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BSc in Chemical and Biochemical Engineering

RISK ANALYSIS ASSOCIATED WITH THE REVALIDATION OF ANALYTICAL METHODS IN HPLC

MASTER IN CHEMICAL AND BIOCHEMICAL ENGINEERING
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ABSTRACT

The pharmaceutical industry is responsible for research, development, production, marketing, and distribution of medicines. It is currently an extremely regulated industry, with focus on accuracy, authenticity, and compliance during drug development. Thus, according to ICH (International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use) analytical methods require, before their implementation, validation.

A risk analysis was carried out, using FMECA (Failure Mode Effect and Criticality Analysis), on the 64 methods whose validation date exceeds 10 years, corresponding to 47% of API (Active Pharmaceutical Ingredient) methods, 33% of preservative methods and the remaining 20% of related substances methods. Of the 30 API methods evaluated, 4 presented an unacceptable risk, 16 presented an acceptable risk and the remaining a level of risk that requires a decision. Regarding the impurity methods, only 1 presented an acceptable risk, 3 presented a risk that requires a decision, and the remaining presented an unacceptable level of risk. Finally, in the preservative dosing methods, 3 were evaluated with an unacceptable risk, 6 acceptable and the remaining not acceptable.

Carrying out a mitigation plan, which consisted of understanding whether the methods needed total or partial revalidation, allowed all methods to reach an acceptable risk level.

Following this analysis, two methods were chosen (assay and related substances) to carry out a total revalidation. Since both present poor performances, it was decided to validate a pharmacopoeia method adapted to the finished product in question.

The analytical methods proved to be specific - with no interference detected in the analyte peaks to be quantified -, precise, exact, and linear, in the concentration ranges evaluated.

A robustness study was also carried out by varying the workflow, %ACN (Acetonitrile) in the gradient, mobile phase pH and column oven temperature. The results of the quantification of the analytes are within specification and are quite similar between tests. Therefore, it was concluded that the method is robust.

Keywords: risk analysis, validation, analytical methods, HPLC, pharmaceutical industry, linearity, robustness.

RESUMO

A indústria farmacêutica é responsável pela pesquisa, desenvolvimento, produção, comercialização e distribuição de medicamentos. Atualmente é uma indústria extremamente regulamentada, com foco principal na precisão, autenticidade e conformidade durante o desenvolvimento de medicamentos. Assim, de acordo com o ICH (Conselho Internacional para Harmonização de Requisitos Técnicos para Produtos Farmacêuticos para Uso Humano) os métodos analíticos requerem, antes da sua implementação, uma validação.

Foi realizada uma análise de risco, utilizando a FMECA (Análise dos Modos de Falha, Efeitos e Criticidade), aos 64 métodos cuja data de validação excede os 10 anos, destes 47% correspondem a métodos de doseamento de API (Ingrediente Farmacêutico Ativo), 33% a métodos de doseamento de conservantes e os restantes 20% a métodos de impurezas. Dos 30 métodos de API avaliados, 4 apresentaram risco não-aceitável, 16 apresentaram risco aceitável e os restantes um nível de risco que requer decisão. Em relação aos métodos de impurezas, apenas 1 apresentou risco aceitável, 3 apresentaram risco que requer decisão e os demais apresentaram um nível de risco não-aceitável. Por fim, nos métodos de doseamento de conservantes, 3 foram avaliados com risco não-aceitável, 6 com risco aceitável e os restantes com risco não-aceitável.

A realização de um plano de mitigação, que consistiu em entender se os métodos necessitavam de revalidação total ou parcial, permitiu que todos os métodos atingissem um nível de risco aceitável.

Após esta análise, foram escolhidos dois métodos (doseamento e impurezas) para realizar uma revalidação total. Dado que ambos apresentam um desempenho fraco, optou-se por validar um método de farmacopeia adaptado ao produto acabado em questão.

Os métodos analíticos mostraram-se específicos - sem interferência detetada nos picos do analito a serem quantificados -, precisos, exatos e lineares, nas gamas de concentração avaliadas.

Foi também realizado um estudo de robustez variando o fluxo de trabalho, %ACN (Acetonitrilo) no gradiente, pH da fase móvel e temperatura do forno da coluna. Os resultados das quantificações dos analitos encontram-se dentro da especificação e são bastante similares entre ensaios. Deste modo, concluiu-se que o método é robusto.

Palavras-chave: análise de risco, validação, métodos analíticos, HPLC, indústria farmacêutica, linearidade, robustez.

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ACRONYMS

API – Active Pharmaceutical Ingredient

ACN – Acetonitrile

DoE – Design of Experiments

DL – Detection Limit

FMECA – Failure Mode Effect and Criticality Analysis

FMEA – Failure Mode and Effect Analysis

FTA – Fault Tree Analysis

HPLC – High Pressure Liquid Chromatography

HACCP – Hazard Analysis and Critical Control Points

ICH - International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use

OOS – Out of Specification

PDE – Permitted Daily Exposure

Ph. Eur. – European Pharmacopeia

QL – Quantification Limit

RSD – Relative Standard Deviation

SD – Standard Deviation

USP – US Pharmacopeia

1

INTRODUCTION

1.1. EDOL

Laboratório EDOL, Produtos Farmacêuticos, S.A. is a privately owned and 100% portuguese company that is part of the EDOL group together with VAPP (Veterinária para Animais de Pequeno Porte), GIDE, S.A. – pharmaceutical consulting services company and, Farmacêutica AUSTRAL – pharmaceutical wholesale distribution service company [1].

Laboratório EDOL started its activity in 1952, starting from a pharmacy, having built the first factory unit in 1962, in Linda-a-Velha. In 2005 it began looking for new markets, starting the process of internationalizing its products and, currently exports to more than 40 countries across Europe, Asia, Africa, and North America. The company's growth led to the construction of a new factory in 2008, in Carnaxide, and in 2022 production began at this new unit, with both factories currently operating [1].

The company develops, produces, and sells medicines, medical devices and cosmetics and body care products, inserted in the areas of ophthalmology, dermatology, gynaecology, and otorhinolaryngology. Being the products presented in the following pharmaceutical preparations: eye drops, ophthalmic ointments, ophthalmic gels, ophthalmic comfort solutions, ear solutions, creams, ointments, lotions, suspensions, and shampoos [1].

