BACHELOR THESIS

ANALYTICAL METHOD VALIDATION FOR THE QUANTIFICATION OF BRODIFACOUM

Faculty of Chemistry and Chemical Technology

Degree in Chemical Engineering

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LIST OF ABBREVIATIONS

EU → European Union

HPLC → High Performance Liquid Chromatography

HPLC-DAD → High-performance liquid chromatography with photodiode-array detection

TFA → Trifluoroacetic acid

MeOH → Methanol

HP water → High Purity water

RSD → Relative standard deviation

R^2 → Squared linear correlation coefficient

ABSTRACT:

The purpose of the study is the validation of a HPLC-DAD method for identifying and quantifying Brodifacoum in a pure standard sample according to SANCO/3030/99 guideline. Brodifacoum is an anticoagulant that reduces blood coagulation ability and it is commonly used for control of rats and mice.

In order to validate this method, parameters such as specificity, linearity, accuracy and precision are investigated. Two different methods, isocratic elution and gradient elution, are carry out in order to get a better precision.

After the validation of the method, the raw material is extracted from real samples and quantified. Finally, the efficiency of the extraction is tested. The identification of Brodifacoum is being performed by HPLC-DAD analysis, comparing the retention time and the shape of the peak of the reference standard with those of the active ingredient in the test sample.

The specificity is tested and it confirms the identity of the compound and provide that there are no interferences. Linearity is also proved since the linear regression coefficient of the line is higher than 0.99, fitting perfectly to a straight line in the working area.

Precision is evaluated analysing the RSD% coefficient, the average and the standard deviation of the peaks. Finally, accuracy is related with the recovery of Brodifacoum in the spike sample. For isocratic method, recovery is 96% and for gradient method it is 105%, high values which are also within the range of the guideline.

Therefore, the method has been proved to be specific, linear, precise and accurate.

1. INTRODUCTION

Brodifacoum was discovered in England in 1975 and its first publication (Hadler and Shadbolt) was quickly followed by reports on its characterisation in the field of rodent control.
In 1978, and following regulatory authority review, Brodifacoum began to receive registrations around the world for rodent control. It was first registered in Indonesia and then in the UK in the same year, while registration in the USA occurred in 1979. Nowadays, Brodifacoum is registered in over 40 countries [(1)] and it is currently the most active known rodenticide within the family of coumarin derivative, second-generation anticoagulant rodenticides. [(2)]

To determine the presence of that compound in samples a HPLC-DAD method is going to be validated. Specificity, linearity, accuracy and precision are the parameters which are investigated.

- Specificity is expressed as the degree of inaccuracy of the method.
- Linearity is the ability of the analytical method to obtain results directly proportional to the concentration.
- Accuracy expresses the closeness of agreement between the value which is accepted as a conventional true value and the value found with the method applied.
- Precision refers to the closeness of agreement between mutually independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment, within short intervals of time.

The extraction from real samples is also carried out and the recovery is calculated.

After the injection of all the solutions in the HPLC chromatograph, the corresponding chromatographs will be generated. From them, the area of the peaks will be studied to validate the parameters mentioned above. In addition, a calibration line is constructed that allows us to relate these peak areas to the concentration and will facilitate the validation of the method.

Finally, the results will be analysed in order to conclude whether the technique is suitable for the detection of this compound or not.

2. THEORICAL PART

2.1. Pesticides

Pesticides are substances composed of different chemical products whose purpose is prevent, destroy, repel or mitigate any pest, use as a plant regulator, defoliant, desiccant or use as a nitrogen stabilizer.
They contain active compounds, which are the ones that control pest, and inert ingredients, which help the correct efficiency and application of the active ingredients. (3)
They can be used in agriculture as well as in domestic life. One example of its application is the use of pesticides for crop protection for centuries.
Although pesticides efficiently repel pests, they also cause a serious negative impact on the environment and on human's health.
Therefore, the excessive use of pesticides may lead to the destruction of biodiversity. (5)
According to al-Saleh IA, member of the Biological and Medical Research Department, at the King Faisal Specialist Hospital and Research Centre in Saudi Arabia, many of the population may be at risk of developing chronic effects. Such as for example cancer diseases, adverse reproductive outcome or immunological effects depending on the type of pesticide they are exposed to. (4)

According to the recent publication of Eurostat, in 2019, the volume of pesticides sold in the EU reached the lowest level since the start of the data series, with just over 333 000 tonnes sold. While in 2020, a bit less than 346 000 tonnes of pesticides were sold in Europe. The highest volumes sold were recorded in four EU Member States, Germany, Spain, France and Italy, which are also the main agricultural producers in the EU. (6)

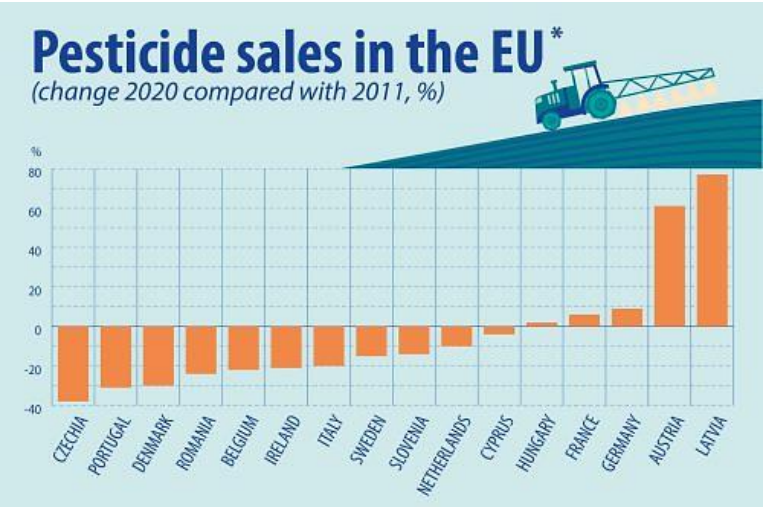

Figure 1. Pesticide sales in the EU during 2020 compared with 2011. (6)

