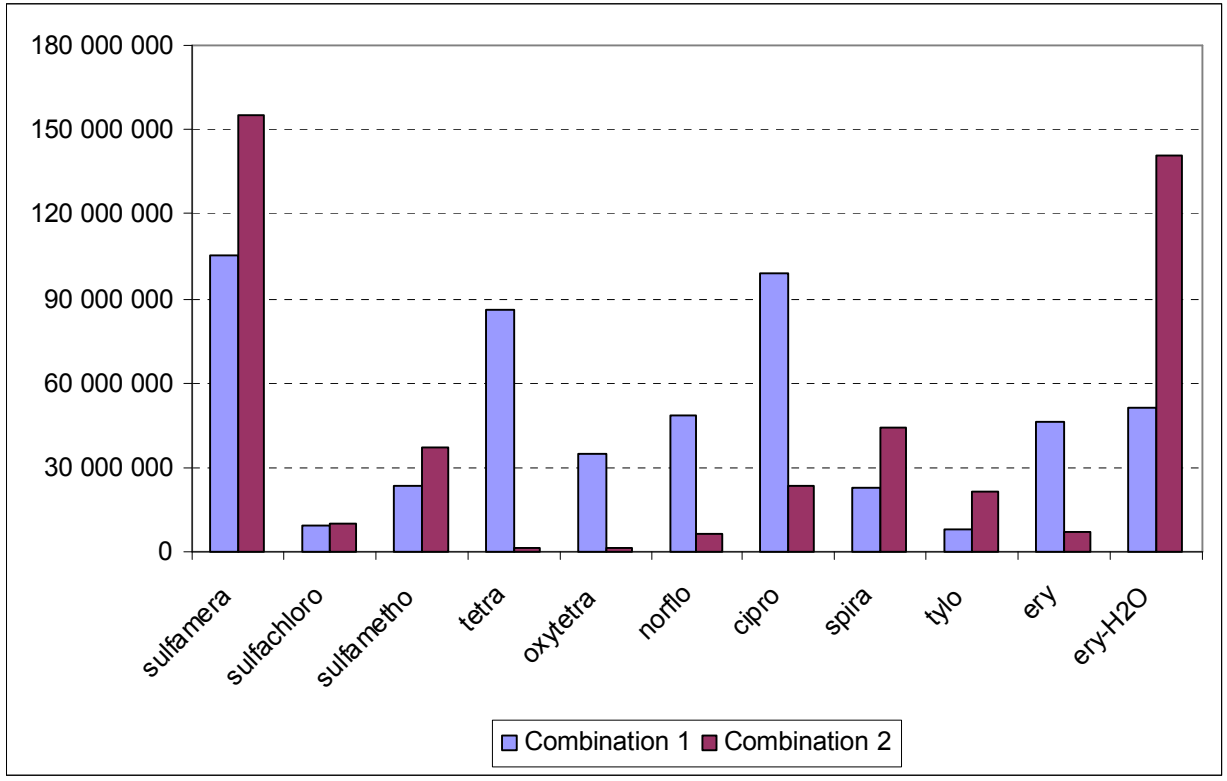
Antibiotics (3)



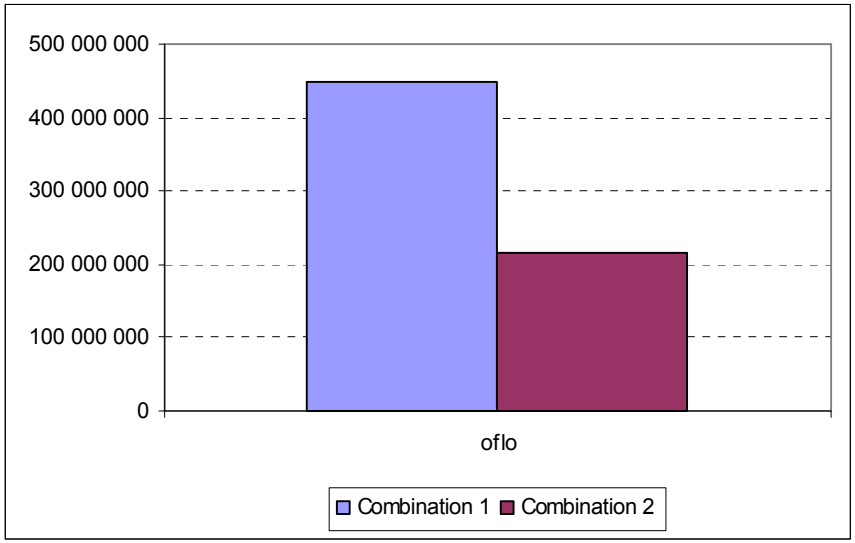
Annexe 11-a : Pharmaceuticals US extraction : comparison of two different solvents
Pharmaceuticals