Currently, Laboratório EDOL is a leading company in the national ophthalmology market and is also a strong presence in Dermatology and Otorhinolaryngology, producing around 5 million units per year [1].

1.2. Motivation

The pharmaceutical industry is responsible for research, development, production, marketing, and distribution of medicines [2]. It is currently an extremely regulated industry, with main focus on accuracy, authenticity and compliance during drug development. Quality control is extremely important so that the quality, safety, efficacy, and credibility of the products are ensured [2,3].

The set of operations whose objective is to verify and ensure that the products are within the required quality standards is known as quality control [2]. This department is responsible for the sampling, analysis and control of the products, from the beginning of their production, with the analysis of the raw material, to the finished product, being also responsible for the control tests during the manufacturing process [3] .

Chemical, physical, and microbiological tests are carried out throughout the manufacturing process. These analytical tests are detailed by the pharmacopoeia in force according to the products destination markets and their respective regulations (US Pharmacopeia – USP – and

European Pharmacopeia – Ph. Eur. – for example), reducing the variability between companies and giving more confidence to the results.

According to ICH (International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use) [4] analytical methods require, before their implementation, validation according to guideline Q2(R1) [5] . The aim of any analytical method is to provide consistent, reliable, and accurate data [6], thus the objective of validating an analytical procedure is to demonstrate that it is suitable for its intended purpose [5] .

1.3. Objectives

This curricular internship, carried out within the scope of the Dissertation in Chemical and Biochemical Engineering, has as its main objective the elaboration of a risk analysis of analytical methods whose data validation exceeds 10 years, in order to assess the need to develop new methods or to revalidate the existing ones. Once the risk analysis is completed, an analytical method is then chosen to be revalidated.

This document is divided into three main parts:

- 1st part – Introduction to the theme and concepts necessary for its development, comprising chapters 1 and 2.
- 2nd part – Methodology, of which chapter 3 is part.
- 3rd part – Presentation and discussion of the final results and conclusions, comprising chapters 4 and 5.

2

STATE OF THE ART

2.1. HPLC – High Pressure Liquid Chromatography

According to O. Coskun (2016), chromatography is a biophysical technique that allows the separation and identification of the components of a mixture for qualitative and quantitative purposes [7] .

In this process, the sample of a mixture is distributed between two phases, one stationary and the other mobile. The separation of the components present in the mixture happens due to the type of interactions between the mobile phase, the stationary phase and the molecules of the mixture themselves [7] . The mobile phase can be gaseous - Gas Chromatography - or liquid - Liquid Chromatography.

Chromatography was discovered at the beginning of the 20th century and during its 100-year evolution went through many different stages, becoming the most widely used analytical separation technique in chemistry and biochemistry [8] . The technique did not undergo any significant evolution until the 1940s, when partition and paper chromatography was developed, which was followed by the development of gas and thin layer chromatography, as well as various size exclusion methods [9] .

In classical column chromatography the columns were not reusable, being packed at each utilization, thus the success of the separation depended on the skill of the operator. Furthermore, the fact that the solvent flow was mainly caused by gravity slowed down the process [9] . Usually several fractions are collected manually and their processing requires time and effort [9] .

With the advent of HPLC, reusable columns began to be used, making it possible to carry out numerous separations in the same column. Therefore, investments were made in packing the columns in order to obtain better results [9] . The developed system involved high pressures, sample injection at such pressures, controlled and rapid solvent flow through the columns and continuous monitoring detectors without the operator intervention [8,9] .

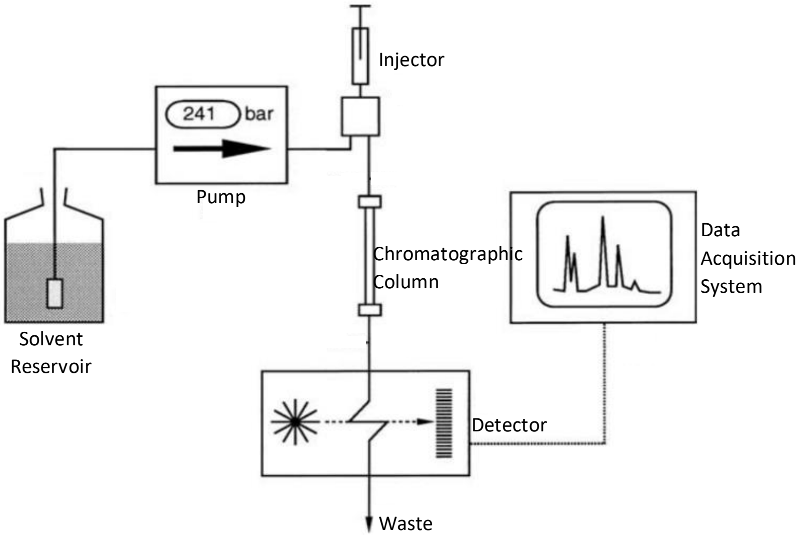

Figure 1. *Schematic diagram of a typical HPLC system, adapted from Meyer, V. R. (2010)* [10] .

2.1.1. HPLC System

The main components of a typical HPLC system are:

- **Pumping system**
  The pumping system supplies the mobile phase with a constant flow. It must be capable of generating a pressure of up to 350 or even 500 bar and it should limit pressure variations as much as possible. The pumps can be equipped with a purge system to eliminate the incorporated air bubbles. It also has to be able to accurately/precisely release a mobile phase of constant composition (isocratic elution) or variable (gradient elution) [10,11] .

- **Injector**
  The injection system must be designed to operate at high pressures. The injection of samples can be done manually or with an automated injection system (autosampler) [11] .

- **Chromatographic column**
  The predominant chromatographic technique, regarding HPLC, is reversed phase chromatography, where a non-polar stationary phase with polar mobile phases is used [8] .

  Columns should be designed so that the sample is in contact with the solid phase as much as possible. There are several ways to increase this contact, for example by simply increasing the column size. Another possibility is the size decreasing of the particles that constitute the solid phase [12]. Decreasing the size of the particles increases the performance of the column as it increases the area of solid phase exposed to the sample and decreases the volume of solvent between the particles. However, decreasing particle size increases column back pressure. Thus, the ideal is to use stationary phases that withstand the high operating pressures [12].

