OPTIMIZATION AND VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF OILS AND GREASE IN WASTEWATER SAMPLES BY INFRARED SPECTROSCOPY

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BACHELOR'S THESIS IN CHEMISTRY

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1.SUMMARY

English

A bad recycling of the vegetable oils produced in the kitchen, catering, restaurants, etc. can cause a great pollution of natural areas, such as rivers, lakes, seas, etc. This pollution can derivate into problems in sanitation systems and wastewater treatment plants. For this reason, an attempt has been made to develop an analytical method for the determination of oils and grease present in these types of waters. The quantification of these substances has been done by means of FT-IR infrared spectroscopy (Nicolet iS5, ThermoFisher Scientific). After optimizing the method, which involves a liquid-liquid extraction, and carrying out a validation process for reproducibility, the results obtained were that the method could not be validated. The main reason for these results is the matrix effect of the wastewater samples used, as their composition differs from one sample to another, and not reproducible results can be obtained.

Key words: wastewater, oils and grease, liquid-liquid extraction, infrared spectroscopy, FT-IR, validation.

Catalan

Un mal reciclatge del olis vegetals emprats a la llar, l'hosteleria, restaurants, pot provocar una gran contaminació de espais naturals, com són els rius, els llacs, els mars, etc. Aquesta contaminació pot causar problemes en els sistemes de sanejament i en les depuradores d'aigües residuals. Es per això, que s'ha intentat desenvolupar un mètode analític per la determinació d'olis i greixos presents en aquestes aigües. La quantificació d'aquestes aigües s'ha dut a terme mitjançant l'espectroscòpia d'infrarojos FT-IR (Nicolet iS5, ThermoFisher Scientific). Després d'optimitzar el mètode, on s'ha realitzat un extracció líquid-líquid, i de realitzar un procés de validació per reproductibilitat, els resultats obtinguts han sigut que no s'ha pogut validar el mètode. La principal raó per justificar aquests resultats es l'efecte matriu de les mostres d'aigües residuals utilitzades, ja que la composició d'aquestes es diferent entre elles i no es poden obtenir resultats reproduïbles.

Paraules clau: aigües residuals, olis i greixos, extracció líquid-líquid, espectroscòpia d'infrarojos, FT-IR, validació.

2. GOAL

The main objective of this bachelor's thesis consists of the development of an analytical method for the extraction and determination of oils and grease (hydrocarbons) present in wastewaters samples. Inside the development of this analytical method, two main procedures will be evaluated:

1- The optimization of the experimental procedure to obtain the maximum analytes as possible.

2- A validation study is performed to check the reproducibility of the method at the concentration levels selected.

3. INTRODUCTION AND FOUNDATIONS

3.1. OILS AND GREASE IN WASTEWATER

Wastewater are impure waters arising from discharges of different origins, such as domestic, urban, and industrial. This water composition is characterized by the pollutant products that are in their origin. Nevertheless, the most common chemicals present in wastewaters are organic compounds (carbohydrates, animal fats, oils, pesticides, etc.) inorganics (chlorides, heavy metals, etc.) and gases (methane and oxygen). [1]

Oils and Grease are organic compounds made of C, H and O atoms and they are usually described as chemical water-pollutants. O&G are a combination of substances including fuels, cooking oils, motor oils, etc. Animal fats and vegetable oils are triacyclglycerides, or also known as glycerine esters, which are long hydrocarbons chains that contain also fatty acids. Then difference between oils and grease is the state of matter in which are found. Greases are differentiated form oils by the solidification degree at room temperature, where grease are found as a solid and oils as liquids. Oils and grease are mainly characterized by being immiscible with water but miscible with many non-polar organic solvents (benzene, hexane). [1,2]

Not only produce negative aesthetic effects, but oils and grease can produce an increase and, consequently, a blocking of sewage systems. Despite that issue, the existence of these problematic substances can evolve to critical damage to the aquatic life and produce ugly and disgusting slimes. [3,4]

3.2. LIQUID – LIQUID EXTRACTION

Analyte determination using any instrumental analysis requires sometimes a specific sample pre-treatment, which is usually selected by the sample nature itself and the source of infromation. Liquid-Liquid extraction is the typical and classical technique for the preparation of organic liquid samples. This separation technique involves the mixing of the most appropriate organic solvent with an equal or higher volume of an aqueous sample solution. It is crucial that the liquids used in the extraction are immiscible. The analytes of interest are transferred or extracted from the aqueous solution to the organic one due to the better and higher affinity of them with the organic layer. The choice of the organic solvent, the extraction time and the composition of the sample matrix affect directly to the selectivity and recovery of the process. When performing a liquid-liquid extraction, the most common case is to find the organic layer as the upper phase and the aqueous one on the bottom of the funnel due to the low density of organic solvents. But in the case of oils and grease determination, a halogenated organic solvent (tetrachloroethylene) is required, which presents a

higher density than water, therefore the organic layer is found under the aqueous layer instead. In *Figure 1* is shown a graphical representation of a complete liquid-liquid extraction.

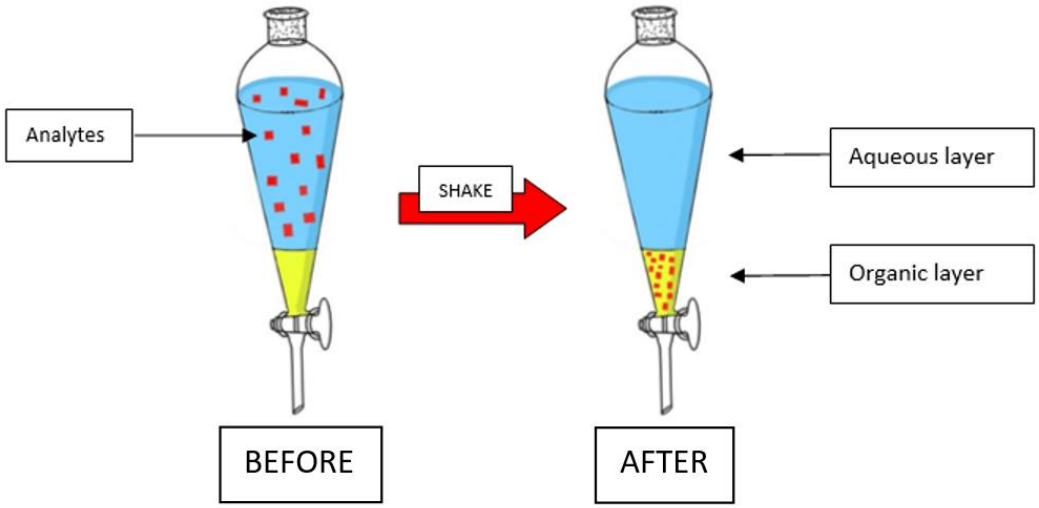


Figure 1. Liquid-Liquid Extraction diagram with a halogenated solvent.

The liquid-liquid extraction that is carried out in the determination of oils and grease uses wastewater samples. These matrices may contain a lot of different compounds, but is very common to find traces of soap, which can produce emulsions in the shaking step of the extraction.

3.3. INFRARED SPECTROSCOPY

Infrared spectroscopy is an analytical technique that allows either the qualitative or quantitative analysis of chemicals. The infrared light is irradiated towards the sample where the interaction between the sample molecules and the IR ray takes place. The different vibrational bond energies of the sample molecules allow the absorption of the infrared radiation at specific wavelengths, which produce the characteristic spectrum of the sample. Identification of molecules is based on the characteristic fingerprint region of the spectrum and the intensity and the position (frequency) of the absorption bands. The signal of these interactions can be studied by emission, reflection, and absorption. These measurements take place in the infrared region, which is the one of the electromagnetic spectrum with longer wavelength and lower frequency than the visible light region. The IR region is divided in different sections according to distance with the visible spectrum:

- FAR - IR (200-10 cm^{-1}): lying adjacent to the microwave region, the far-infrared has the lowest energy which allow the determination of lattice vibrations or the study of rotational spectroscopy.

- MIDDLE - IR (4000-200 cm^{-1}): The mid-infrared region may be used to study the fundamental vibrations which allow the determination of chemical structures. It is the most used infrared sub-region.

- NEAR – IR (12800-4000 cm^{-1}): Due to its high energy, the near infrared can excite into overtones or combination vibrations. [5]

Inside the infrared spectroscopy, the most useful and effective technique is the Fourier Transform Infrared (FTIR) spectroscopy, which is very common to use it in industrial and academic laboratories to determine the chemical structure of individual compounds or the composition of mixtures. FT-IR spectrometers offers a high number of benefits in comparison with conventional disperse spectrometers. The main difference between both spectrometers is the presence of the Michelson interferometer. This device allows the division of the infrared radiation into two different rays, which are generated by a beam splitter containing a refractive glass plate. 50% of the emitted light is reflected into a movable plane mirror, while the other 50% of light is transmitted towards a fixed plane mirror. Both beams are reflected and transmitted back to the glass plate, where a combined radiation is emitted towards the detector, passing through the sample compartment. Figure 2 represents the structure of the Michelson interferometer. [6]

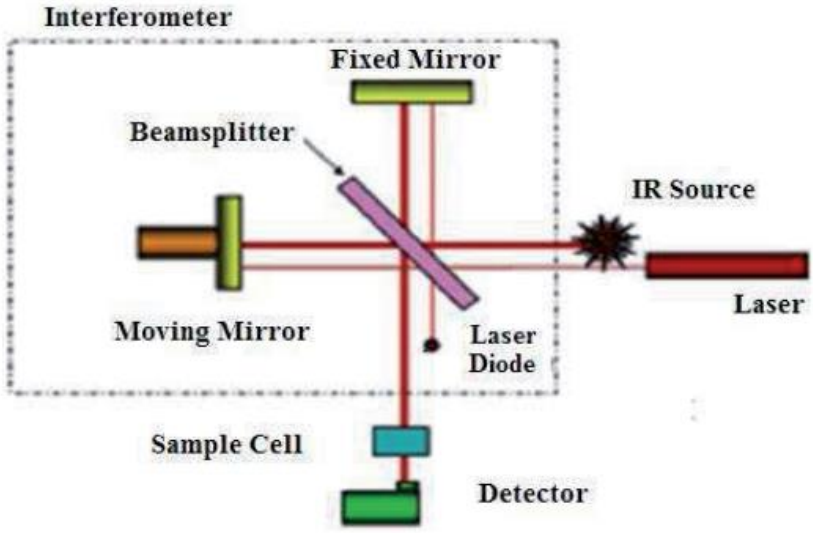


Figure 2. Michelson Interferometer diagram [6]

The signal that arrives to the detector generates the interferogram, which consist of an interference pattern. The equipment apply the Fourier transformed algorithm in order to transform the interference signal into an infrared spectrum.