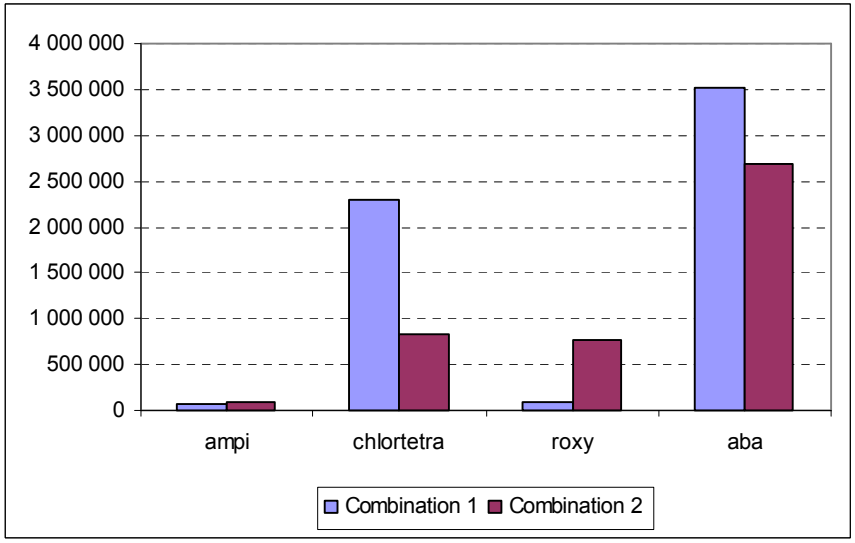


Annexe 11-b : Antibiotics US extraction : comparison of two different solvents
Antibiotics (1)

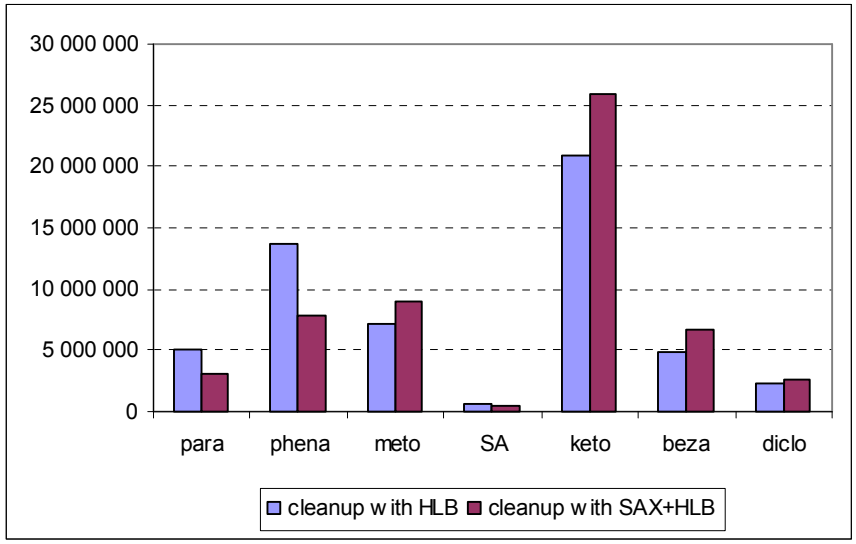


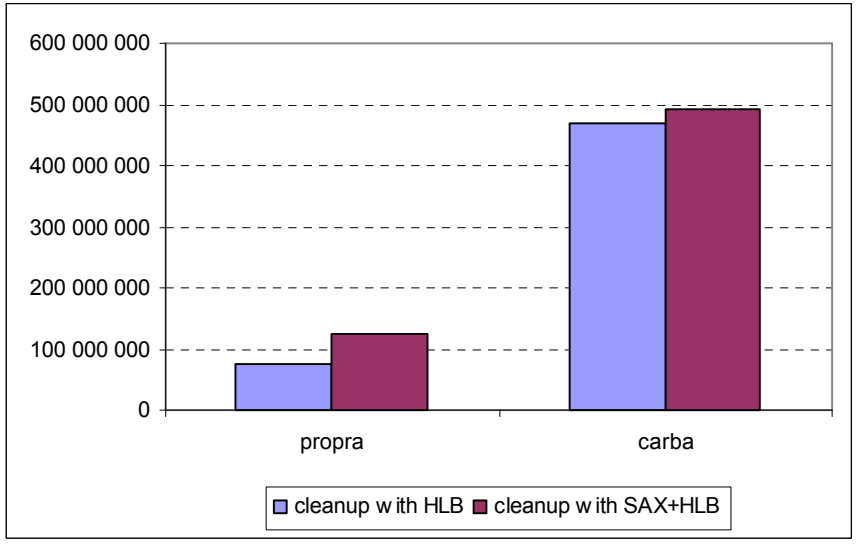
Antibiotics (2)





Annexe 12-a: Pharmaceuticals US cleanup : comparison of two different protocols of cleanup
Pharmaceuticals





Annexe 12-b: Antibiotics US cleanup : comparison of two different protocols of cleanup
Antibiotics