  The most commonly used column packings for reverse phase separation are silica particles bonded to carbon chains (octadecyl (C-18), octyl (C-8)), phenyl, or cyanopropyl [9]. These modified silica particles can be used to separate most molecules of interest, being the most used in HPLC methods [12].

- **Detector**

The detector must be able to recognize when a component is eluted from the column. To do so, it must monitor the change in the composition of the mobile phase and convert it into an electrical signal. The most used detectors are ultraviolet/visible (UV/Vis) spectrophotometers, including diode array detectors. Fluorescence spectrophotometers, differential refractometers (RI), electrochemical detectors (ECD), light scattering detectors (ELSD), charged aerosol detectors (CAD), mass spectrometers (MS), radioactivity detectors, multi-angle light scattering (MALS) or other detectors [10,11].

- **Data acquisition and processing system**

Computer program that allows the collection and processing of data collected by the detector.

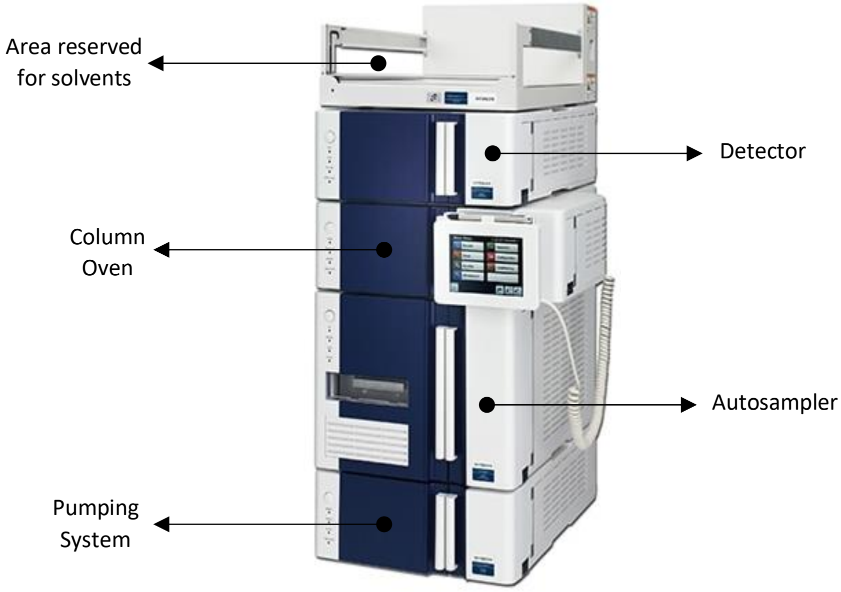

Figure 2. *Diagram of the HITACHI Chromaster equipment, adapted from HITACHI* [13].

2.1.2. Chromatographic Parameters

The chromatographic separation results in a chromatogram, which is generally a graphical representation of the detector's response, as a function of time, volume or distance, ideally presented as a sequence of Gaussian peaks on a baseline [14].

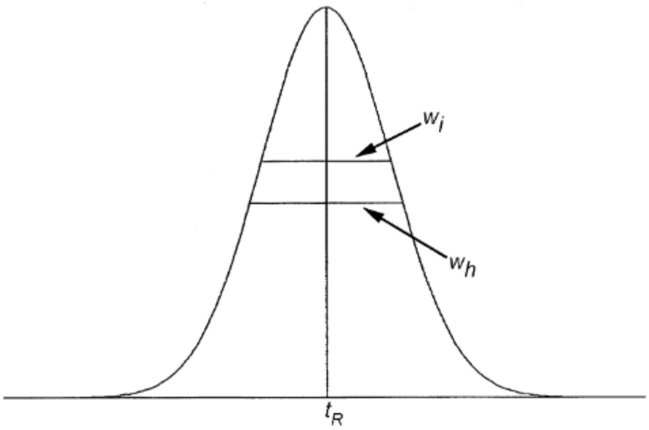

Figure 3. *Example of a Gaussian peak, taken from Ph. Eur. (2005)* [14] *.*

A peak can be defined by its area (*A*), or by its height (*h*) and its width at half height (w_h), or by its height and its width at the inflection points (w_i) [14] . For Gaussian peaks it is possible to establish the following relation:

$$w_h = 1.18w_i \qquad (1)$$

Analysing the chromatograms obtained, considering several parameters – chromatographic parameters – it is possible to evaluate the separation.

- **Retention Time and Volume (t_R / V_R)**

  The retention time corresponds to the period between the sample injection and the detection of the signal maximum [10]. The retention time of a component is always constant under identical chromatographic conditions [10]. However, small changes in the operating variables of the method can seriously affect the retention time [15]. One of the most common causes of retention time variation is the concentration of organic in the mobile phase, with small changes in column temperature and mobile phase flow also causing variations [15].

  From the retention time, the retention volume can be calculated:

  $$V_R = t_R v \qquad (2)$$

  Where v is the mobile phase flow rate.

- **Symmetry Factor**

  The symmetry factor (A*s*) of a peak is a parameter that characterizes the shape of the peak [16]. According to the European Pharmacopoeia (EP), it must present values between 0,8 and 1,5 [17], with 1,0 being assigned to fully symmetrical peaks.

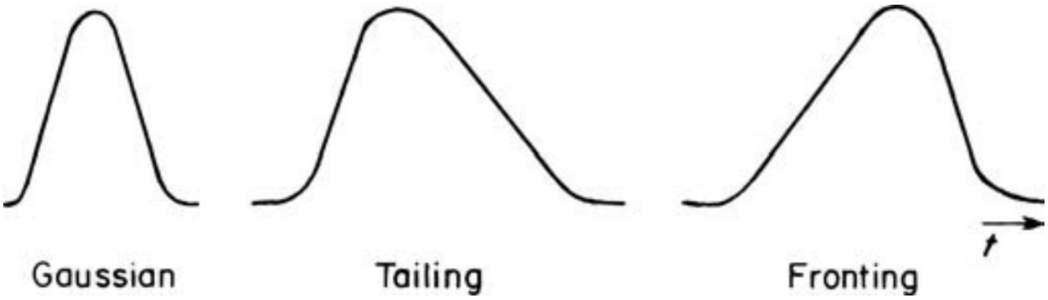

Figure 4. *Peak shapes, taken from Meyer, V. R. (2010)* [10].