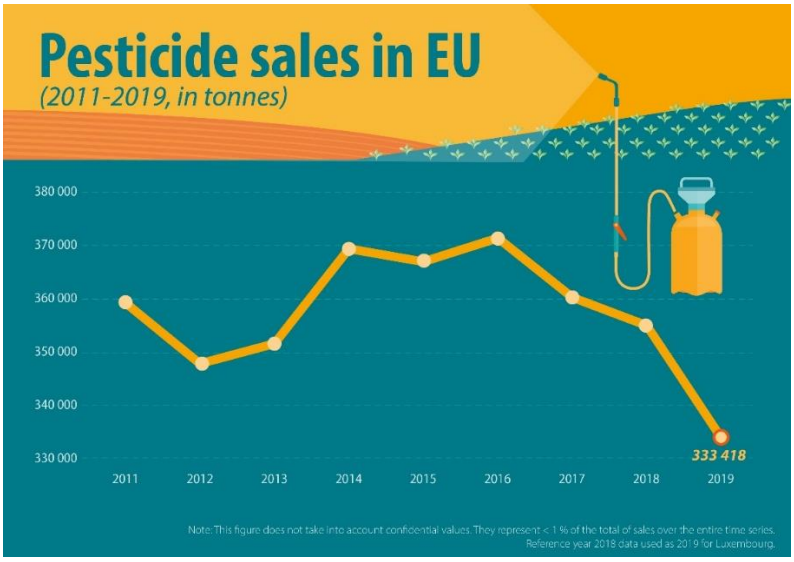

Figure 2. Pesticide sales in EU in tonnes from 2011 to 2019. [7]

Pesticides are becoming a major issue which concerns the entire world. In order to cut down the amounts of pesticides, United Nations have developed the Sustainable Development Goal indicators.

The 2030 Agenda for Sustainable Development, adopted by all United Nations Member States in 2015, provides a shared blueprint for peace and prosperity for people and the planet, now and into the future. [8]

There are 17 goals that countries must fulfil by the end of 2030. Among all the objectives, the second one is most closely related to pesticides. Through Goal 2, countries promise to "End hunger, achieve food security and improved nutrition and promote sustainable agriculture". One of the measures taken is get focus on ecological production.

Ecological production is a general system of agricultural management and food production. It combines the best environmental practices, a high level of biodiversity, the preservation of natural resources, the application of high animal welfare standards and production in accordance with the preferences of certain consumers for products obtained from natural substances and processes.

The European Union countries with the highest percentage of ecological production were Austria (25.69%), Estonia (22.41%) and Sweden (20.31%). On the other hand, the countries with the lowest shares were Malta (0.62%), Ireland (1.66%) and Bulgaria (2.30%). [9]

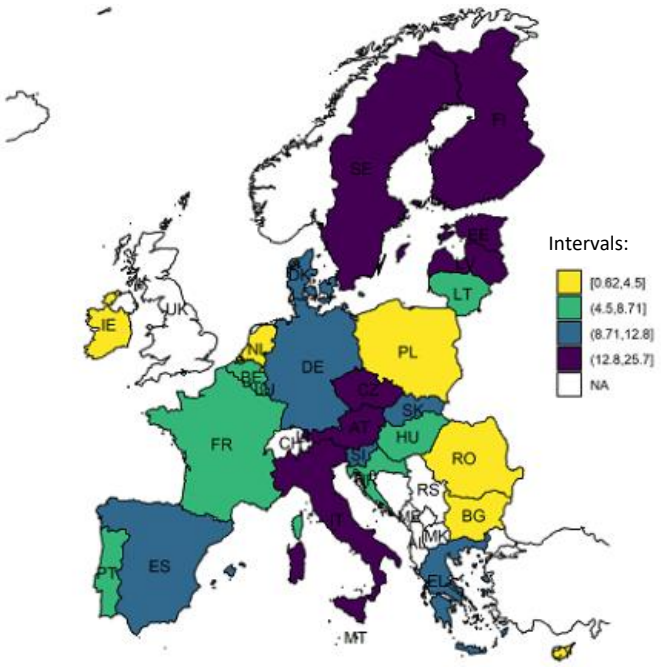

Figure 3. Proportion of agricultural area under ecological agriculture in 2020(%).

2.2. Brodifacoum

Brodifacoum is a second generation single-dose anticoagulant rodenticide. Its IUPAC name is 3-[3-(4'-bromobiphenyl-4-yl)-1,2,3,4- tetrahydro-1-napthyl]- 4-Hydroxycoumarin and its molecular weight is 523.42 g/mol.

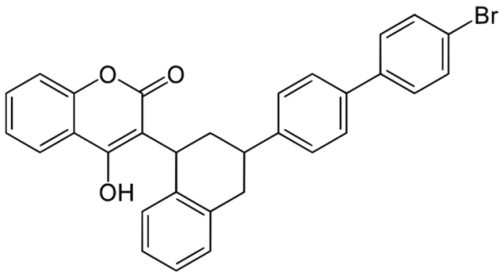

Figure 4. Brodifacoum molecular formula.

Like other coumarin derivatives, it is a vitamin K antagonist. It functions inhibiting the blood ability of clotting, by blocking the regeneration of vitamin K in the liver. Organisms die due to massive internal haemorrhages after several days of ingestion of a lethal dose.