Compared with the disperse spectrometer, some of the advantages that presents the FT-IR spectroscopy are the increase of the signal to noise ratio of the spectrum, the higher accuracy of the selected wavelengths, the shorter analysis time due to the high number of scans performed in a single analysis and the high resolution of the infrared spectrum. [7]

3.4. VALIDATION OF ANALYTICAL METHODS

According to "Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics"[8] and the ISO/IEC 17025:2017[9] standard (standard that specify the requirements for the competence evaluation of assay and calibration laboratories), the definition of Validation is *"confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled"*. [8] Method validation is required when it is necessary to demonstrate that the performance of the method is adequate for the specified purpose, when the method is not standardized, when it is developed by the self-laboratory and when modifying the standard method. While performing the method validation, several characteristics are usually evaluated, in which are included selectivity, LOD, LOQ, trueness (in terms of bias or recovery), precision (in terms of repeatability (single laboratory approach), intermediate repeatability or reproducibility (interlaboratory approach)), uncertainty and robustness. To validate an analytical method, the laboratory chooses which of these parameters are determined.

In the case of oils and grease determination, the quality of the results obtained from the analytical method will be studied through uncertainty, trueness, and precision. Trueness is determined through the recovery of the results and precision is determined by means of reproducibility.

- Uncertainty: The uncertainty of an analytical method is described as an interval associated to the measurement result which establish the values that can be considerably assigned to quantification. When determining the uncertainty of the method, all the components that can give uncertainty must be considered.

- Precision: It can be defined as the closeness of the results obtained in the method to each other. Typically, the precision is expressed by the variance coefficient, which is directly related to the standard deviation of the results obtained.

- Trueness: It expresses the closeness between the results obtained after applying the analysis procedure and the reference value. Method trueness can be given in terms of recoveries. [8]

4. EXPERIMENTAL PART

The experimental procedure has been extracted from an ASTM D7066-04 standard[10,11]. The main idea of the experimental part consists in the preparation of an external calibration curve through standards solutions. Those are prepared from a stock solution made of 3 different hydrocarbons dissolved in tetrachloroethylene. Then, the sample pretreatment, which consists of a liquid-liquid extraction, is optimized by analyzing several spiked wastewater samples while checking the recovery values. Finally, it is tried to validate the method in terms of reproducibility.

4.1. REAGENTS:

Table 1. Reagent's Chart

Reagent	**Chemical Formula**	**Purity (%)**	**Toxicity**	**Manipulation**
Isooctane[12]	C_8H_{18}	99.984	- Flammable - Harmful - Health Hazard - Environmental Hazard	-Lab coat, goggles, vinylic gloves. -Under the fume hood.
n-Hexadecane[13]	$C_{16}H_{34}$	99.4	- Health Hazard	-Lab coat, goggles, vinylic gloves. -Under the fume hood.
Benzene[14]	C_6H_6	99.983	- Flammable - Harmful - Health Hazard	-Lab coat, goggles, vinylic gloves. -Under the fume hood.
Tetrachloroethylene[15]	C_2Cl_4	≥99.9	- Harmful - Health Hazard - Environmental Hazard	-Lab coat, goggles, vinylic gloves. -Under the fume hood.
Hydrochloric acid[16]	HCl	37	- Harmful - Corrosive	-Lab coat, goggles, vinylic gloves. -Under the fume hood.
Sodium Sulphate anhydrous	Na_2SO_4	99.5	_	-Lab coat, goggles.
Sodium Chloride	NaCl	99.9	_	-Lab coat, goggles.

4.2. LABORAROTY MATERIAL AND EQUIPMENTS:

Laboratory Material:

- Volumetric Flasks: 10ml, 25ml, 50ml, 100ml, 200ml and 1L.
- Micropipette: 10-100µl and 100-1000µl.
- Beakers.
- Separatory Funnel 250ml.
- Conical Funnel 100mm.
- Glass Pasteur Pipettes.
- Filter Paper.
- 15ml Volumetric Pipette.
- 100ml Erlenmeyer.
- Spatula.
- Parafilm.

Equipments:

- FT-IR Nicolet iS5 with transmission accessory and DGTS detector (ThermoFischer Scientific).
- OMNIC and TQ Analyst software.
- Analytical Balance.

4.3. PREVIOUS CONSIDERATIONS:

Before starting with the experimental procedure, several aspects taken from the ASTM D7066-04 standard must be considered:

1- Tetrachlorethylene (TCE) is used as the solvent for the analytical method, and since it has carcinogen properties it is necessary to work under the fume hood and wearing nitrile gloves to avoid risks.
2- TCE is a chemical compound that gets easily contaminated by other chemicals, therefore it is preferable to work in a place where there is no contamination.
3- For the drying step, it is necessary to have dried sodium sulfate, therefore, a small amount of the anhydrous salt is heated in the oven at 100ºC for 1 hour and afterwards, it is cooled down in the desiccator until its use.
4- All the laboratory material must be pre-conditioned with the solvent and the waste TCE is thrown away in the organic solvent container.
5- It is extremely necessary that the material employed for the determination of oils and grease is glass to avoid contamination from the plastic material.
6- The cleaning of the material must be done in a specific way. The material that can be dried in the oven is cleaned with an alkaline soap. While the volumetric material (which cannot be placed in the oven) must be cleaned with a small volume of solvent followed by isopropanol, and it is dried at room temperature.
7- The stock solutions can be stored at fridge temperature for a period of one month. [17]

4.4. STOCK SOLUTION:

The preparation of the external calibration curve for the quantification of oils and grease is created through standards solutions with similar behavior and nature of the substances present in the samples. A reference stock solution is created with three hydrocarbons. These organic compounds are isooctane, n-hexadecane, and benzene.

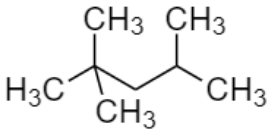


Figure 3. Isooctane

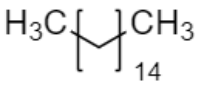


Figure 4. n-hexadecane

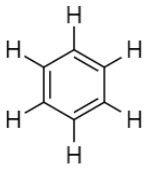


Figure 5. Benzene

These organic compounds are chemicals containing uniquely C-C (single and double) and C-H bonds; therefore, they can be used as reference oil mixture. The stock solution contains a 37,5% w/w of isooctane, 37,5% w/w of n-hexadecane and a 25% w/w of benzene. Then, it is dissolved in tetrachloroethylene.

The stock solution is prepared by weighting in a beaker each analyte in the analytical balance, then, it is introduced in the corresponding volumetric flask containing a small amount of tetrachloroethylene. The solution is mixed gently and when the three analytes are in, the volumetric flask is filled up to the mark. The stock solution contains a total hydrocarbon concentration of 100000 mg/L, approximately.

Table 2. Theoretical weights of each analyte in the stock solutions.

Stock solution	Final volume (ml)	Weight isooctane (mg)	Weight n-hexadecane (mg)	Weight benzene (mg)
1	1000	37500	37500	25000

Since the real amount of each analyte that has been weight is not exactly the ones in *Table 2*, it is necessary to recalculate the real concentration of each one, considering the purity of the substances and the real weight of the substances. To do so, the following equation is used:

$$[Analyte\ ] = \frac{P*R}{V} \qquad (1)$$

Where,

P = real weight of the analyte in mg.

R = purity of the reagent in 1%.

V = Volume in L.

Table 3 shows the real concentrations of each analyte and the total concentration of hydrocarbons in the stock solution.

Table 3. Real concentrations of the hydrocarbons in stock solution 1.

	Isooctane	n-Hexadecane	Benzene
Real weight (mg)	37,509.0	37,500.9	25,005.0
Purity (%)	99.984	99.4	99.983
Real Concentration (mg/L)	37,503.0	37,275.9	25,000.8
Total Hydrocarbon Concentration (mg/L)	99,779.6		

4.5. STANDARDS AND CALIBRATION CURVES:

As in any analytical determination, calibration curves are required to perform the quantification of the sample's concentration. It is decided to build an external calibration curve from 48 to 400 mg/L, since in this range the linearity is maintained. For the validation process, as it is wanted to study the reproducibility of the method, two calibration curves are built, one of each analyst, who will use his or her stock solution.

Five standards of different concentration are prepared to create the calibration curve. The concentrations used are: 48, 100, 250, 300 and 400 ppm. Since the volume of stock solution that must be pipetted is so small, a diluted stock solution of 5000 mg/L is prepared by adding 0,5 ml of the original stock solution into 10 ml of tetrachloroethylene. The volume of diluted stock solution (V_1) that must be added is calculated with expression (2). The values can be found in *Table 4.*

$$C_1 \times V_1 = C_2 \times V_2 \quad (2)$$

Where,

C_1 is the approximated concentration of the stock solution.

V_1 is the volume of stock solution added.

C_2 is the concentration of the standard.

V_2 is the final volume of the standard solution.

Table 4. Values of the volume added in the standards solutions.

[ ] of the standard (mg/L)	Final Volume (ml)	Added volume (µl)
400	10	800
300	10	600
250	10	500
100	25	500
48	10	96

The volumes are transferred with a micropipette in pre-conditioned volumetric flasks which are filled up to the mark with tetrachloroethylene.

4.6. SAMPLE PRESERVATION:

The ASTM D7066-4 standard is followed to obtain the preservation and the treatment of the sample. Wastewaters that are going to be used for the corresponding tests and the validation process require a specific preservation. They must be acidified at a pH of 2 or lower, using hydrochloric acid and checked with the pH-meter and conserved between 0 - 4ºC in the fridge until they are analyzed.

4.7. SAMPLE TREATMENT:

One consideration that must be taken into account before starting the pretreatment is the conditioning of the material. All the laboratory material must be cleaned with a small amount of tetrachloroethylene. The sample matrix that is being used is wastewater, which are samples containing a lot of organic, inorganic, and biological compounds. When starting with the extraction procedure, it could be seen that wastewater samples contained a lot of solid particles in suspension, as shown in *Figures 6 and 7*, so it was required to filtrate the water to obtain cleaner extractions.

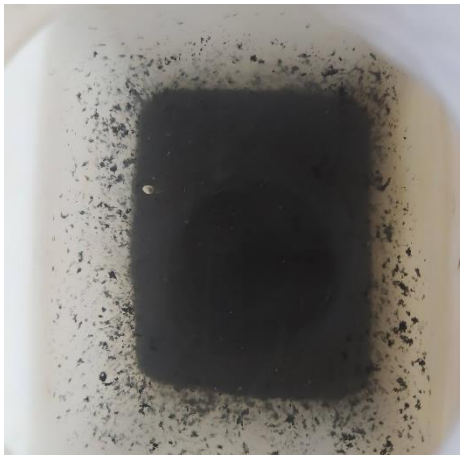


Figure 6. Wastewater samples 1

Figure 7. Wastewater sample 2

With a volumetric flask, 200mL of the filtrated wastewater sample are measured. The flask is shaken manually and introduced into a pre-conditioned 250ml separatory funnel with PTFE stopcock. With a volumetric pipette, 15 ml of solvent are measured and transferred into the 200mL flask, in order to collect the maximum amount of sample. This tetrachloroethylene is then transferred to the funnel with the aqueous phase.

Figure 8. Wastewater sample extraction containing a high volume of emulsions.