The symmetry factor of a peak is calculated by the following expression:

$$A_s = \frac{w_{0.05}}{2d} \qquad (3)$$

Where $w_{0.05}$ is the width of the peak at one-twentieth of its height and *d* is the distance between the perpendicular drawn from the maximum of the peak and the point located on the ascending branch of the same peak at one-twentieth of its height [14].

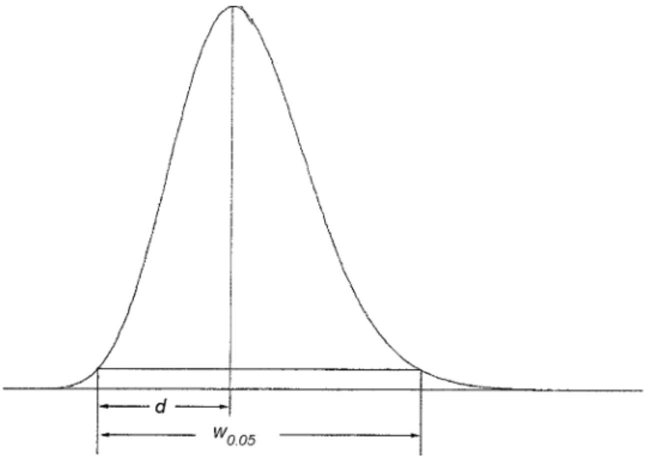

Figure 5. *Indication of how to calculate the symmetry factor, taken from Ph. Eur. (2005)* [14].

In addition to being more difficult to integrate very asymmetrical peaks, the asymmetry of the peaks reduces the column plate number, affecting the resolution. Therefore, the cause of the asymmetry must always be identified and eliminated [10] .

A poorly packed or deteriorated column, extra-column volumes, a column overload, and an incompatibility of sample with the stationary and/or mobile phase, are some of the causes for peak asymmetry [10] .

- **Column Efficiency and Apparent Number of Theoretical Plates**

The apparent number of theoretical plates is a parameter used in all types of chromatography to measure column efficiency [18], by measuring the peak dispersion on the HPLC column [19]. A column with a greater number of theoretical plates is capable of separating mixtures with very similar components [10].

$$N = 5.54 \left(\frac{t_R}{w_h}\right)^2 \qquad (4)$$

Where t_R and w_h must be expressed in the same unit (time, volume or distance) [14].

The apparent number of theoretical plates depends on the component to be analysed, the column used (packaging and length) and the mobile phase flow rate conditions [10,14].

- **Relative Retention**

The relative retention (*r*), or separation factor, is a measure of the chromatographic system's potential for separating two compounds and is affected by stationary and mobile phases [10]. It's calculated from the expression:

$$r = \frac{t_{R2} - t_M}{t_{R1} - t_M} \qquad (5)$$

Where t_{R1} is the retention time of the standard peak, t_{R2} is the retention time of the peak of interest and t_M is the retention time or distance over the baseline between the injection point and the perpendicular lowered from the maximum of the peak corresponding to an unretained component [14].

The uncorrected relative retention (r_G) is given by:

$$r_G = \frac{t_{R2}}{t_{R1}} \qquad (6)$$

Unless otherwise indicated, the relative retention values indicated in the monograph correspond to the uncorrected relative retention.

If $r = 1$, then no separation occurs, since the retention times are identical [10].

- **Resolution**

The resolution (Rs) between two peaks of similar height can be calculated by [14]:

$$R_S = \frac{1.18(t_{R2} - t_{R1})}{w_{h1} + w_{h2}} \qquad (7)$$

Where $t_{R1} < t_{R2}$, corresponding to the retention times between the injection point and the perpendiculars drawn from the maxima of two adjacent peaks. And w_{h1} and w_{h2} are the peak widths at half height [14].

A resolution greater than 1,5 corresponds to separation to the baseline, and a value of 1,0 corresponds to peaks that are not completely separated [10].

The resolution is clearly dependent on the separation factor, since this represents the selectivity of the chromatographic system, where the value is higher when the analysed components interact differently with the mobile and/or stationary phase [10,19]. The separation factor can easily be improved by changing the constituents and/or pH of the mobile phase, but also by changing the column chemistry [19] .

The retention factor (*K*) is a parameter that measures the retention of an analyte in the chromatographic column, and it also influences the resolution [19]. A high *K* value indicates that the sample is highly retained. The most effective way to change the retention factor, in reverse phase chromatography, is to alter the amount of organic solvent in the mobile phase [19], changing its "strength" [10].

Finally, increasing column efficiency (number of theoretical plates) narrows the peaks, thus improving resolution [10]. As previously mentioned, one of the causes for weak efficiency is the column itself therefore, acting on column conditions is one way to change the resolution [19].

- **Signal-to-Noise Ratio**

The aspect of the baseline noise is one of the most useful indicators of the system overall performance [20]. However, there is always a minimum noise level despite good grounding and the use of the most modern equipment [10].

The signal-to-noise ratio (*S/N*) influences the quantification accuracy and is calculated from the next equation [14]:

$$S/N = \frac{2H}{h} \qquad (8)$$

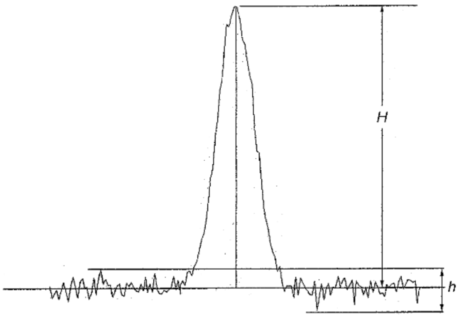

Figure 6. *Indication of how to calculate signal-to-noise-ratio, taken from Ph. Eur. (2005)* [14] .