The active substance effectiveness depends on the grade of exposure. Nevertheless, for effective and comprehensive control of rats and mice, including those strains that may be resistant to other anticoagulant compounds, a bait concentration of 50 mg/kg is proposed. [(10)]

In regards to Brodifacoum's physical-chemical properties, it is not a hazardous chemical compound. It is an off-white powder, which is stable in the solid form. Its solubility in water is very low (less than 10 mg/litre at 20 °C and pH 7) and it is slightly soluble in benzene and soluble in acetone. [11] It is not highly flammable and it shows no self-ignition below its melting point, either. In addition, it does not show oxidizing or explosive properties. [10]
The safety data sheet is presented in order to know the GHS Hazard Statements, the Precautionary Statement Codes and the pictograms.

Pictograms	
Signal	Danger
GHS Hazard Statements	H300: Fatal if swallowed [Danger Acute toxicity, oral] H310: Fatal in contact with skin [Danger Acute toxicity, dermal] H330: Fatal if inhaled [Danger Acute toxicity, inhalation] H360D: May damage the unborn child [Danger Reproductive toxicity] H372: Causes damage to organs through prolonged or repeated exposure [Danger Specific target organ toxicity, repeated exposure] H400: Very toxic to aquatic life [Warning Hazardous to the aquatic environment, acute hazard] H410: Very toxic to aquatic life with long lasting effects [Warning Hazardous to the aquatic environment, long-term hazard]
Precautionary Statement Codes	P203, P260, P262, P264, P270, P271, P273, P280, P284, P301+P316, P302+P352, P304+P340, P316, P318, P319, P320, P321, P330, P361+P364, P391, P403+P233, P405, and P501

Table 1. Safety data sheet for Brodifacoum. [12]

2.3. High Performance Liquid Chromatography (HPLC)

HPLC is a chromatography technic that is used for the separation of organic compounds (preparative purpose) or for measuring the proportion of the components in the mixture (analytic purpose). [13] It analyse compounds of different properties from the low up to very high molecular mass substances. [14] This specific technic is most commonly used when the compounds to be analysed are low volatile and/or thermolabile. [13]
The components of the mixture distribute into two phases, the mobile phase and the stationary phase, both phases are immiscible. The mobile phase is liquid while the stationary phase is composed by a solid located inside a long and narrow column. Solid particles are much smaller in HPLC, which requires high pressure to force the elution solvent (mobile phase) through the column. [15]

The instrumentation for HPLC consists of a column, a sample injection system, a solvent reservoir, a pump, a detector, and a recorder or computer readout. The sample is introduced in the equipment through an automated injection system (autosampler) that injects a tiny amount of sample solution into the column. There is generally a short guard column before the more expensive main column. The guard column retains fine particles and strongly adsorbed compounds that would degrade the main column. The length of the main column can range from 5 to 30 cm, with an inner diameter of 1–5 mm for analytical HPLC of 0.01–1.0-mg samples. [15]

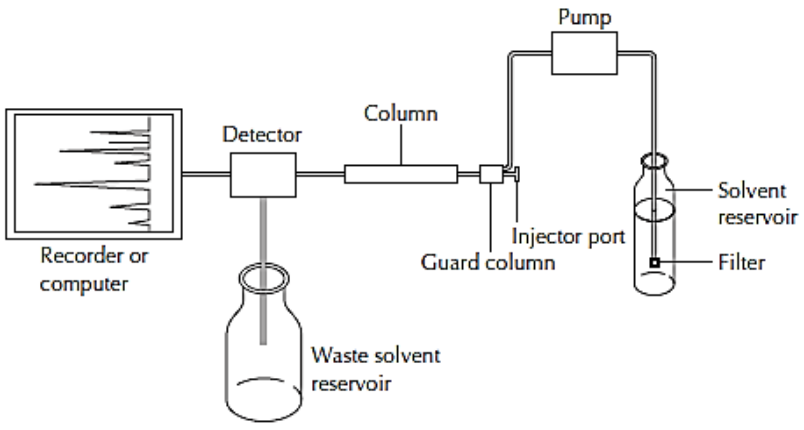

Figure 5. Schema of a typical high-performance liquid chromatograph. [15]

Depending on the relative polarity of the stationary and the mobile phase, we can difference two types of HPLC chromatography:

- **Reverse-phase chromatography.** It is used when samples have high polarity. The stationary phase is a thin layer of a nonpolar or hydrophobic organic compound, such for example derivatized silica gel C18, C8, C5. The mobile phase is polar like water or mixtures of water-methanol or water- acetonitrile. [13]

In reverse-phase chromatography, more polar compounds elute first because the mobile-phase solvent is more polar than the nonpolar stationary phase. Furthermore, an increase in the polarity of the mobile phase increases the elution time. [15]

- **Normal-phase chromatography.** It is useful for medium-low polarity samples. The stationary phase is highly polar and the most common used is silica gel because it allows the separation of compounds with a wide range of polarities. By contrast, the mobile phase is a nonpolar solution as for example n-hexane or CH2Cl2.
  In addition, the retention mechanism is dipole-dipole interactions.
  In comparison with the reverse-phase chromatography, less polar compounds elute first since they are the most soluble compounds in the mobile phase and an increase in the polarity of the mobile phase leads to a decrease in the elution time. [13]

2.4. High-performance liquid chromatography with photodiode-array detection

High-performance liquid chromatography with photodiode-array detection (HPLC-DAD) provides two identification parameters: retention and UV spectral data. [16]

The diode-array detector is a microprocessor controlled multi-channel detector that scans the samples with a beam of light that varies across the entire ultraviolet and visible light spectrum. It measures the amount of each wavelength of light that is absorbed against the time each chemical species was eluted from the column. Together these spectral and retention data help identify the different components within the sample, and the quantities they are in.