Once the two immiscible phases are together, the funnel is shaken vertically and gently for 2 minutes, regularly opening the stopcock to avoid overpressure. At that time, the two layers are allowed to separate. Since the extraction is performed with a halogenated organic solvent, which has a higher density than water, the organic phase will be found in the lower part of the funnel. When performing this step, it was possible to see that a very big emulsion appeared, therefore, it was required to optimize that technique.

Different options for reducing emulsions were tried, such as adding table salt (NaCl), acidifying the water sample with hydrochloric acid (which takes part of the sample preservation), stirring manually and with a glass rod, and letting the sample to sit up. After performing several tests, it could be seen that the most effective way to reduce the emulsion was to let the sample

sit for up to an hour while stirring gently with a glass rod the emulsion. With that step, the emulsion is not completely eliminated, but reduced quite a lot, therefore a considerable volume of organic solvent is extracted. In *Figure 9*, it can be seen how the emulsion is reduce after letting the sample sit up for 1 hour. [18]

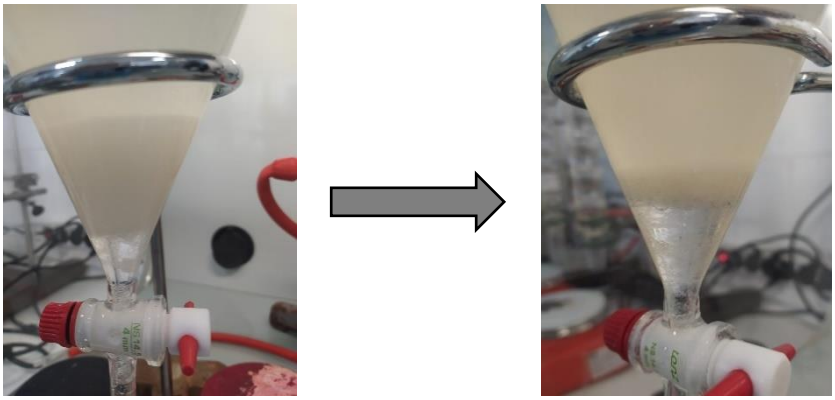


Figure 9. Evolution of the emulsions after 1 hour of letting the sample to sit up.

When the emulsion layer is reduced, the organic layer is extracted and collect in a cleaned 100ml Erlenmeyer flask. It is necessary to cover the flask with Parafilm to avoid the solvent evaporation. The extraction process is repeated two more times.

Once the three extracts of 15ml of tetrachloroethylene are recovered, a dehydrating agent is added to remove the water that may have fallen into the flask. In our case, the selected dehydrating agent is anhydrous sodium sulfate (Na_2SO_4). There is not an exact amount of sodium sulfate to be used, but it is added until the salt is not any more compacted and powder is observed, such as in *Figures 10 and 11*.

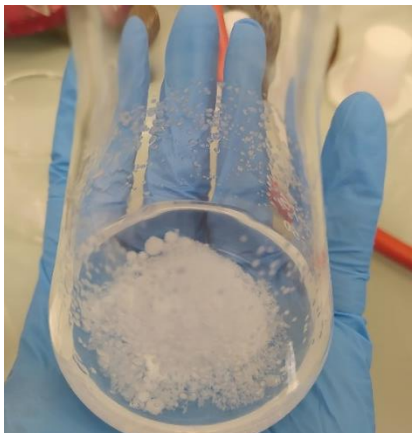


Figure 10. Drying step with NaSO4.

Figure 11. Compacted Na2SO4.

The following step consist of a gravity filtration to remove the sodium salt. A conical funnel is placed in a stand and a multi-folded filter paper is prepared. The paper is rinsed with a small volume of tetrachloroethylene and the organic phase is filtered. The filtered extract is collected in a pre-cleaned 50ml volumetric flask and filled up to the mark with tetrachloroethylene.

4.8. SPIKED SOLUTIONS

After optimizing the sample treatment, several spiked solutions containing the high, medium, and low ranges of the calibration curve (360, 200 and 48 mg/L of oils and grease) were tested to check how was the recovery of the method. According to the ASTM standard, recoveries around 63-100% were obtained for the three levels.[10] In our case, for the lower-level, it was possible to recovered only a 54% of the analytes added, while for the medium and higher-levels recoveries larger than 80% were observed. From that analysis, it is decided to validate the method just for the medium and high concentration levels, due to the recovery values.

4.9. INFRARED SPECTROSCOPY:

As mentioned before, oils and grease are hydrocarbons constituted only be carbon and hydrogen, consequently, these molecules only absorb the infrared radiation at this bond energies where the vibration take place. The infrared bands of interest are the ones corresponding to the stretching of the C-H bonds of the alkane and aromatic compounds. The aromatic C-H bonds are seen at frequency values around 3100-3000 cm^{-1} while the aliphatic C-H bonds are observed between 2950-2800 cm^{-1}. According to this information, only the region of frequencies from 3200 cm^{-1} to 2600 cm^{-1} from all the Middle infrared spectrum is collected.

Figure 12. FT-IR Nicolet iS5 with transmittance accessory

The collection of the standards and samples spectra is performed by means of mid-infrared spectroscopy, specifically using the equipment FT-IR Nicolet iS5 with a DTGS (deuterated triglycine sulfate) detector and a transmittance accessory[19]. The OMNIC software is used to obtain the spectra of the solutions and the TQ Analyst is used to build the calibration curve.

One-centimeter optical pathlength Quart-IR cuvette is used to analyze the different solutions. Before starting with the collection of the data, several parameters related to the FT-IR software (OMNIC) must be specified:

<u>OMNIC parameters:</u>

# of scans = 32

Background Handling = After 1000 min

Analysis time = 47 s

Gain = Auto gain

Resolution = 4 cm^{-1}

Spectral Range = 3200-2600 cm^{-1}

Firstly, it is extremely necessary to be sure that the cuvette is correctly cleaned and dried, due to it can affect directly to the results. Moreover, as the solvent evaporates easily, the cover of the transmittance accessory must be open, therefore the atmosphere is homogenized during the analysis.

Then, a background spectrum is collected by measuring pure tetrachloroethylene. After that, the same solvent is analyzed again, but now it is collected as a sample spectrum, specifically as a standard 0, instead of a background. With that data collection, it is possible to see the quality of the solvent.

The standards solutions are analyzed in increasing order of concentration by cleaning the cuvette twice with the solution to be analyzed. Each standard solution is collected four times without moving or changing the standard in the cuvette. The procedure for the sample solutions is the same as for the standards.

With the collection of all the spectra of the standard solutions, it is obtained the calibration curve using TQ Analyst. The 20 spectra (4 for each standard) are uploaded to the software, and the value of the theoretical concentration must be specified. The calibration curve is built by the real concentration (equipment lecture) respect the theoretical concentration.

4.10. VALIDATION STUDY:

When studying and developing an analytical method, the results obtained by a laboratory need to provide a certain level of validity. Validation is a testing method which needs to confirm that the developed method behaves in a correct and adequate way along the different concentration ranges of the method and the type of matrices.

In the case of Oils and Grease, the validation is carried out by means of reproducibility, which involves reproducing the whole method process changing the analysts, the analysis dates, the concentration levels, the sample matrix, etc. The validation process is based on the repetition of the optimized analytical method for each concentration range for 10 days. In *Table 5* appears the variables along the validation process.

Table 5. Reproducibility variables

Analysts	1	2
Concentration range	High (360 mg/L)	Medium (180 mg/L)
Analysis Date	02/05/2022	13/05/2022
Calibration curve	Analyst 1	Analyst 2
Matrix	Wastewater	

In order to know if the method is valid, the parameters that are going to be determined are trueness, precision, and uncertainty. According to the ASTM D7066-04 standard, where an interlaboratory study is performed, the reference values of these parameters are 31% for precision, 20% for trueness and 35% for uncertainty for both concentration levels.

Since the reproducibility of the method is tested by different analysts, two different calibration curves are required. The calibration curve of analyst 2 is done in the same way as the one of analyst 1 but changing the reference stock solution. Due to the short stability and the small volume used for standards and samples, the final volume of the reference stock solution #2 has been reduced to 50 ml instead of 1L, in order to save chemicals.

Table 6 shows the amount of each analyte that has been used for this stock solution and the final concentration of hydrocarbons, by using equation 1.

Table 6. Data of stock solution #2

	Isooctane	n-Hexadecane	Benzene
Real weight (g)	1.8782	1.8775	1.2575
Purity (%)	99,984	99,4	99,98
Real Concentration (mg/L)	37,558.0	37,324.7	25,145.7
Total Hydrocarbon Concentration (mg/L)	100,028.4		

The calibration curve of analyst 2 is performed in the same way as analyst 1 and this new stock solution is used for the validation process in all the wastewater samples.

Before starting with the validation, it is necessary to calculate the volume of stock solution that is going to be added in the spiked samples, considering the dilution factor between the aqueous and organic layers.

- HIGH RANGE: The total hydrocarbons concentration selected to study the high concentration level is 360 mg/L.

$$50\ ml\ org.phase\ \times \frac{360\ mg\ hydrocarbons}{1000\ ml\ org.phase} \times \frac{1}{0{,}2\ L\ aq.phase} = 90 \frac{mg}{L}\ aq.phase$$

$$200\ mL\ aq\ phase \times \frac{90\ mg\ hydrocarbons}{1000\ ml\ aq\ phase} \times \frac{1000\ ml\ stock\ sol.}{100{,}000\ mg\ hydrocarbons} = 0{,}18\ mL = 180\ \mu L\ of\ stock\ sol.$$

To obtain 360 ppm of oils after the liquid-liquid extraction, 180 µL of stock solution is added to the wastewater sample.

- MEDIUM RANGE: The total hydrocarbons concentration selected to study the medium concentration level is 180 mg/L.

$$50\ ml\ org.phase\ \times \frac{180\ mg\ hydrocarbons}{1000\ ml\ org.phase} \times \frac{1}{0{,}2\ L\ aq.phase} = 45 \frac{mg}{L}\ aq.phase$$

$$200\ mL\ aq\ phase \times \frac{45\ mg\ hydrocarbons}{1000\ ml\ aq\ phase} \times \frac{1000\ ml\ stock\ sol.}{100{,}000\ mg\ hydrocarbons} = 0{,}09\ mL = 90\ \mu L\ of\ stock\ sol.$$

To obtain 180 ppm of oils after the liquid-liquid extraction, 90 µL of stock solution is added to the wastewater sample.

Since the low level is excluded, 2 samples per day will be analyzed, including the analysis of the blank solutions. The planning for the validation study is the following:

Table 7. Validation planning.

DAY	ANALYST	SAMPLE 1	SAMPLE 2	DAY	ANALYST	SAMPLE 1	SAMPLE 2
1	1	HIGH	MEDIUM	6	1	HIGH	MEDIUM
2	2	HIGH	MEDIUM	7	2	HIGH	MEDIUM
3	1	HIGH	MEDIUM	8	1	HIGH	MEDIUM
4	2	HIGH	MEDIUM	9	2	HIGH	MEDIUM
5	1	HIGH	MEDIUM	10	1	HIGH	MEDIUM

On days when the type of wastewater is changed, it is necessary to carry out a sample blank, in which the hydrocarbon standard is not added. To be considered valid, the blank must not contain more than one third of the addition, i.e., it must not contain more than 66.67 mg/l of oils and fats in the organic phase for the medium range.