Where *H* is the height of the peak corresponding to the component considered in the chromatogram obtained with the indicated standard solution, measured between the summit of the peak and the extrapolated baseline of the observed signal over a distance equal to 20 times the width of the peak at half height, and *h* the background noise amplitude in a chromatogram obtained after injection of a blank, observed at a distance equal to 20 times the width at half height of the peak of the chromatogram obtained with the indicated standard solution, and if possible centred on the place where the peak would be located [14] .

Abnormalities in the baseline noise can be attributed to a variety of factors, however, the mobile phase is the more common factor, due to impurities, bubbles and, change in flow rate [10,20] .

- **Repeatability**

The repeatability of the response is expressed, in percentage, by the relative standard deviation (RSD) calculated from the results of a series of consecutive measurements carried out by injection or application of a standard solution, through [14] :

$$RSD_{\%} = \frac{100}{\bar{y}} \sqrt{\frac{\Sigma(y_i - \bar{y})^2}{n-1}} \qquad (9)$$

Where *n* is the number of individual values, y_i the individual values and $\bar{y}$ the average of the individual values.

2.1.3. System Suitability

As previously mentioned, there are several factors that affect the chromatographic system, such as the properties of the mobile and stationary phases and the system parameters (temperature and pressure, for example). Thus, to ensure that the system is suitable for the analysis to be carried out, the system suitability is evaluated before and during the performance of any analysis [21].

Both USP and ICH agree on the importance of performing system suitability tests, but neither establishes the tests to be performed, leaving it up to the chromatographer [22]. Resolution, column efficiency, peak symmetry, and system repeatability (RSD of peak area and retention time) are usually evaluated [23]. Such parameters are evaluated through several injections of a standard solution.

To guarantee the correct preparation of the standard solution, and thus attribute a greater degree of confidence to the results obtained, it must be prepared in duplicate to carry out a counter-verification. In Laboratório EDOL, Produtos Farmacêuticos, S.A., this verification is done through the response factor (*RF*), which must present values between 98-102% for API and preservative assay and, between 95-105% for related substances.

$$RF = \frac{R_c}{R_w} \times \frac{C_w}{C_c} \times 100 \qquad (10)$$

Where R_c and R_w are the average response of control and work standards and, C_w and C_c are the concentrations of working and control standards.

2.2. Risk Management

As reported by ICH, quality risk management is a systematic process for the assessment, control, communication, and review of risks to the quality of the drug product across the product lifecycle, being the combination of the probability of harm occurring and the severity of that harm the definition of risk [24]. To assess the risk, the consequences or damage associated with the event under consideration must also be considered.

Risk assessment is not an easy or straightforward process, since the probability of occurrence and the consequence of occurrence are parameters that are not directly measured and must be estimated by judgment, statistics, or other procedures [25]. In this way, subjectivity directly affects risk management and the decisions made, which is why this subjectivity must be minimized as much as possible [24].

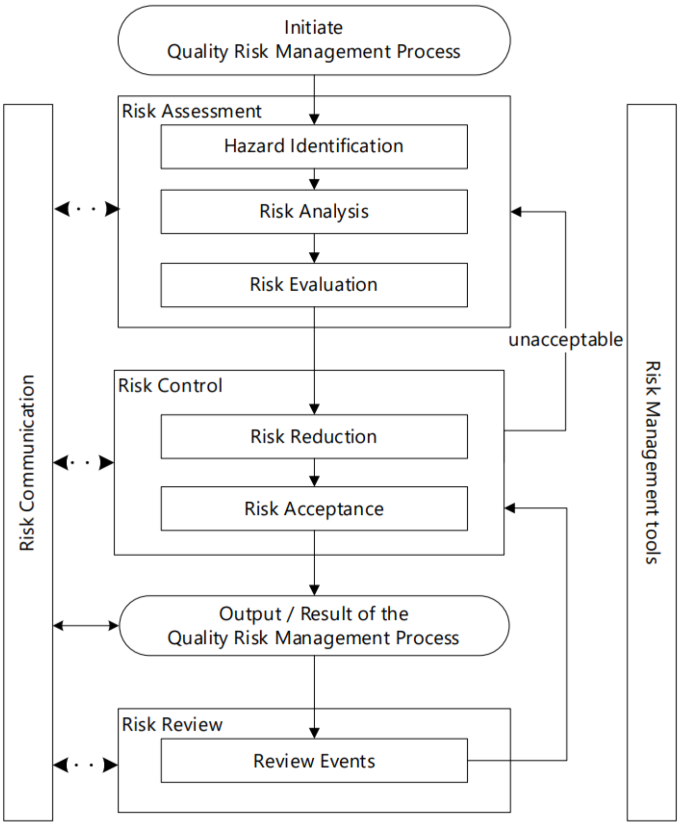

Figure 7. *Typical quality risk management process, taken from ICH* [24] .

2.2.1. Risk Management Tools

There are several tools/methodologies that contribute to obtaining knowledge and detail about the assessment to be considered. The tool or set of tools used depends on case to case. Below are some of the methodologies that can be applied.

- **Flowcharts**
- **Process Mapping**
- **Cause and Effect Diagrams**

- **Failure Mode and Effect Analysis (FMEA) and Failure Mode Effect and Criticality Analysis (FMECA)**

FMEA is a systematic method that allows to identify, evaluate and prevent possible failure modes, and their effects, in processes before they occur, being used in various production industries and at various stages of the product life cycle [26] .

The FMEA can be extended to incorporate an analysis of the degree of severity of the consequences, their probabilities of occurrence and detectability, thus becoming Failure Mode Effect and Criticality Analysis (FMECA). FMECA evaluate the risk failure through the risk priority number (RPN), which is the product of the occurrence (O), severity (S) and detection (D) of a failure [26].

- **Fault Tree Analysis (FTA)**

FTA is an analytical technique that assumes failure of the functionality of a product or process [27]. This tool evaluates system or sub-system failures one at a time but can combine multiple causes of failure by identifying causal chains [24]. The results are presented in a graphical representation of logical relationships between the undesired event and the basic fault events [27].

- **Hazard Analysis and Critical Control Points (HACCP)**

HACCP is a systematic, proactive, and preventive tool for assuring product quality and safety, being an approach that applies technical and scientific principles to analyse, evaluate, prevent, and control the risk or adverse consequences of hazards due to the design, development, production, and use of products [24,28] .