The principle of measuring the amount of light absorbed by a sample works in the same way for DAD and UV detectors. Nevertheless, the UV detector is set up to measure specific parts of the spectrum at any one time, while the DAD can measure the entire spectrum at once. This difference makes the DAD method more efficient than conventional UV detection. [17]

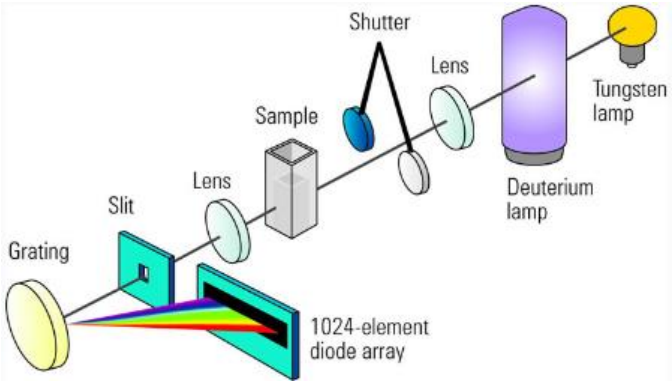

Figure 6. Photodiode array detection schematic. [18]

2.5. Parameters of validation under investigation

2.5.1. Specificity

Specificity is the ability of the method to unequivocally estimate the analyte in presence of the other components of the final product. According to SANCO/3030/99 rev. 5 guidelines, it is necessary to confirm the identity of the compound and provide that there are no interferences > 3%. In order to demonstrate this parameter, it is necessary to prepared the solvent solution, the test sample solution, the placebo solution and the raw material solution. [19]

2.5.2. Linearity

Linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of the analyte in the sample. The linearity of the method is usually assessed at 50%, 75%, 100%, 125% and 150% of the active ingredient Brodifacoum according to the literature. However, sometimes it is difficult to achieve that amount of the active ingredient.

The concentration shall be plotted against the corresponding area (sum of the area of each homologue). Finally, the linear regression coefficient of the line fitting the data (R) is calculated along with its equation. The R value should be higher to 0.99. [19]

2.5.3. Accuracy

Accuracy is calculated by the percentage recovery of the assay obtained adding known amounts of the active ingredient to the reconstituted placebo (reconstituted sample). [19]

2.5.4. Precision

The precision is evaluated performing the analysis of 5 independently samples and calculating the corresponding average and standard deviation. 5 injections of five standards solutions and 4 injections of four sample solution were done to evaluate the instrumental precision.

3. EXPERIMENTAL PART

3.1. Chemical products

- Ultrapure water
- TFA ≥99.9% (HiPerSolv CHROMANORM® for LC-MS)
- MeOH (Ultra) (Gradient HPLC Grade 'BAKER HPLC ANALYZED', for use in Liquid Chromatography (HPLC & UHPLC) & Spectrophotometry)
- Acetone (for HPLC, ≥ 99.8%, Honeywell)
- Brodifacoum (PESTANAL™, analytical standard, > 98%, Sigma-Aldrich)
- White and blue rat baits (real sample)

3.2. Instruments and materials

- 25mL and 50mL beakers
- Pasteur pipette
- 10mL, 20mL, 50mL and 1L volumetric flasks
- 15mL and 50mL conical test tubes
- Syringes
- Filters
- 100-1000µL automatic micropipettes
- Glass bottle for the mobile phase reagents
- Small vials
- Mortar and pestle
- Analytical Balance
- Ultrasonic bath
- HPLC chromatography (HPLC Agilent Technologies model 1100, Agilent technologies, USA)

3.3. Experimental procedures

3.3.1. Mobile phase

It is necessary to prepare two mobile phases. One is called mobile phase A and the other one is called mobile phase B.
Mobile phase A is composed of ultrapure water and 0.1% of TFA. So to reach that concentration, it is taken 500 mL of ultrapure water and 0.5 mL of TFA. However, mobile phase B is composed by methanol instead of water, but it also has 0.1% of TFA. It is necessary to introduce in the glass bottle 500 mL of MeOH and 0.5 mL of TFA.

3.3.2. Solvent

The solvent has the same composition as mobile phase B. It has got methanol and 0.1% of TFA. It is necessary to introduce in the glass bottle 200 mL of MeOH and 0.2 mL of TFA.

3.3.3. Raw material

20.7 mg of Brodifacoum (>98%) are weighted. Then, they are dissolved into a 20 mL volumetric flask with acetone, obtaining a solution with a concentration of 1.035 mg/mL.

3.3.4. Standard sample

The standard sample is prepared from the solution of 20.7 mg/ 20 mL of acetone, diluting it with solvent (MeOH + 0.1% TFA) to get a concentration of 10 µg/mL. One possibility is to prepare first a 100 µg/mL dilution by taking 2 mL from the Brodifacoum and acetone solution and diluting to 20 mL with solvent. From this last solution 2 mL are taken and diluted to the desired concentration in a 20 mL volumetric flask.

3.3.5. Adequacy of the equipment

Before injecting the samples, it is necessary to set up the equipment. First of all, the mobile phases are passed through the equipment to eliminate the air that may be presented in the tubes. Next, the flow of the mobile phases is allowed to pass through the column to remove any impurities that may remain. Once the equipment has stabilised and the pressure is adequate, the samples are placed for injection.

In this case, the solvent (MeOH + 0.1% TFA) and the 10 µg/mL Brodifacoum solution are injected. In this way, we check if the equipment is able to detect our compound.