5. RESULTS AND DISCUSSION

In this section, it can be found the results obtained by applying the optimized method during the validation dates and the necessary calculations to check if the method is valid in terms of reproducibility. In addition, the creation of the calibration line, with its corresponding validation, is discussed.

5.1. BLANK SOLUTION:

Before starting with the collection of the standard/sample solutions with the software OMNIC, a tetrachloroethylene background spectrum is required. This spectrum is used to subtract the solvent of the sample solution.

The spectrum of tetrachloroethylene is collected as a standard 0 in order to check the purity of the solvent. This spectrum can be seen in *Figure 13.*

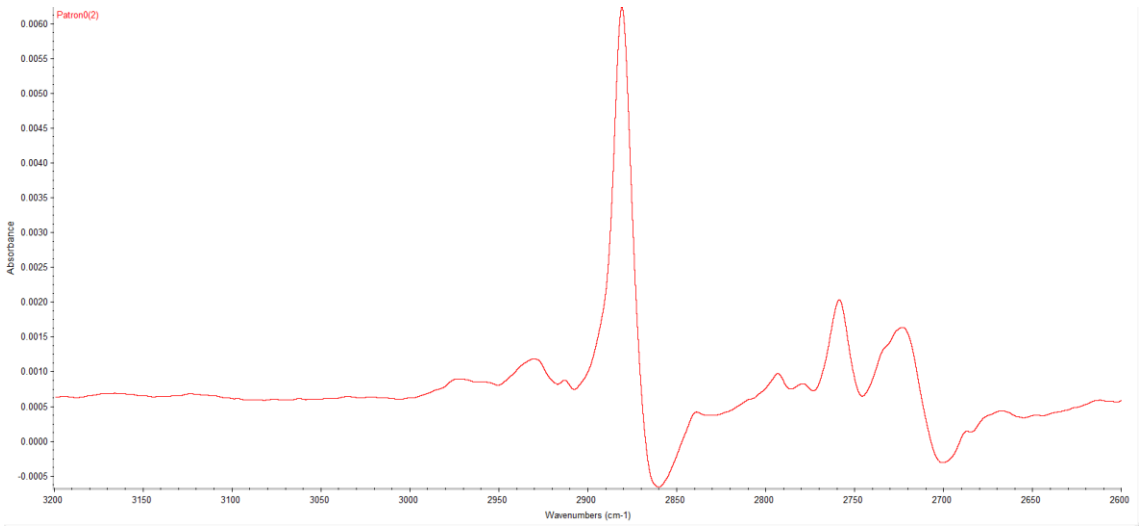


Figure 13. Spectrum of tetrachloroethylene

As a background of the same tetrachloroethylene has been made, the expected spectrum should give no signal/absorbance. On the contrary, as it can be seen in *Figure 13*, the used tetrachloroethylene presents a weak band around 2875 cm^{-1}. The main reason of the presence of this peak is due to tetrachloroethylene is not 100% pure. By checking the solvent certificate, the purity of TCE is 99.93%, therefore, this band could correspond to solvent impurities.

5.2. CALIBRATION CURVE:

With a background spectrum of the solvent, the five standards (48, 100, 250, 300 and 400 ppm) are analyzed in the FT-IR equipment. In *Figure 14*, it can be seen different spectra corresponding to one repetition of each standard solution belonging to the calibration curve of Analyst 1.

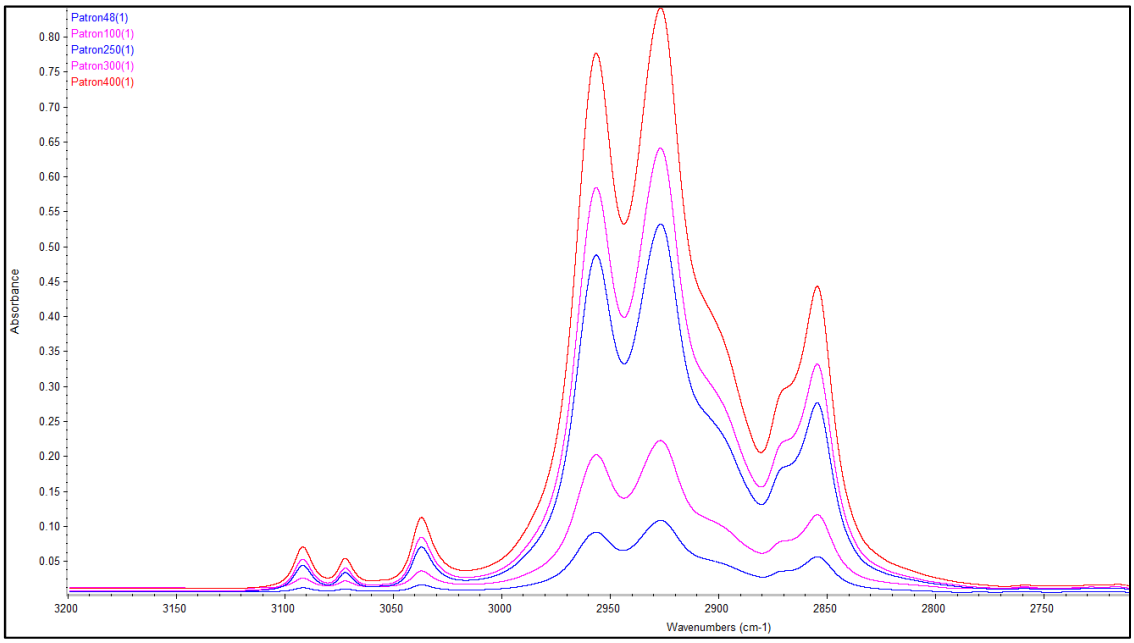


Figure 14. Spectra of the calibration curve of analyst 1.

The three peaks that appear around 3100-3000cm^{-1} belong to the aromatic C-H bonds of benzene while the remaining bands correspond to the aliphatic C-H bonds of isooctane and n-hexadecane.

Once the spectra are collected with the FT-IR equipment with the transmission accessory, the real concentration of each standard must be calculated before constructing the calibration curve. It must be considered the real concentration of the stock solution #1 and expression 3.

$$C_{2,theor} = \frac{C_{1,theor} \times V_1}{V_2} \quad (3)$$

Where,

$C_{1,\,theo}$ is the real concentration of stock solution

$C_{2,\,theo}$ is the real concentration of standard solution

V_1 is the volume of the stock solution added

V_2 is the volume of the volumetric flask

The real values of the standards are shown in *Table 8.*

Table 8. Theoretical concentration of hydrocarbons in the standards solutions.

	Approximated [ ] (mg/L)	**Theoretical [ ] (mg/L)**
Stock solution	100000	99778.89
Intermediate solution	5000	4988.94
Standard 1	48	47.89
Standard 2	100	99.78
Standard 3	250	249.45
Standard 4	300	299.34
Standard 5	400	399.12

Once the standards spectra are collected and their real concentrations are determined, the calibration curve for the quantification of oils and grease can be created with the TQ Analyst software. Each repetition of the standard solutions (in total, 20 spectra) are uploaded to the program considering the band that appear in the blank spectrum. Due to this peak can interfere in the calibration curve, the spectral region for the calibration must be divided. The two regions that are selected can be seen in *Figure 15.*

Index	Region Type	Location	% or Ht	Baseline Type	Point 1	Point 2
1	Area	3,111.00		Two points	3,111.00	2,771.00
		2,896.00		Fixed location		
2	Area	2,865.00		Two points	3,111.10	2,771.00
		2,771.00		Fixed location		

Figure 15. Region data for the calibration curve.

With these parameters selected, the calibration curve is generated with the classical least square method, which is based on the Lambert-Beer's method. The law states that the concentration of a solution is directly proportional to the attenuation of the light in the path-length established, in other words, it is directly related to the absorbance (signal). The software itself takes the absorbance as area of the spectral range selected and by means of internal calculations of the program, it returns a calibration line representing the estimated concentration (calculated) with respect to the theoretical concentration of the standard (actual).

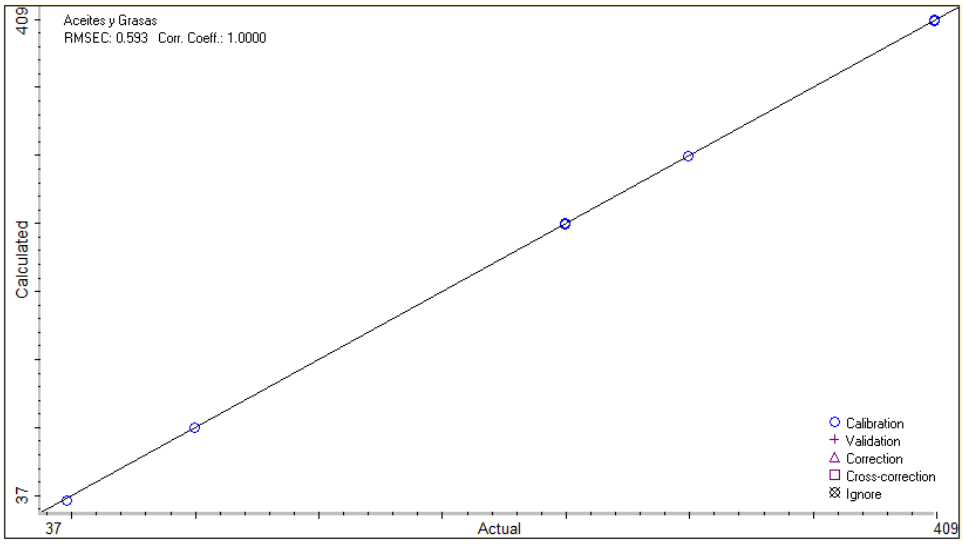


Figure 16. External calibration curve

Figure 16 shows the graphical representation of the calibration curve. It is plotted the real concentration against the expected concentration. Two parameters need to be evaluated to know if the calibration curve created is valid for the determination of oils and grease on real samples. These parameters are the determination coefficient (R^2) and the standard deviation of the standard solutions.

The R^2 coefficient, which evaluates the adjustment to the lineal model proposed, is given by the software, and it has a value of 1 or very close to 1 (>0.99999) due to the program only gives 4 decimals. To validate the calibration curve, the value of this coefficient must be greater than 0,99. In this case, the curve is valid by checking the coefficient of determination. In addition to this, the residuals and the standard deviation of each pattern are also studied. The standard deviation of each solution is calculated with expressions 4 and 5:

$$Residual = [\,]_{expected} - [\,]_{real} \quad (4)$$

$$\%\ Deviation = \left|\frac{Residual}{[\,]_{real}}\right| \times 100 \quad (5)$$

Table 9. Results of the standards solutions of the calibration curve.