- **Statistical Support tools**

In order to make decision making more reliable and facilitate the evaluation of the data set, there are several statistical tools that are very useful in risk management. For example, control charts, design of experiments (DoE), histograms, pareto charts, process capability analysis, etc [24] .

2.2.2. Hazard Identification

A hazard is defined as an intrinsic trait or attribute that has the potential to harm the process and, consequently, the end user [29]. To identify these harms and their consequences, historical information, theoretical analysis, brainstorming sessions, etc., are generally used [25,29]. The questions to which we generally try to find answers are: what might go wrong? What is the probability it will go wrong? What are the consequences? [24]

2.2.3. Risk Analysis

Risk analysis consists on the estimation of the risk associated with the identified hazards [24] . It is a qualitative or quantitative process that takes into account the probability of occurrence and severity of harms, with some tools also determining detectability (ability to detect the harm) [24,29]. Risk analysis provides guidance on where the greatest vulnerabilities lie.

After the risk analysis, the risk evaluation is carried out, which consists of comparing the risk obtained against a given risk criteria.

2.2.4. Risk Control

After risk analysis, decisions need to be made. The decision to reduce and/or accept risks is part of risk control [24] . The objective of risk control is to reduce the risk to an acceptable level, for cases that have been classified as "not acceptable", and to this end there are several strategies [25] :

- Mitigation

  Consists of defining actions to reduce the level of risk and can be done by reducing the probability of occurrence and/or increasing detectability, since, generally, the severity cannot be changed.
- Eliminate

  Define actions to ensure that the risk does not occur.
- Transfer

  Delegate risk responsibility to another entity.

For cases where the risk is classified as "acceptable" there are two options: not taking any measures to control it, deliberately accepting its existence; or monitor it in order to identify any changes that could alter the risk classification [25] .

2.2.5. Risk Reanalysis

After implementing the mitigation plan, a risk review is carried out, which in principle will lead to a new risk classification. If unacceptable classification values continue to exist, the mitigation plan must be re-evaluated [24].

The risk management process must be a continuous process and must continue to be used and updated. The results obtained must be reanalysed considering new knowledge and experience [24].

2.3. Validation of Analytical Methods in HPLC

According to ICH, the purpose of validating an analytical method is to demonstrate that it is suitable for the intended purpose [5]. Validation of an analytical method confirms that it is specific, linear, precise, accurate and sensitive [30].
All methods used must be duly validated and the necessary validation parameters depend on the type of method to be validated [30].

Table 1 shows the parameters to be validated according to the type of method, according to the ICH [5].

***Table 1**. Parameters to be validated, adapted from ICH (2005) [5].*

	Identification	Related Substances	Assay
Accuracy	-	+	+
Precision			
Repeatability	-	+	+
Intermediate Precision	-	+	+
Specificity	+	+	+
Detection Limit	-	+	-
Quantitation Limit	-	+	-
Linearity	-	+	+
Range	-	+	+

- characteristic is not normally evaluated
+ characteristic is normally evaluated

It is important to mention that this list of parameters is mandatory to validate, but other parameters can and should be validated, being the decision on the parameters to be validated up to the person responsible for validating the method.

- **Specificity / Selectivity**

The main objective of validating this parameter is to ensure that the analytical method can differentiate and quantify the analyte under study in the presence of other expected components in the sample matrix, and that the result is not affected by the presence of these components [5,31].

- **Linearity**

The linearity assessment is a really important parameter to validate for methods of API assay and quantification of impurities [32].

According to ICH [5]: the linearity of an analytical method corresponds to its ability to obtain results, in a given work set, which are directly proportional to the concentration of the analyte in the sample.

The evaluation of the linearity of the method is made considering the following statistical methods:

1) Correlation coefficient of the straight line (r^2): must be greater than 0,9950.
2) Residual analysis: it should be verified visually that the distribution of the residuals is random [33].
3) Mandel Test: this test compares linear and polynomial regressions. Generally, polynomial regression fits better than linear regression, therefore, the aim of this

test is to demonstrate that linear regression does not present a significant difference compared to polynomial. For this, an F test is used with a null hypothesis that there are not significant differences between the residual variances of both regressions [34].

4) Rikilt Test: This test serves to evaluate the relative response factors (RRF) obtained for the method, and these should not vary significantly throughout the working range [35,36] . Internally, these values must be between 98.0 and 102.0% for assay methods and between 90.0 and 110% for related substance methods.

- **Accuracy**

The accuracy of an analytical procedure expresses the proximity between the real value, or the value accepted as the reference value, and the value obtained by the method [5]. It is an essential parameter, however with little meaning in the absence of precision [37]. Accuracy must be determined taking into account the working range established for the method and must be reported as the percentage recovery obtained between the concentrations obtained by the method and the previously known nominal concentrations [5,35].

- **Precision**

All measurements contain random error or noise that cannot be removed, and one way to assess this is by taking a series of measurements from the same sample and calculating the standard deviation, or variance, or coefficient of variation [38] .

The precision of an analytical method expresses the degree of dispersion between a series of measurements obtained from multiple sample preparations prepared from the same homogeneous sample, under the prescribed conditions [5].

Precision can be assessed at three levels, namely repeatability, intermediate precision, and reproducibility. Repeatability expresses precision under the same operating conditions in a short time interval and is subdivided into two levels: system repeatability (dispersion obtained for a series of signals obtained for the same sample) and analytical repeatability (dispersion obtained for a series of sample results prepared from the same batch) [5]. Intermediate precision translates the dispersion obtained considering the influence of intra-laboratory variations according to the intended use of the procedure, with the operator, equipment and day varying [39]. Finally, reproducibility expresses the precision obtained between laboratories.

- **Detection / Quantitation Limits**

No method is capable of accurately measuring analyte concentrations down to zero; there must be a sufficient concentration to produce a signal that can be distinguished from noise [40] . Therefore, the detection limit (DL) corresponds to the lowest concentration of analyte present in a sample that is detectable by the method. However, this concentration is not necessarily quantifiable with an adequate level of accuracy and precision [5,35]. The quantitation limit (QL) corresponds to the lowest concentration of analyte that can be quantified with accuracy and precision [5,35] .