In some cases, we carry out the experiment with two different methods, one was by isocratic elution while the other one was by gradient elution. Two methods are used to improve the resolution since in the chromatograph exits several peaks that sometimes are overlapped. So to avoid this and improve the resolution to identify our compound the two methods are carry out. The specific settings for each method are set out below.

Instrument	HPLC
Detector	UV/DAD
Column	Phenomenex Gemini 5u (C18), 150x4.6mm, 5 µm
Detection wavelength	265 nm, Bw 4 nm
Reference wavelength	360 nm, Bw 100 nm
Injection volume	20 µm
Flow	1 mL/min
Run time	12 min
Mobile phase B	MeOH + 0.1% TFA
Isocratic	15% A, 85% B
Retention time	Brodifacoum: ~ 9.26 min

Table 2. Instrumental settings for HPLC/UV validated method by isocratic elution.

Instrument	HPLC
Detector	UV/DAD
Column	Phenomenex Gemini 5u (C18), 150x4.6mm, 5 µm
Detection wavelength	265 nm, Bw 4 nm
Reference wavelength	360 nm, Bw 100 nm
Injection volume	20 µm
Flow	1 mL/min
Run time	18 min
Gradient	0 min → 65% MeOH, 35% H2O 12 min → 90% MeOH, 10% H2O 15 min → 90% MeOH, 10% H2O 15 min → 65% MeOH, 35% H2O 18 min → 65% MeOH, 35% H2O
Retention time	Brodifacoum: ~ 15.35 min

Table 3. Instrumental settings for HPLC/UV validated method by gradient elution.

Figure 7. Laboratory HPLC equipment (HPLC Agilent Technologies model 1100, Agilent technologies, USA).

3.3.6. Calibration curve

It is necessary to create a calibration line that relates the concentration to the area provided by the equipment. For this purpose, different solutions with different concentrations are prepared in the working range: 10 µg/mL, 7 µg/mL, 5 µg/mL, 3 µg/mL and 1 µg/mL.

Firstly, a 100 µg/mL dilution is prepared from which the other dilutions will be prepared. This solution is prepared from the solution of 20.7 mg/ 20 mL of acetone. You take 2 mL from that solution and dilute them into a 20 mL volumetric flask with solvent.

The table below shows the specific volume that is necessary to take from the 100 µg/mL dilution and transferred into a 20 mL volumetric flask for further dilution with solvent.

Concentration (µg/mL)	Volume (mL)
10	2
7	1.4
5	1
3	0.6
1	0.2

Table 4. Volume that is necessary to take from the 100 µg/mL dilution.

3.3.7. Recovery test

A blank sample, a spiked sample and a real sample needs to be prepared.

For preparing blank sample, around 5 g of white rat baits were weight. This baits should not have Brodifacoum.
For the spiked sample we add the same material as for the blank sample but it is mixed with 1.25 mL of standard 100 µg/mL solution. The final weight was 6.35 g.
Finally, in the real sample, containing Brodifacoum and other additives, 5.595 g of blue rat baits were weighed.
Once the samples have been weighed, the extraction is carried out. 20 mL of solvent are also added to each beaker in order to cover the solid sample. These three beakers are introduced into an ultrasonic bath for 30 minutes. After 30 minutes we transferred the liquid phase into a volumetric flask and we repeat again the extraction. 20 mL of solvent are added again to every beaker and putting them into the ultrasonic bath for 30 minutes. When time is running out, the liquid phase obtained is added to the previous one in the volumetric flask and the solid phase can be discarded. The liquid of the volumetric flask is diluted to 50 mL with solvent.
Afterwards the liquid is filtered and transferred into small vials in order to analyse them with HPLC chromatography.

In order to check if we can get a higher concentration in the sample another test is developed. Around 3.646 g are weight in a beaker and the oat pieces are removed, reaching a weight of 3.285 g. The extraction process is carried out and the same steps are repeated again.

Figure 8. White rat baits used for the blank sample and the spiked sample.

Figure 9. Blue rat baits used for preparing the real sample.

Figure 10. Liquid extract obtained from the extraction process.

3.3.8. Repeatability

Repeatability is defined such as the measure of the ability of the method to generate similar results for multiple preparations of the same sample. [20]
Therefore, 5 independently standard solutions of 1 µg/mL and another 5 independently standard solutions of 10 µg/mL are prepared in order to check the repeatability of the procedure.
On the other hand, 4 beakers with approximately 5 g of blue rat baits are also prepared and the extraction process is carried out.

4. RESULTS AND DISCUSSION

4.1. Chromatograms

Figures 11 and 12 below present the response of detector toward solvent, so we only see a first peak at the beginning corresponding to the mixture of MeOH and TFA. The chromatogram of the isocratic elution is shorter, while the one for gradient elution is longer, as explained above.

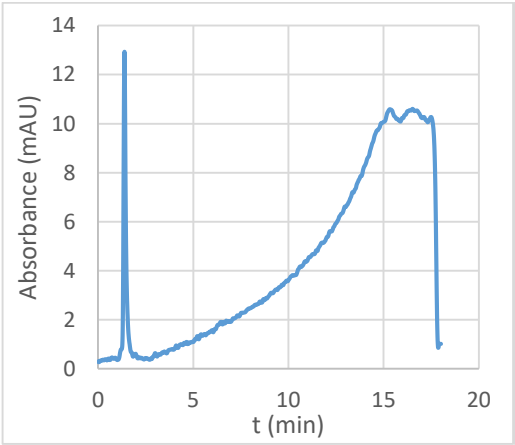

Figure 11. Solvent chromatogram with gradient elution.