	[ ] expected	[ ] real	Residual	% Deviation
Standard 1	47.89	46.33	1.560	3.367
Standard 2	99.78	99.69	0.095	0.095
Standard 3	249.45	249.52	0.115	0.046
Standard 4	299.34	299.37	0.230	0.077
Standard 5	399.12	399.12	0.245	0.061

By the internal convention of the laboratory, the maximum values permitted for the standard deviations are:

- 15% → For the limit of quantification of the curve.
- 10% → For the remaining points of the curve.

As it can be seen, all the standards behave correctly, and the values of the standard deviations do not exceed the maximum values allowed. Therefore, this calibration curve is valid for the analysis method.

The validation process is performed by creating two calibration curves, one for each analyst. The discussion of the calibration curve of analyst 2 can be found in the ANNEX section.

5.3. VALIDATION

For the validation of the method, different wastewater matrices from different sites have been used. The analytical method developed and optimized in section 4 is used on these samples and analyzed in the FT-IR apparatus. The spectra obtained are directly quantified in the OMNIC software by selecting the corresponding calibration line. Samples made by analyst 1 are quantified with the calibration line of analyst 1 and samples made by analyst 2, with his calibration line.

In order to perform the relevant validation calculations, it is necessary to calculate the actual concentration that has been added to the wastewater taking into account the actual concentration of the stock solution and the dilution factor.

- HIGH LEVEL: It has been added 0,18 ml of stock solution into a 200ml of wastewater sample, which after the extraction is concentrated 4 times.

$$0.18 \text{ mL stock sol.} \times \frac{100{,}028.4 \text{ mg hydrocarbons}}{1000ml\ stock\ sol.} \times \frac{1}{0{,}2\ L\ final\ volume} = 90{,}0256 \frac{mg}{L}\ aq.phase$$

$$200ml\ aq.phase \times \frac{90.0256\ mg}{1000ml} \times \frac{1}{0{,}05\ L\ final\ volume} = \mathbf{360.1022 \frac{mg}{L}\ organic\ phase}$$

- MEDIUM LEVEL: It has been added 0,09 ml of stock solution into a 200ml of wastewater sample, which after the extraction is concentrated 4 times.

$$0.09 \text{ mL stock sol.} \times \frac{100{,}028.4 \text{ mg hydrocarbons}}{1000ml\ stock\ sol.} \times \frac{1}{0.2\ L\ final\ volume} = 45{,}0128 \frac{mg}{L}\ aq.phase$$

$$200ml\ aq.phase \times \frac{45.0128\ mg}{1000ml} \times \frac{1}{0.05\ L\ final\ volume} = \mathbf{180.0511 \frac{mg}{L}\ organic\ phase}$$

Some of the spectra that belong to the blank samples are shown in *Figure 17*. The wastewater sample analyzed on day 7 had a high content of oils and grease with respect to the other blanks, but it does not overpass 1/3 of the addition concentration.

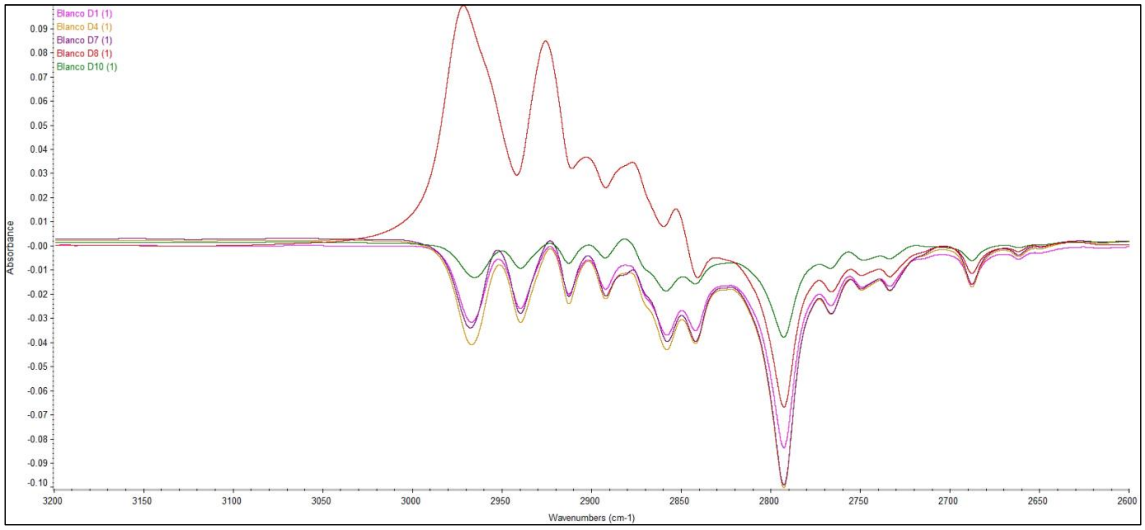


Figure 17. Blank sample spectra.

The spectra and the results related to the analysis of the spiked solution with the medium and high concentration ranges are shown below. The different samples are quantified through OMNIC software, and the results obtained for each range are shown in *Table 10* and *Table 11*. The recovery values are calculated with the following expression:

$$R\ (\%) = \frac{Sample\ response - Blank\ response}{Theroetical\ concentration} \times 100 \qquad (6)$$

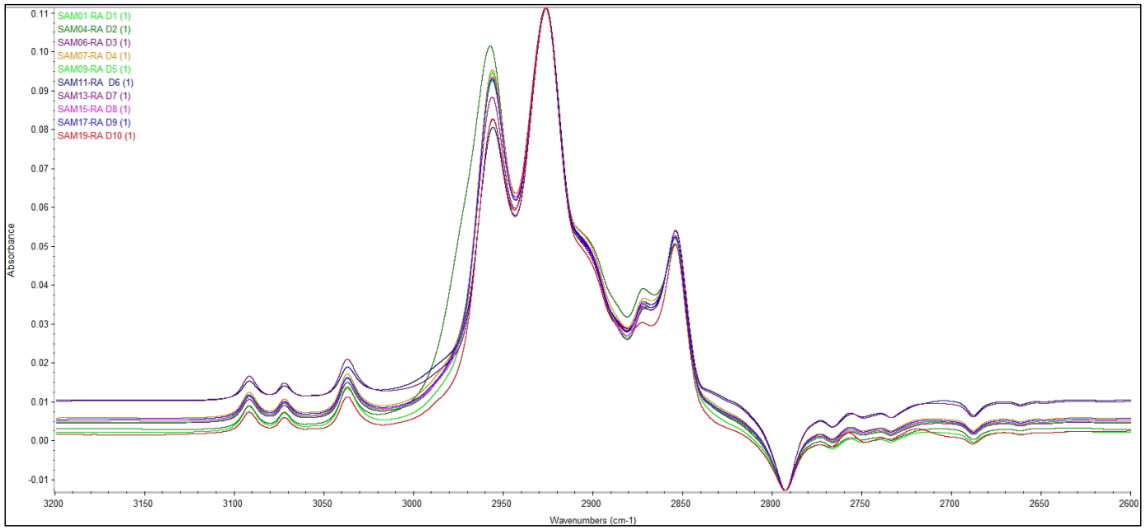


Figure 18. Validation spectra of the high concentration spiked solutions.

Table 10. Validation results of the high concentration level.

SAMPLE	ANALYST	BLANK RESPONSE (mg/L)	SAMPLE RESPONSE (mg/L)	RECOVERY (%)
1	1	0	206.45	57
2	2	0	305.94	85
3	1	0	258.81	72
4	2	0	227.12	63
5	1	0	248.22	69
6	2	0	118.00	33
7	1	0	185.42	52
8	2	54.59	255.97	56
9	1	54.59	305.75	70
10	2	0	45.80	13

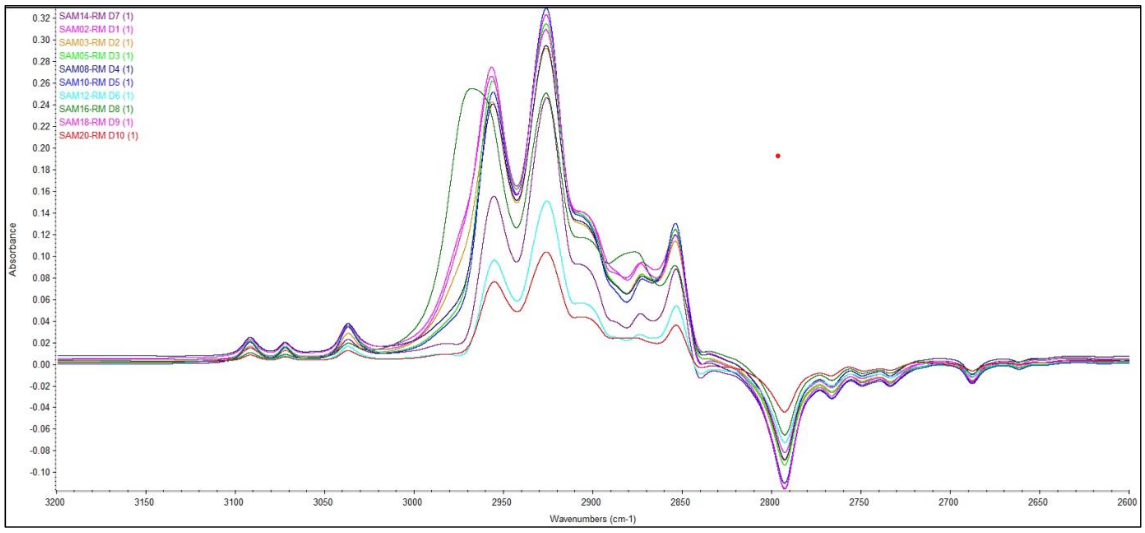


Figure 19. Validation spectra of the spiked solution with the medium concentration level.

Table 11. Validation results of the medium concentration range.

SAMPLE	ANALYST	BLANK RESPONSE (mg/L)	SAMPLE RESPONSE (mg/L)	RECOVERY (%)
1	1	0	137.83	77
2	2	0	125.12	70
3	1	0	128.52	71
4	2	0	116.79	65
5	1	0	129.32	72
6	2	0	57.60	32
7	1	0	95.95	53
8	2	54.59	146.49	51
9	1	0	155.68	87
10	2	0	42.27	24

Once the validation process is finished, the results obtained above are introduced in an Excel sheet, where the precision, trueness and uncertainty are calculated automatically. However, these calculations are specified and detailed below.

5.3.1. PRECISION CALCULATIONS:

Precision is calculated by means of the coefficient of variation (CV), or also known as relative standards deviation (RSD) of the results obtained during the validation. Expression 7 and 8 are used to obtain the values of precision for the high and medium levels. [20,21]

$$CV = 100 * \frac{s}{\chi} \tag{7}$$

Where,

s is the standard deviation of the results,

$$s = \sqrt{\frac{n\,(\sum y^2) - (\sum y)^2}{n\,(n-1)}} \tag{8}$$

χ is the mean value of the results,

Taking into account these expressions and the validation results, the value of CV for high and medium range are calculated:

Table 12. Precision results.