These limits can be estimated in different ways: based on signal-to noise, based on accuracy and precision measurements and based on the standard deviation of a linear response and a slope [5,35] .

Considering the based on the standard deviation of a linear response and a slope approach:

$$DL = \frac{3.3\sigma}{S} \qquad (11) \qquad\qquad QL = \frac{10\sigma}{S} \qquad (12)$$

Where σ is the standard deviation of the response and S the slope of the calibration curve.

- **Robustness**

The robustness of an analytical method is a measure of its ability to maintain results unchanged in the face of small but deliberate variations in method parameters and provides an indication of its reliability during routine use [5,35]. Generally, the study of the robustness of a method is carried out during the development of the method, as it is essential for its optimization. This study is also very important regarding troubleshooting problems, since knowledge of the factors that affect a particular method can be very useful when the method is not performing as expected [41].

Factors such as column oven temperature, flow rate, wavelength, mobile phase composition, injection volume, column manufactured, etc., can be used to understand their effects on analytical parameters [41].

3

METHODOLOGY

3.1. Risk Analysis Rational

Risk identification: classify a nonconforming product as conforming, or vice versa, due to the inadequacy of the analytical method used.

3.1.1. Probability / Occurrence

Regarding the risk assessment of probability, several factors related to the historical performance of the method, the current state of validation of the method and also the state of the art of the method itself will be taken into account.

Table 2. *Risk level for probability / occurrence, taken from company internal documents.*

Probability / Occurrence	Risk Level
Remote probability of occurrence	1
Low probability of occurrence	3
Average probability of occurrence	5
High probability of occurrence	7
Very high probability of occurrence	9
Almost certain probability of occurrence	11

- **Method Performance**

To evaluate the performance of the method, historical data was collected for each method regarding column efficiency, symmetry factor, resolution, OOS's and RSDs of peak areas, retention times and between samples.

The evaluation of these parameters, if there is sufficient historical data, was carried out through the analysis of the process capacity (Cpk) and the probability/occurrence classification was made considering the Cpk value obtained for each one of them. In general, the higher the Cpk value, the better. A value lower than 1.0 indicates that the process is not capable, between 1.0 and 1.3 it is considered somewhat capable and greater than 1.3 is considered capable. A Cpk greater than 2.0 implies that the process only uses 50% of the specification width, significantly lowering the risk of an error occurring [42].

However, this analysis of the process capacity can only be applied to data that follow a normal distribution, so first several statistical tests were performed to verify the normality of the data distributions. The chosen tests were: qq-plot [43–45], Shapiro-Wilk, and Kolmogorov-Smirnov. Data whose tests indicate that they do not follow a normal distribution was submitted to a box-cox transformation [46] so that the Cpk analysis can also be performed.

Table 3. *Probability / occurrence rational – method performance.*

	Probability / Occurrence					
	Criteria			**Specification**	**Classification**	**Rational**
Method Performance	Column Efficiency	20 or more points	Capacity Analysis (Cpk)	2.0 ≤ Cpk	1	In the case where there are enough points (20 or more) the capacity analysis is carried out, both for normal data distributions and for non-normal distributions (after data transformation). In case there is not enough historical data, a worst-case scenario approach is assumed, and the highest level of risk is assigned. The RSD between samples does not apply to impurity methods and, on the contrary, the resolution only applies to impurities. About resolution, if the method does not refer to this parameter, a risk level of 11 will be assigned, as this is an important parameter in this type of method.
				1.3 ≤ Cpk < 2.0	3	
				1.0 ≤ Cpk < 1.3	7	
				0.0 ≤ Cpk < 1.0	9	
				Cpk < 0.0	11	
		Less than 20 points		Insufficient historical data	11	
	Symmetry Factor	20 or more points	Capacity Analysis (Cpk)	2.0 ≤ Cpk	1	
				1.3 ≤ Cpk < 2.0	3	
				1.0 ≤ Cpk < 1.3	7	
				0.0 ≤ Cpk < 1.0	9	
				Cpk < 0.0	11	
		Less than 20 points		Insufficient historical data	11	
	Peak Area RSD	20 or more points	Capacity Analysis (Cpk)	2.0 ≤ Cpk	1	
				1.3 ≤ Cpk < 2.0	3	
				1.0 ≤ Cpk < 1.3	7	
				0.0 ≤ Cpk < 1.0	9	
				Cpk < 0.0	11	
		Less than 20 points		Insufficient historical data	11	
	Retention Time RSD	20 or more points	Capacity Analysis (Cpk)	2.0 ≤ Cpk	1	
				1.3 ≤ Cpk < 2.0	3	
				1.0 ≤ Cpk < 1.3	7	
				0.0 ≤ Cpk < 1.0	9	
				Cpk < 0.0	11	
		Less than 20 points		Insufficient historical data	11	
	Samples RSD	20 or more points	Capacity Analysis (Cpk)	2.0 ≤ Cpk	1	
				1.3 ≤ Cpk < 2.0	3	
				1.0 ≤ Cpk < 1.3	7	
				0.0 ≤ Cpk < 1.0	9	
				Cpk < 0.0	11	
		Less than 20 points		Insufficient historical data	11	
	Resolution	20 or more points	Capacity Analysis (Cpk)	2.0 ≤ Cpk	1	
				1.3 ≤ Cpk < 2.0	3	
				1.0 ≤ Cpk < 1.3	7	
				0.0 ≤ Cpk < 1.0	9	
				Cpk < 0.0	11	
		Less than 20 points		Insufficient historical data	11	
	OOS due to lack of method performance			0	1	The existence of an out-of-specification result due to an error in the analytical method represents a high risk.
				1 or more	11	

In case there is not enough historical data, a worst-case scenario approach is assumed, and the highest level of risk is assigned.

Resolution is a parameter that was only considered for impurity methods and, in case the method does not present resolution, it was classified with the highest level of risk, since it is an extremely important parameter in this type of methods.

Finally, the existence of an out-of-specification result (OOS) due to analytical error was evaluated. To this end, records of OOSs from recent years was collected.

The classification to be attributed to the performance of the method corresponds to the arithmetic mean of the classifications attributed to all parameters.