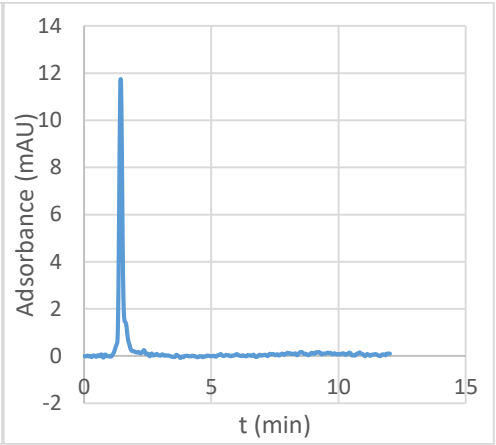

Figure 12. Solvent chromatogram with isocratic elution.

Nevertheless, in figures 13 and 14, you can distinguish two different peaks. It makes sense since the chromatographs correspond to the standard solution that only contains solvent and Brodifacoum.

First of all, nearly at the starting time, a big peak is found. This peak corresponds with the solvent as we have already checked in the previous chromatographs.

The second peak that you can distinguish around 9.26 min for isocratic method or 15.32 min for gradient method, corresponds with Brodifacoum.

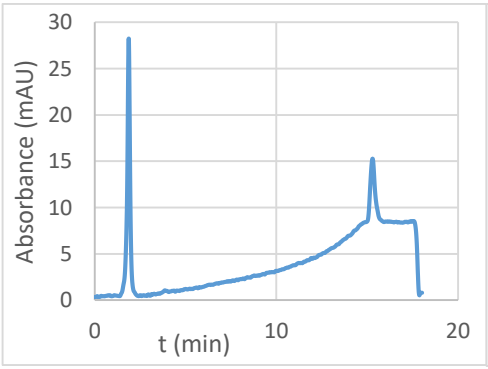

Figure 13. Standard solution chromatogram with gradient elution.

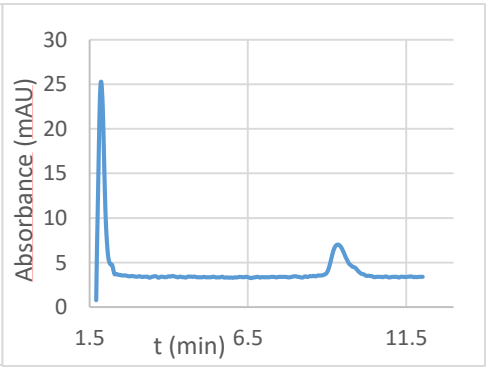

Figure 14. Standard solution chromatogram with isocratic elution.

In the figure 15 the chromatographs for the real sample and the spiked sample are presented.

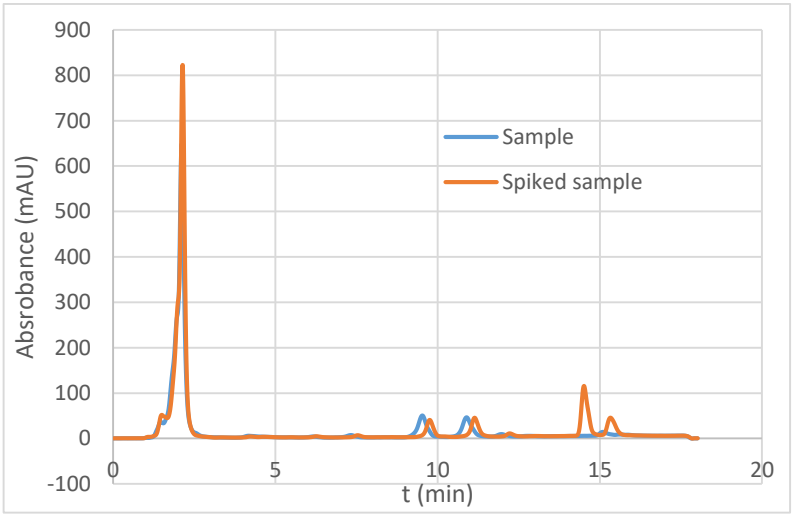

Figure 15. Sample and spiked sample chromatogram with gradient elution.

In gradient elution method, the resolution is higher so that we can distinguish a larger number of peaks, as can be seen in figure 15. The first big peak corresponds with the solvent, then, Brodifacoum peak can be seen at around 15.32 min and finally there are extra peaks that correspond with impurities that are presented in the real samples.

The Brodifacoum peak in the real sample is much smaller than in the spiked sample (figure 16) thus the concentration is smaller.

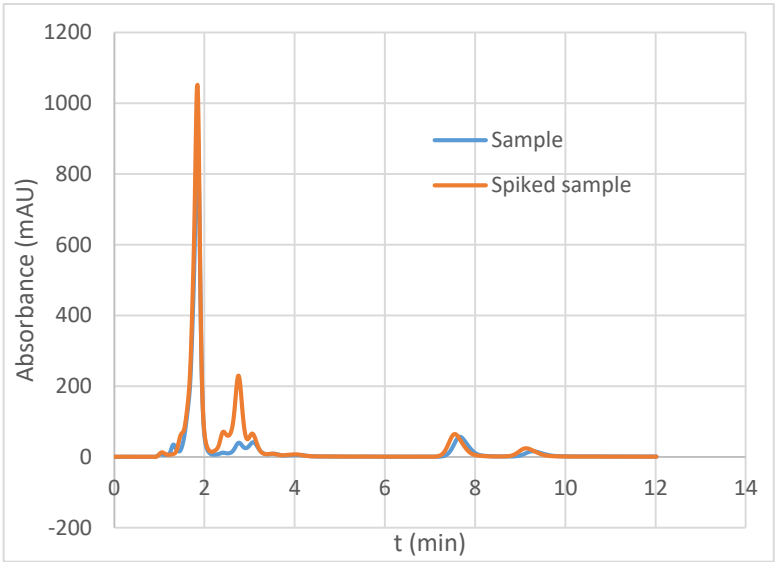

Figure 16. Sample and spiked sample chromatogram with isocratic elution.