	HIGH CONCENTRATION LEVEL	**MEDIUM CONCENTRATION LEVEL**
Mean (mg/L)	204.8290	108,0980
Std deviation (mg/L)	75.1917	36.0156
CV (%)	36.7095	33.3175

Comparing the values of the precision obtained during the validation process, it can be seen that the results exceed the reference values extracted from the ASTM D7066-04 standard, where it was established a maximum value of precision of 32. It can be concluded that the method has determined results that are not precise with respect to each other.

5.3.2. TRUENESS CALCULATION:

In the case of trueness, it is calculated by using the recovery values of each sample. Expression 9 shows how to calculate the accuracy. [20,21]

$$Accuracy = \frac{\sum(100-R)}{n} \tag{9}$$

Substituting R by the recovery values of the ten analyses, the trueness results obtained for the high and medium concentration ranges are:

Table 13. Trueness results.

	HIGH CONCENTRATION LEVEL	MEDIUM CONCENTRATION LEVEL
ACCURACY (%)	43.1192	39.9626

As in the precision calculations, the accuracy values exceed the reference values taken from ASTM D7066-04 standard, which means that the method is not accurate.

5.3.3. UNCERTAINTY CALCULATIONS:

The uncertainty of an analytical method is associated to the dispersion of the results obtained in the measurements. This dispersion can be attributed to the measurand (concentration). When calculating the uncertainty, different sources that can produce uncertainty must be considered, such as the volumetric equipment, the reference values, the uncertainties relate to masses, the matrix effect, etc. In analytical chemistry purposes, the uncertainty of a method is expressed in terms of expanded uncertainty (U_{EXP}) or relative expanded uncertainty ($U_{REL,\ EXP}$). The expanded uncertainty is calculated by multiplying the combined uncertainty by the coverage factor *k*, which has a value of 2 when considering a confidence interval of 95%. Combined uncertainty (U_c) is estimated by linking all the uncertainty sources of the method. This value must be calculated using the variance propagation law, which formula is: [20,21]

$$U_{tot}{}^2 = \sum_i \left(\frac{\partial f}{\partial f y_i}\right)^2 U_i{}^2 \quad (10)$$

Considering the uncertainty sources that may affect directly to the analytical method of the determination of oils and grease in wastewater samples, the final expression to calculate the combined uncertainty is the following:

$$U_{Comb} = \sqrt{U_{MR}^2 + U_{mean}^2 + U_{repro}^2 + U_{correc}^2} \quad (11)$$

Where,

U_{MR} is the uncertainty of the reference material,

U_{mean} is the uncertainty related the mean value of the results,

U_{repro} is the uncertainty of reproducibility,

U_{correc} is the uncertainty of the correction.

- ADDITION UNCERTAINTY

One of the sources that can give uncertainty to the measurement is the addition of reference material. For this reason, the uncertainty related to the addition of stock solution in the sample matrices must be calculated, for both concentration levels. But firstly, it is necessary to calculate the uncertainty of the stock solution.

The reference material that is used for this analytical method is a stock solution prepared with three different organic compounds, for that reason, the individual uncertainty of each analyte is calculated and after that, the stock solution uncertainty.

In that case, the principal sources of uncertainty in the preparation of the stock solution 2 are weight (P), purity (R), and volumetric material (V). The expression of the uncertainty of each analyte has the following shape:

$$U_C = \sqrt{U_P^2 * \left(\frac{R}{V}\right)^2 + U_R^2 * \left(\frac{P}{V}\right)^2 + U_V^2 * \left(\frac{P \times R}{V^2}\right)^2} \quad (12)$$

Where,

U_P belongs to the uncertainty related to the experimental weight of each analyte.

U_R belongs to the uncertainty of the reagent's purity.

U_V belongs to the uncertainty of the volumetric material that is used to prepare the stock solution.

WEIGHT UNCERTAINTY: the calculation of this type of uncertainty is given by expression 13.

$$U_P = \sqrt{U_{Cal}^2 + U_{Res}^2 + U_{drift}^2 + U_{Corr}^2} \quad (13)$$

Where,

U_{Cal} is related to the uncertainty of the analytical balance,

U_{Res} is related to the resolution of the analytical balance,

U_{Drift} is related to the drift of the analytical balance,

U_{Corr} is related to the correction of the analytical balance.

The preparation of the stock solution is carried out by weighting the reagents in the same analytical balance, therefore, the uncertainty related to this equipment will be identical for each of the three chemical substances.

In order to calculate U_P, first it is required to obtain the individual values of the other components (U_{Cal}, U_{res}, U_{drift}, U_{corr}):

> U_{Cal}:

$$U_{Cal} = \frac{I}{K} \quad (14)$$

Where,

I is the uncertainty of the analytical balance, which is specified in the balance certificate.

K has a value of 2, which corresponds to a confidence interval of 95%.

The value of U_{cal} is **0,5 mg.**

> U_{Res}:

$$U_{Res} = \frac{Resolution}{2\sqrt{3}} \quad (15)$$

Considering that the value of the resolution of the analytical balance is 0,1 mg, the value of U_{Res} is **0,0288675 mg.**

> U_{Drift}:

$$U_{Drift} = \frac{Drift}{\sqrt{3}} \quad (16)$$

The drift of an analytical balance is given by the difference between two different calibrations done by two external entities. In our case, the drift is 0 and therefore, the uncertainty.

> U_{Corr}:

$$U_{Corr} = \frac{Correction}{\sqrt{3}} \quad (17)$$

The value of the correction of the analytical balance is 5 mg, therefore the value of the U_{corr} is **2,8868 mg.**

With these individual values, the uncertainty related to the analytical balance can be calculated with expression 13.

$$U_p = \sqrt{0{,}5^2 + 0{,}0289^2 + 2{,}8868^2}$$

$$U_p = 2{,}9299\ mg$$

<u>VOLUMETRIC MATERIAL UNCERTAINTY:</u> The uncertainty of the volumetric material (U_v) is given by the volume of stock solution. This uncertainty value is the same for the three analytes.

$$U_V = \frac{Vf \times Correction}{\sqrt{3}} \quad (18)$$

$$U_V = \frac{0{,}05 \times 0{,}005}{\sqrt{3}}$$

$$\mathrm{U_V} = 0{,}000144\ \mathrm{L}$$

With the value of U_P and U_v calculated, it is necessary to calculate the values of U_r (purity) for each analyte. The purity in 1 percent must be considered. The equations to calculate the uncertainty of the purity can be seen below (expression 19):

$$U_R = \frac{1-R\ (1\%)}{\sqrt{3}} \quad (19)$$

- ISOOCTANE (99,984%):

$$U_R = \frac{1-0,99984}{\sqrt{3}} = 0,0000924$$

- n-HEXADECANE (99,4%)

$$U_R = \frac{1-0,994}{\sqrt{3}} = 0,00346$$

- BENZENE (99,983%):

$$U_R = \frac{1-0,99983}{\sqrt{3}} = 0,0000982$$

With the values of Up, Uv and Ur, and considering the amount of analyte weighted, purity and final volume of solution, the uncertainty of each analyte of the reference stock solution can be calculated through equation 12.

ISOOCTANE:

$$U_{C,ISO} = \sqrt{2.9299^2 * \left(\frac{0.99984}{0.05}\right)^2 + 0.0000924^2 * \left(\frac{1878.2}{0.05}\right)^2 + 0.000144^2 * \left(\frac{1878.2 \times 0.99984}{0.05^2}\right)^2}$$

$$U_{C,ISOOCTANE} = \textbf{123,2868 mg/L}$$

n-HEXADECANE

$$U_{C,HEX} = \sqrt{2.9299^2 * \left(\frac{0.994}{0.05}\right)^2 + 0.00346^2 * \left(\frac{1877.5}{0.05}\right)^2 + 0.000144^2 * \left(\frac{1877.5 \times 0.994}{0.05^2}\right)^2}$$

$$U_{C,n-HEXADECANE} = \textbf{178,6675 mg/L}$$

BENZENE

$$U_{C,BEN} = \sqrt{2.9299^2 * \left(\frac{0.99983}{0.05}\right)^2 + 0.0000982^2 * \left(\frac{1257.5}{0.05}\right)^2 + 0.000144^2 * \left(\frac{1257.5 \times 0.99983}{0.05^2}\right)^2}$$

$$U_{C,BENZENE} = \textbf{93,3157 mg/L}$$

Due to the concentration of the stock solution is calculated by adding the individual concentration of the three analytes, the uncertainty of the stock solution is estimated by applying the square root of the sum of squares of the Uc of each analyte.

$$U_C = \sqrt{123{,}2868^2 + 178{,}6675^2 + 93{,}3157^2}$$

$$U_C = \mathbf{236.2827\ mg/L}$$

U_c represents the uncertainty that is generated when creating the stock solution. But, when performing the validation study, different volumes of this solution are pipetted, which also has an uncertainty called the addition uncertainty. With the value of U_C, it is possible to calculate the addition uncertainty of the high and medium concentration range. Equation 9 shows how to calculate the addition uncertainty.

$$U_{Cad} = \sqrt{U_C^2 * \left(\frac{V_{added}}{V_f}\right)^2 + U_{V_{added}}^2 * \left(\frac{C_i}{V_f}\right)^2 + U_V^2 * \left(\frac{V_{added} \times C_i}{{V_f}^2}\right)^2} \quad (20)$$

Where,

U_C is the uncertainty of the stock solution,

$U_{V,added}$ is the uncertainty related to the volume of stock solution added, and it is calculated with the following expression:

$$U_{V_{added}} = \frac{V_{added} \times Tolerance\ (Vf)}{\sqrt{3}} \quad (21)$$

U_V is the uncertainty of the final volume of wastewater (200ml), and is calculated with equation

$$U_{V_f} = \frac{V_f \times Tolerance\ (V_{V_{added}})}{\sqrt{3}} \quad (22)$$

The results of the addition uncertainty of each concentration level are shown in *Table 14.*

Table 14. Addition uncertainties

	HIGH CONCENTRATION LEVEL	**MEDIUM CONCENTRATION RANGE**
Final volume (ml)	200	
Tolerance V_f (ml)	0.15	
Tolerance V_{added} (ml)	0.005	
U_C (mg/L)	236.2827	
Volume of stock sol. added (ml)	0.18	0.09
$U_{V,added}$ (ml)	0.0156	0.0078
U_V (ml)	0.5774	0.5774
U_{Cad} (mg/L)	**7.804**	**3.902**

- COMBINED UNCERTAINTY:

The next stage of the validation process is to compute the combined uncertainty (U_C). Once the addition uncertainty of each concentration level is determined, the combined U_C can be calculated by expression 23.

$$U_{Comb} = \sqrt{U_{MR}^2 + U_{mean}^2 + U_{repro}^2 + U_{correc}^2} \quad (23)$$

Where,

U_{MR} is the uncertainty of the reference material, which is calculated above,

U_{mean} is the uncertainty related the mean value of the results,

U_{repro} is the uncertainty of reproducibility of the method,

U_{correc} is the uncertainty of the correction.