In the isocratic elution method, the resolution is worse, which is why we see fewer peaks compared to the gradient elution. In figure 16 it is also possible to see the first large peak corresponding to the solvent, then the Brodifacoum peak can be seen at about 9.26 min, and finally there is an additional peak corresponding to impurities in the sample. Here, the area of both samples, the spiked one and the real one, seems to be very similar, while in the gradient method the areas differ more.

4.2. Linearity

In figures 17 and 18 the relation between the area and the concentration is represented within the working range. The working range is defined from the lower concentration of 1 mg/L until the higher concentration which is 10 mg/L for both methods.

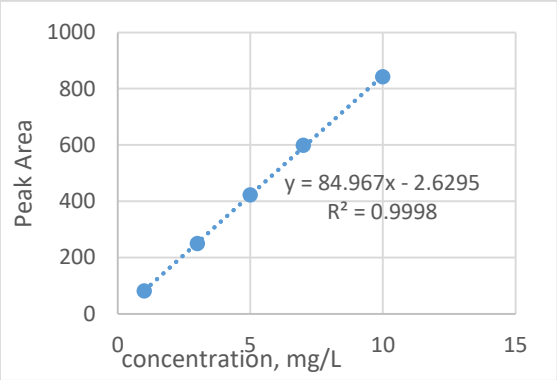

Figure 17. Brodifacoum calibration curve for isocratic elution.

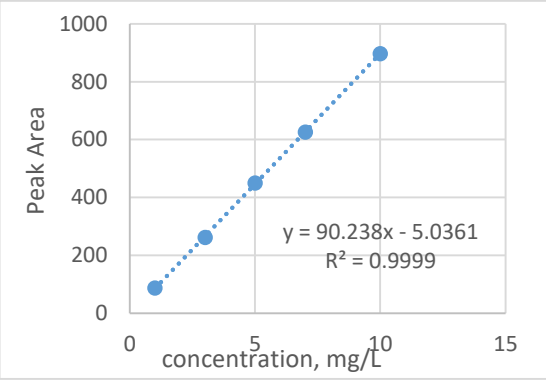

Figure 18. Brodifacoum calibration curve for gradient elution.

As we can see in the graph for isocratic elution, the linear regression coefficient of the line (R) is 0.9998. According to the guideline from the literature, it must be higher to 0.99 so we can say that our data distributes as a line within the working range. The same happens for the gradient elution, the linear regression coefficient of the line (R) is 0.9999 that is also higher to 0.99. Therefore, the relation between the concentration and the area of the peaks follows a linear distribution within the working range.

Stability has not been checked, consequently, the calibration curve is repeated every single time you analyse new samples.

4.3. Repeatability

The precision is evaluated performing the analysis of 5 independently prepared solutions of 1 mg/L and 10 mg/L and calculating the corresponding RSD% for both methods and concentrations.

	C = 1 mg/L Area	C = 10 mg/L Area
Std 1	123.7	642.2
Std 2	105.5	626.8
Std 3	119.9	612.2
Std 4	117.2	714.5
Std 5	132.0	539.8
AVERAGE	119.7	627.1
STANDARD DESVIATION	9.7	62.7
RSD (%)	8	10

Table 5. Repeatability for isocratic elution.

	C = 1 mg/L Area	C = 10 mg/L Area
Std 1	138.8	614.2
Std 2	118.0	601.5
Std 3	130.4	598.0
Std 4	123.9	700.2
Std 5	144.2	531.4
AVERAGE	131.1	609.06
STANDARD DESVIATION	10.7	60.3
RSD (%)	8	10

Table 6. Repeatability for gradient elution.

Although two methods were tested, the RSD value (%) is the same for both, as isocratic elution and gradient elution yield 8% for a concentration of 1 mg/L. Similarly, for the most concentrated solution, 10% is obtained for the isocratic method and for the gradient method, which can also be considered the same. Moreover, the higher the concentration, the larger the peak area, and therefore a higher %RSD is obtained. However, the deviations obtained are expected values.

4.4. Real samples

Repeatability is tested for the real samples weighting around 5 g of blue rat baits. Then, the average, the standard deviation and the RSD (%) is calculated

from the concentration in ppm. Another test is done in order to check if the concentration of Brodifacoum is higher removing the oat pieces (table 9).

	Mass (g)	Volume (mL)	Area	Concentration (mg/L)	Mass (mg)	mg/g	ppm
Sample 1	5.0	50	156.2	1.8	0.089	0.018	18
Sample 2	5.1	50	161.9	1.8	0.092	0.018	18
Sample 3	5.0	50	153.7	1.8	0.088	0.017	18
Sample 4	5.1	50	157.3	1.8	0.089	0.018	18

Table 7. Peak area, mass and concentration in ppm of each sample obtained from the gradient elution calibration line.

AVERAGE	17.8
STANDARD DESVIATION	0.3
RSD (%)	1.8

Table 8. Average, standard deviation and RSD (%) for the real sample repeatability.

The data show practically no variation compared to the average value, since the standard deviation is 0.3. Thus, despite some experimental errors due to the lack of precision in weighing, the four values are quite similar. Nevertheless, there are two solutions with the same weight but with different peak areas. This could be because there are different integrals for the same peak, which differ depending on where the peak boundaries are taken from.