The correction uncertainty must be only considered when the value of the compatibility index (IC) is higher than 2, which means that there is significant difference between the results obtained. The expression to calculate the IC is the following:

$$IC = \frac{|V_R - V_M|}{(U_{repro})^2 + (U_{mean})^2} \quad (24)$$

But before computing the IC, it is necessary to find the values of U_{mean} and U_{repro} with equations 25 and 26.

$$U_{mean} = \frac{S_{repro}}{\sqrt{Nº\ of\ measurements}} \quad (25)$$

$$U_{repro} = \frac{S_{repro}}{\sqrt{Nº\ of\ daily\ measurements}} \quad (26)$$

The results of these three parameters can be found in *Table 15.*

Table 15. Results of Umean, Urepro and IC.

	HIGH CONCENTRATION RANGE	MEDIUM CONCENTRATION RANGE
Srepro (mg/L)	75.1917	36.0156
Umean (mg/L)	23.7777	11.3891
Urepro (mg/L)	75.1917	36.0156
IC	6.2045	5.9767

The value of IC is greater than 2, which means that there are significant difference and the uncertainty related to the correction must be considered. The U_{corr} is determined with the following equation:

$$U_{corr} = \frac{V_R - V_{mean}}{\sqrt{3}} \quad (27)$$

Where V_R is the theoretical concentration added and V_{mean} is the average value of the results obtained during the validation. After that, the combined uncertainty of both levels is calculated with expression 23.

Table 16. Combined uncertainty results.

	HIGH CONCENTRATION RANGE	MEDIUM CONCENTRATION RANGE
V_R (mg/L)	360.1022	180.0511
V_{mean} (mg/L)	204.8290	108.0980
U_{correc} (mg/L)	89.6471	41.5422
U_{Com} (mg/L)	**119.6523**	**56.2833**

EXPANDED AND RELATIVE UNCERTAINTIES:

The last step on the uncertainty calculation is the relative expanded uncertainty through the expanded one, which are calculated with the expressions 28 and 29.

$$U_{exp} = t_{student(0,05;efective\ degrees)} \times U_{com} \quad (28)$$

$$U_{rel,exp} = \frac{U_{exp}}{\bar{x}} \times 100 \quad (29)$$

Despite the most common procedure is to consider the value of $t_{student}$ as 2, which means that a confidence interval of 95% is selected, it is necessary to select the $t_{student}$ value according to the number of effective degrees too. The number of effective degrees is calculated with expression:

$$\nu_{efect} = \frac{{U_{Com}}^4}{\frac{{U_{mean}}^4}{n-1} + \frac{{U_{repro}}^4}{n_I - 1}} \quad (30)$$

Taking the values of the mean, reproducibility and combined uncertainties obtained above, the values of the effective degree for the medium range is 53 and 57 for the high range. Using the t student table that can be found in the ANNEX, the values of the t student coefficient are taken by selecting an α of 0,05 and the corresponding effective degrees.

t student (57 effective degrees) = 1,6720 t student (53 effective degrees) = 1,6741

The results of the expanded and relative expanded uncertainty are shown in *Table 17.*

Table 17. Relative expanded uncertainty results.

	HIGH CONCNETRATION RANGE	MEDIUM CONCENTRATION RANGE
Expanded Uncertainty (mg/L)	200.0586	94.2239
Relative Expanded Uncertainty (%)	97.6711	87.1652

According to the UNE-EN ISO 17025 standard, the value of the uncertainty of a measurement must be expressed with two decimal significant numbers. Therefore, the final uncertainty results are 98% for the medium concentration, and 88% for the high one, as in validations, the uncertainty is rounded upwards.

Comparing the uncertainty results obtained in that validation procedure with the ones extracted form the ASTM D7066-04 standard, it is seen that the uncertainty if the whole method exceeds the reference values. Since the true value of a measurement cannot be precisely determined, uncertainty is the doubt that the result carries with it. A large value of the relative uncertainty implies that the result is much more doubtful and that the range of the measurement is much larger.

6. CONCLUSION

English

After developing and optimizing the analytical method to the maximum, the conclusions that have been reached are that it is a rather complex method, experimentally, due to the fact that it requires a very specific process and uses a very complicated analytical separation technique. From the results obtained from the validation, where the precision, accuracy and uncertainty of the method have been studied, it can be seen that the method for the determination of oils and grease in wastewater samples gives imprecise and inaccurate results, due to the low recoveries of some samples. Furthermore, the uncertainty values are too high, which means that the results obtained in the different analyses are not of high quality and can lead to confusion. As a general conclusion, this analytical method has not been validated in terms of reproducibility.

The main reason why these results could have been obtained is due to the matrix effect of the wastewater samples. When the liquid-liquid extraction was carried out, the treatment of the emulsions did not have the same effect along the different samples of wastewater, which in some cases produced a poor recovery of the analytes in the organic phase. One option to obtain better results could be the use of an alternative analytical method that does not involve liquid-liquid extraction as the main technique, since this involves major problems in the recovery depending on the type of sample. Furthermore, to be more respectful of the environment and health, it is better to avoid the use of chlorinated solvents, as they are toxic and require specific treatment after the analysis.

Català

Després de desenvolupar i optimitzar el mètode analític al màxim, les conclusions a les que s'han pogut arribar es que és un mètode bastant complex experimentalment, degut a que necessita un procés molt específic i s'utilitza una tècnica de separació molt complicada analíticament. A partir dels resultats obtinguts de la validació, on s'han estudiat la precisió, l'exactitud i la incertesa del mètode, es pot veure que el mètode per la determinació d'olis i greixos en mostres d'aigües residuals dona resultats imprecisos i inexactes, degut a les baixes recuperacions

d'algunes mostres. A més, els valors de la incertesa son massa elevats, el que produeix que els resultats que es puguin obtenir en els diferents anàlisis no siguin de qualitat i puguin portar a confusions. Com a conclusió general, aquest mètode analític no s'ha pogut validar per termes de reproductibilitat.

La principal raó per la qual es podrien haver obtingut aquests resultats es degut a l'efecte matriu de les mostres. A l'hora de fer l'extracció líquid-líquid, el tractament de les emulsions optimitzat no tenia el mateix efecte en les diferents mostres d'aigües residuals, cosa que produïa en alguns casos una pobre recuperació dels analits a fase orgànica. Una opció per obtenir millor resultats podria ser l'ús d'un altre mètode analític que no impliqui com a tècnica principal una extracció líquid-líquid, ja que aquesta comporta grans problemes en les recuperacions depenent del tipus de mostra. A més, per tal de ser més respectuós amb el medi ambient i amb la salut, es millor evitar l'ús de dissolvents clorats, ja que són tòxics i necessiten un tractament específic després del anàlisis.

7. ANNEX

In that section, it is described the calculation of the second stock solution that is prepared and the calibration curve that Analyst 2 has built for the validation process. Moreover, the t student table is attached.

7.1. CALIBRATION CURVE #2

To construct the second calibration curve, the stock solution of analyst 2 is specified in section 4.10. The calibration curve has been prepared in the same way as Analyst 1, using the same material, standards concentration, solvent, except for this new stock solution. Then, these new solutions are analyzed in the FT-IR spectrometer applying the same spectral parameters.

- INFRARED SPECTRA:

The spectra that are obtained from these standard solutions are shown in Figure 20:

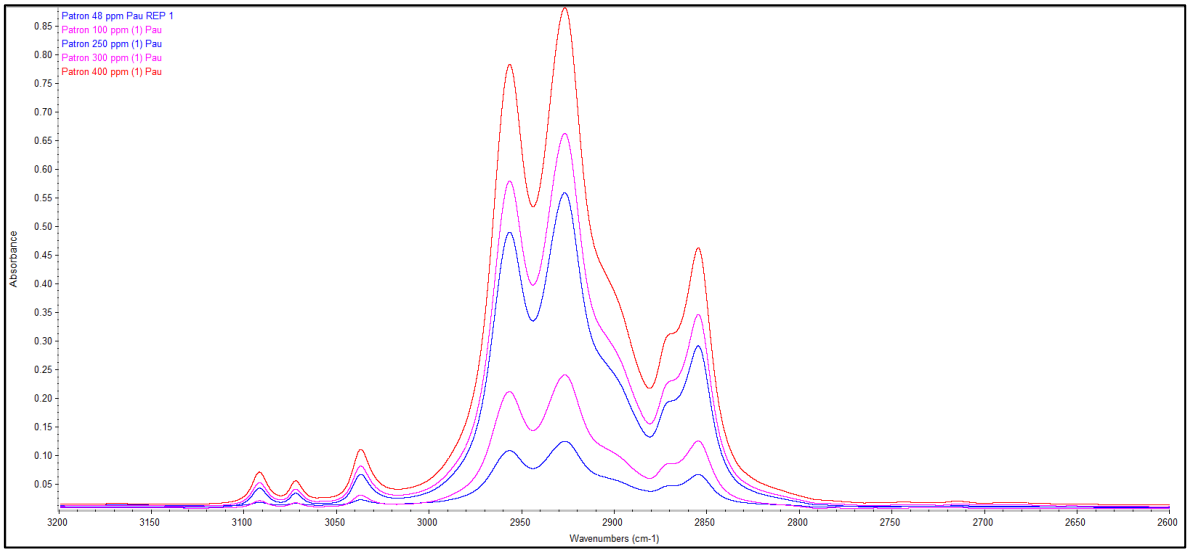


Figure 20. Calibration curve of Analyst 2.

- CALCULATION OF THE REAL STANDARD CONCENTRATION:

Considering that the new stock solution contains 100.027,66 mg/L of total hydrocarbons, the concentrations are calculated with that value and expression 3 (section 4):

Table 18. Theoretical concentration of the calibration standards.