	Mass (g)	Volume (mL)	Area	Concentration (mg/L)	Mass (mg)	mg/g	ppm
Sample without oat	3.3	50	81.8	1	0.048	0.015	15

Table 9. Peak area, mass and concentration in ppm of the sample without oat pieces obtained from the gradient elution calibration line.

Comparing the concentration (ppm) of the samples with oats to those without oats in this section is not possible since the mass of the sample is really different. However, what can be confirmed is that as less mass you have the area is smaller since the concentration is also reduced.

4.5. Recovery

First of all, the theoretical Brodifacoum concentration in ppm is calculated.

$$m_{sample} = 6.35 - 125 \cdot 0.792 = 5.36\ g$$

$$theoretical\ mass = \frac{1.25\ mL \cdot 100\ {}^{\mu g}/_{mL}}{5.36\ g} = 23.321\ \frac{\mu g}{g}\ (ppm)$$

Once the samples have been injected, we obtain the area. The concentration can be calculated using the calibration line.

	Mass (g)	Volume (mL)	Area	Concentration (mg/L)	Mass (mg)	mg/g	ppm
Blank sample	5.0	50	395.1	4.7	0.2	0.047	47
Spiked sample	5.4	50	645.1	7.6	0.4	0.071	71
Real sample	5.6	50	133	1.6	0.1	0.014	14

Table 10. Peak area, mass and concentration in ppm of each sample obtained from the gradient elution calibration line.

From the data obtained, the recovery is calculated with the different masses. Firstly, the mass which contains the spiked sample is calculated and then the experimental mass is obtained subtracting the total mass of Brodifacoum found in the sample. Finally, the recovery is calculated dividing the experimental mass in ppm and the theoretical mass in ppm calculated before.

$$m_{found} = 0.381\ mg$$

$$m_{in\ the\ sample} = \frac{0.234 \cdot 5.36}{5.019} = 0.2499\ mg$$

$$m = 0.381 - 0.2499 = 0.131\ mg \rightarrow ppm = 24.480$$

$$\boldsymbol{recovery}(\%) = \frac{24.480}{23.321} \cdot 100 \approx 105\%$$

Figure 19. Calculation of recovery from mass data.

	Mass (g)	Volume (mL)	Area	Concentration (mg/L)	Mass (mg)	mg/g	ppm
Blank sample	5.0	50	406.8	4.6	0.2	0.045	45
Spiked sample	5.4	50	652.1	7.3	0.4	0.068	68
Real sample	5.6	50	137.5	1.6	0.1	0.014	14

Table 11. Peak area, mass and concentration in ppm of each sample obtained from the isocratic elution calibration line.

$$m_{found} = 0.364\ mg$$

$$m_{in\ the\ sample} = \frac{0.364 \cdot 5.36}{5.019} = 0.244\ mg$$

$$m = 0.364 - 0.244 = 0.12\ mg \rightarrow ppm = 22.465$$

$$\boldsymbol{recovery}(\%) = \frac{22.465}{23.321} \cdot 100 \approx 96\%$$

Figure 20. Calculation of recovery from mass data.

There is small difference between the isocratic method and the gradient method, obtaining a lower value for the isocratic method that could be due to the improvement of resolution.

According to the guideline, the recovery rate should be between 70%-130%. So for both methods, recovery value is within the recommended limit.

In addition, as can be seen in tables 10 and 11, the blank sample presents Brodifacoum although it should not have any of the active compound.

5. CONCLUSION

After taking data with the HPLC chromatograph of the Brodifacoum solutions and carrying out the extraction of the active compound in real samples, the data obtained were analysed. The experimental part was carried out by using two different methods, gradient elution and isocratic elution. The purpose of the application of two methods was to improve the resolution in order to obtain better results.

Two mobile phases were used. Mobile phase A composed by HP water and 0.1% TFA and mobile phase B composed of MeOH and 0.1% TFA. For the isocratic method, 15% of mobile phase A and 85% of mobile phase B are used. Therefore, the retention time of Brodifacoum is around 9.26 minutes. On the other hand, for gradient elution the proportion of the different mobile phases varies with time resulting in a retention time of 15.35 min.

Consequently, specificity, linearity, accuracy and precision has been proved to fulfil the requirements of the guidelines and quantify the amount of Brodifacoum in the test item.

As shown before, according to SANCO/3030/99 rev. 5 guidelines, in order to validate specificity, it is necessary to confirm the identity of the compound and provide that there are no interferences. The method proved that does not exit interferences since the peaks of Brodifacoum can be distinguish in every chromatogram independently of the elution used.

Linearity is also proved because the linear regression coefficient of the line is higher than 0.99 for each method, what means that our data fits perfectly to a straight line in the working area.

In addition, precision is evaluated analysing the RSD% coefficient, the average and the standard deviation of the peaks. Although two methods have been tested, for both, RSD (%) value is 8% for a concentration of 1 mg/L and 10% is obtained for a concentration of 10 mg/L. The average is quite different, for isocratic elution 119.7 is obtained while for gradient elution 131.1, for a concentration of 10 mg/L. Something similar happens with the average of 10 mg/L, for isocratic elution is 627.1 and for gradient elution the value is 609.1. The higher the concentration is, the larger the peak area is and therefore higher RSD% and average is obtained.

For the real samples, the %RSD coefficient is 1.8% and the average is 17.84.

Moreover, accuracy is related with the recovery of Brodifacoum in the spike sample. For isocratic method, recovery is 96% and for gradient method is 105%. Recovery value for both methods is within 70%-130%, as suggest the guidelines.

In conclusion, it can be said that the method has been validated successfully due to the validation of the investigated parameters.

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