	Approximated [ ] (mg/L)	**Theoretical [ ] (mg/L)**
Stock solution	100000	100027.66
Intermediate solution	5000	5001.38
Standard 1	48	48.01
Standard 2	100	100.03
Standard 3	250	250.07
Standard 4	300	300.08
Standard 5	400	400.11

- CALIBRATION CURVE

Uploading the spectra obtained by the standard solutions and introducing the real concentration values into TQ Analyst, the calibration curve #2 is built considering the same spectral regions as in calibration curve 1. The calibration curve obtained is the following:

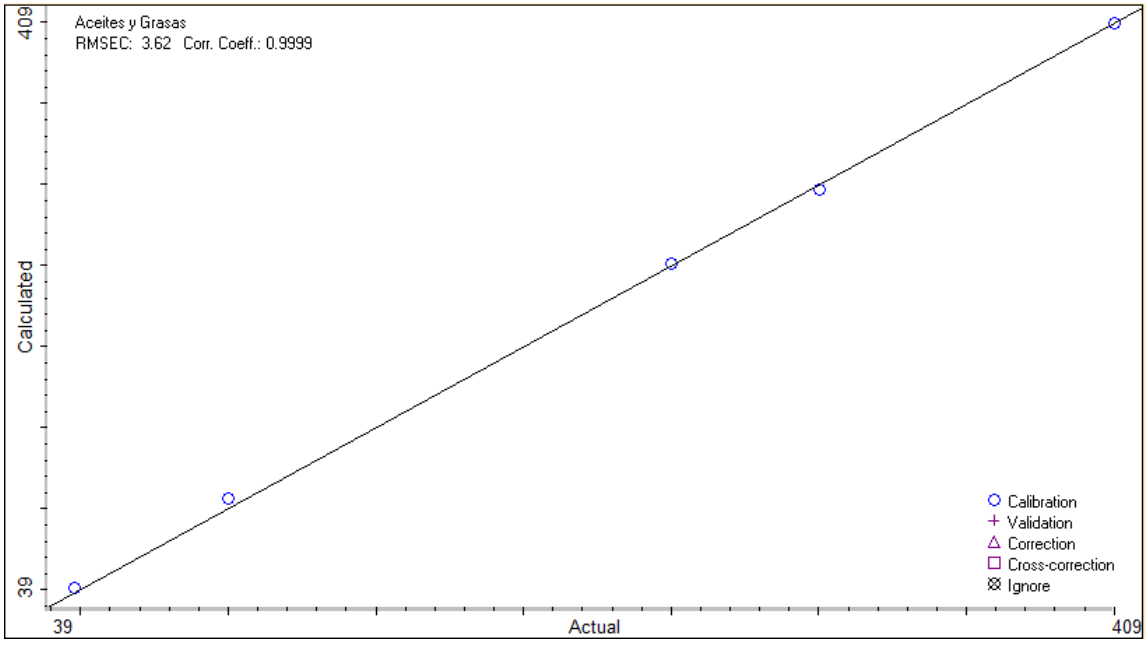


Figure 21. External calibration curve of analyst 2.

As it was done in the first calibration curve, the determination coefficient and the standard deviation of each standard must be evaluated. It can be seen that the R^2 is 0,9999 which is larger than the reference value, 0,99, and the calculations of the residuals and the deviation are done with expressions 4 and 5, which are found in section 5.3. These results are shown in *Table 19.*

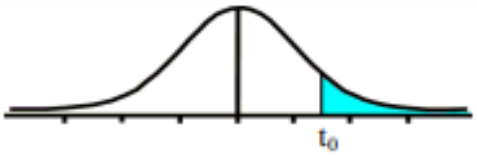
Table 19. Results of the standards solutions of the calibration curve.

	[ ] expected	[ ] real	Residual	% Deviation
Standard 1	47.89	46.33	1.560	3.367
Standard 2	99.78	99.69	0.095	0.095
Standard 3	249.45	249.52	0.115	0.046
Standard 4	299.34	299.37	0.230	0.077
Standard 5	399.12	399.12	0.245	0.061

The calibration curve corresponding to analyst 2 fits according to the laboratory convention.

7.2.t-STUDENT TABLE:

Tabla t-Student



Grados de libertad	0.25	0.1	0.05	0.025	0.01	0.005
1	1.0000	3.0777	6.3137	12.7062	31.8210	63.6559
2	0.8165	1.8856	2.9200	4.3027	6.9645	9.9250
3	0.7649	1.6377	2.3534	3.1824	4.5407	5.8408
4	0.7407	1.5332	2.1318	2.7765	3.7469	4.6041
5	0.7267	1.4759	2.0150	2.5706	3.3649	4.0321
6	0.7176	1.4398	1.9432	2.4469	3.1427	3.7074
7	0.7111	1.4149	1.8946	2.3646	2.9979	3.4995
8	0.7064	1.3968	1.8595	2.3060	2.8965	3.3554
9	0.7027	1.3830	1.8331	2.2622	2.8214	3.2498
10	0.6998	1.3722	1.8125	2.2281	2.7638	3.1693
11	0.6974	1.3634	1.7959	2.2010	2.7181	3.1058
12	0.6955	1.3562	1.7823	2.1788	2.6810	3.0545
13	0.6938	1.3502	1.7709	2.1604	2.6503	3.0123
14	0.6924	1.3450	1.7613	2.1448	2.6245	2.9768
15	0.6912	1.3406	1.7531	2.1315	2.6025	2.9467
16	0.6901	1.3368	1.7459	2.1199	2.5835	2.9208
17	0.6892	1.3334	1.7396	2.1098	2.5669	2.8982
18	0.6884	1.3304	1.7341	2.1009	2.5524	2.8784
19	0.6876	1.3277	1.7291	2.0930	2.5395	2.8609
20	0.6870	1.3253	1.7247	2.0860	2.5280	2.8453
21	0.6864	1.3232	1.7207	2.0796	2.5176	2.8314
22	0.6858	1.3212	1.7171	2.0739	2.5083	2.8188
23	0.6853	1.3195	1.7139	2.0687	2.4999	2.8073
24	0.6848	1.3178	1.7109	2.0639	2.4922	2.7970
25	0.6844	1.3163	1.7081	2.0595	2.4851	2.7874
26	0.6840	1.3150	1.7056	2.0555	2.4786	2.7787
27	0.6837	1.3137	1.7033	2.0518	2.4727	2.7707
28	0.6834	1.3125	1.7011	2.0484	2.4671	2.7633
29	0.6830	1.3114	1.6991	2.0452	2.4620	2.7564
30	0.6828	1.3104	1.6973	2.0423	2.4573	2.7500
31	0.6825	1.3095	1.6955	2.0395	2.4528	2.7440
32	0.6822	1.3086	1.6939	2.0369	2.4487	2.7385
33	0.6820	1.3077	1.6924	2.0345	2.4448	2.7333
34	0.6818	1.3070	1.6909	2.0322	2.4411	2.7284
35	0.6816	1.3062	1.6896	2.0301	2.4377	2.7238
36	0.6814	1.3055	1.6883	2.0281	2.4345	2.7195
37	0.6812	1.3049	1.6871	2.0262	2.4314	2.7154
38	0.6810	1.3042	1.6860	2.0244	2.4286	2.7116
39	0.6808	1.3036	1.6849	2.0227	2.4258	2.7079
40	0.6807	1.3031	1.6839	2.0211	2.4233	2.7045
41	0.6805	1.3025	1.6829	2.0195	2.4208	2.7012
42	0.6804	1.3020	1.6820	2.0181	2.4185	2.6981
43	0.6802	1.3016	1.6811	2.0167	2.4163	2.6951
44	0.6801	1.3011	1.6802	2.0154	2.4141	2.6923
45	0.6800	1.3007	1.6794	2.0141	2.4121	2.6896
46	0.6799	1.3002	1.6787	2.0129	2.4102	2.6870
47	0.6797	1.2998	1.6779	2.0117	2.4083	2.6846
48	0.6796	1.2994	1.6772	2.0106	2.4066	2.6822
49	0.6795	1.2991	1.6766	2.0096	2.4049	2.6800

50	0.6794	1.2987	1.6759	2.0086	2.4033	2.6778
51	0.6793	1.2984	1.6753	2.0076	2.4017	2.6757
52	0.6792	1.2980	1.6747	2.0066	2.4002	2.6737
53	0.6791	1.2977	1.6741	2.0057	2.3988	2.6718
54	0.6791	1.2974	1.6736	2.0049	2.3974	2.6700
55	0.6790	1.2971	1.6730	2.0040	2.3961	2.6682
56	0.6789	1.2969	1.6725	2.0032	2.3948	2.6665
57	0.6788	1.2966	1.6720	2.0025	2.3936	2.6649
58	0.6787	1.2963	1.6716	2.0017	2.3924	2.6633
59	0.6787	1.2961	1.6711	2.0010	2.3912	2.6618
60	0.6786	1.2958	1.6706	2.0003	2.3901	2.6603
61	0.6785	1.2956	1.6702	1.9996	2.3890	2.6589
62	0.6785	1.2954	1.6698	1.9990	2.3880	2.6575
63	0.6784	1.2951	1.6694	1.9983	2.3870	2.6561
64	0.6783	1.2949	1.6690	1.9977	2.3860	2.6549
65	0.6783	1.2947	1.6686	1.9971	2.3851	2.6536
66	0.6782	1.2945	1.6683	1.9966	2.3842	2.6524
67	0.6782	1.2943	1.6679	1.9960	2.3833	2.6512
68	0.6781	1.2941	1.6676	1.9955	2.3824	2.6501
69	0.6781	1.2939	1.6672	1.9949	2.3816	2.6490
70	0.6780	1.2938	1.6669	1.9944	2.3808	2.6479
71	0.6780	1.2936	1.6666	1.9939	2.3800	2.6469
72	0.6779	1.2934	1.6663	1.9935	2.3793	2.6458
73	0.6779	1.2933	1.6660	1.9930	2.3785	2.6449
74	0.6778	1.2931	1.6657	1.9925	2.3778	2.6439
75	0.6778	1.2929	1.6654	1.9921	2.3771	2.6430
76	0.6777	1.2928	1.6652	1.9917	2.3764	2.6421
77	0.6777	1.2926	1.6649	1.9913	2.3758	2.6412
78	0.6776	1.2925	1.6646	1.9908	2.3751	2.6403
79	0.6776	1.2924	1.6644	1.9905	2.3745	2.6395
80	0.6776	1.2922	1.6641	1.9901	2.3739	2.6387
81	0.6775	1.2921	1.6639	1.9897	2.3733	2.6379
82	0.6775	1.2920	1.6636	1.9893	2.3727	2.6371
83	0.6775	1.2918	1.6634	1.9890	2.3721	2.6364
84	0.6774	1.2917	1.6632	1.9886	2.3716	2.6356
85	0.6774	1.2916	1.6630	1.9883	2.3710	2.6349
86	0.6774	1.2915	1.6628	1.9879	2.3705	2.6342
87	0.6773	1.2914	1.6626	1.9876	2.3700	2.6335
88	0.6773	1.2912	1.6624	1.9873	2.3695	2.6329
89	0.6773	1.2911	1.6622	1.9870	2.3690	2.6322
90	0.6772	1.2910	1.6620	1.9867	2.3685	2.6316
91	0.6772	1.2909	1.6618	1.9864	2.3680	2.6309
92	0.6772	1.2908	1.6616	1.9861	2.3676	2.6303
93	0.6771	1.2907	1.6614	1.9858	2.3671	2.6297
94	0.6771	1.2906	1.6612	1.9855	2.3667	2.6291
95	0.6771	1.2905	1.6611	1.9852	2.3662	2.6286
96	0.6771	1.2904	1.6609	1.9850	2.3658	2.6280
97	0.6770	1.2903	1.6607	1.9847	2.3654	2.6275
98	0.6770	1.2903	1.6606	1.9845	2.3650	2.6269
99	0.6770	1.2902	1.6604	1.9842	2.3646	2.6264
100	0.6770	1.2901	1.6602	1.9840	2.3642	2.6259
∞	0.6745	1.2816	1.6449	1.9600	2.3263	2.5758

Figure 19. t student table. [22]

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