- **State of the Art – Method Validation**

The state of the art of method validation will be evaluated according to the different parameters that constitute the method validation process. To this end, the existence (or not) of each of these will be confirmed in the method validation report. An analytical method whose validation includes all the parameters listed below presents greater confidence in the results obtained.

The existence of validation parameter corresponds to the lowest risk level and non-validation to the highest. However, parameters such as linearity and accuracy lack an intermediate level in case parameter validation has been performed, but the working range used was not adequate.

The classification to be attributed to the method validation state of the art can be calculated through the arithmetic mean of the classifications attributed to all parameters or using a weighted average, where the most important parameters have greater weight. Using this weighted average will lead to a slightly lower rating when compared to the arithmetic mean, as the most important parameters are in fact almost always validated. Thus, the most conservative approach will be to consider the arithmetic mean.

Table 4. *Probability / occurrence rational - state of the art of method validation.*

	Probability / Occurrence				
	Criteria		Specification	Classification	Rational
Method Validation - State of the Art	Selectivity		Yes	1	Having carried out the validation of each of the parameters, the lowest risk level is assigned. In case the parameter under study has not been validated, the highest level of risk is assigned. For linearity and accuracy, it is necessary to check the working range used and, if this is not adequate, an intermediate risk level is assigned. Both detection / quantitation limits and relative response factor concern impurity methods only.
			No	11	
	Linearity	Correlation coefficient of the line	Yes	1	
			Yes, with limitations	3	
			No	11	
		Residual analysis	Yes	1	
			Yes, with limitations	3	
			No	11	
		Mandel Test	Yes	1	
			Yes, with limitations	3	
			No	11	
		Rikilt Test	Yes	1	
			Yes, with limitations	3	
			No	11	
	Precision	System repeatability	Yes	1	
			No	11	
		Analytical repeatability	Yes	1	
			No	11	
		Intermediate precision	Yes	1	
			No	11	
	Accuracy		Yes	1	
			Yes, with limitations	3	
			No	11	
	Detection and Quantification limits*		Yes	1	
			No	11	
	Robustness		Yes	1	
			No	11	
	Forced degradation tests		Yes	1	
			No	11	
	Solutions stability		Yes	1	
			No	11	
	Variances homogeneity		Yes	1	
			No	11	
	Relative response factor*		Yes	1	
			No	11	

*Only if applicable

- **State of the Art - Method**

The fact that a current method presents a very complete validation does not mean that it isn't outdated, so it is also necessary to evaluate the state of the art of the method. For this, two factors will be considered: the technology used and knowledge of the substance.

Regarding technology, the existence of more advanced technology in the company than the one currently used in the method was evaluated. If it exists, a risk level corresponding to 9 is attributed, since the use of an older technology (less sensitive) may result in a greater probability of errors in the results.

The substance knowledge level was applied to impurity methods only, as the fact that if more known impurities were identified since the time when the method was developed indicates that the method is outdated and is assigned the highest risk.

Table 5. *Probability / occurrence rational - state of the art of the method.*

	Probability / Occurrence			
	Criteria	**Specification**	**Classification**	**Rational**
Method State of the Art	Most advanced technology	Yes	9	The existence of more advanced technology in the company than that currently used in the method corresponds to a risk level of 9.
		No	1	
	Substance Knowledge	Equal	1	In the case where the level of knowledge of the substance remains the same as it was when the method was developed, 1 is assigned. In the case where the level of knowledge has been updated, a value of 11 is assigned, since the method is outdated.
		Bigger	11	

The state of the art of the method classification for API and preservative assay methods is the same as the rating obtained for the “most advanced technology” parameter. For impurity methods, the arithmetic mean of the two parameter ratings is performed.

Finally, the classification assigned to the probability / occurrence corresponds to the arithmetic mean of the three parameters considered: method performance, state of the art of the method validation and, estate of the art of the method.

3.1.2. Severity

The risk associated with the severity was evaluated differently for the different types of methods to be analysed.

Table 6. *Risk level for severity, taken from company internal documents.*

Severity	Risk Level
Insignificant severity	1
Minor severity	3
Low severity	5
Average severity	7
High severity	9
Very high severity	11

- **Analyte Type**

For API assay methods, the risk is associated with the toxicity of the API, and the greater this is, the greater the risk. In this way, the permitted daily exposure (PDE) of the active substance will be assessed.

In preservative assay methods, the factor that must be evaluated is the microbiological requirement of the drug product. Preservatives are used to prevent the growth of bacteria, fungi, and other microorganisms [47], therefore, the use of preservatives in a sterile product serves to maintain its sterility. Thus, the occurrence of an error in the results of a method of this type is more serious in a sterile product.

Regarding impurity methods, a conservative approach will be taken, considering the highest risk level, since, if there is any error in the results obtained from an unknown impurity, not knowing its structure, its effects will also be unknown.

Table 7. *Severity rational – analyte type.*

Severity				
Criteria	**Specification**		**Classification**	**Rational**
Analyte Type	API	600 ≤ (PDE x 1000)	1	The severity of an error occurring in an API dosing method increases with decreasing PDE.
		400 ≤ (PDE x 1000) < 600	3	
		100 ≤ (PDE x 1000) < 400	5	
		15 ≤ (PDE x 1000) < 100	7	
		1 ≤ (PDE x 1000) < 15	9	
		(PDE x 1000) < 1	11	
	Preservative	Sterile	11	An error in the results of a preservative dosage test can compromise the function of the preservative, which, in the case of a sterile product, is very serious.
		Non-sterile	7	
	Impurity		11	In impurity methods, assuming a conservative approach, the highest risk level will be considered, since, if there is any error in the results obtained from an unknown impurity, not knowing its structure, its effects will also be unknown.

- **Product Use Frequency and Type**

For all types of methods, the type of product and the frequency of use of the product are also considered, because the severity of the occurrence of an error depends on the duration of the treatment and on the type of intervention in which the product will be used.

Table 8. *Severity rational - product use frequency and type.*

Severity			
Criteria	**Specification**	**Classification**	**Rational**
Product Use Frequency	Short (occasional use)	5	The severity of the occurrence of an error in a method is greater the greater the frequency of use of the product.
	Average (about 1 month)	9	
	Long (more than 1 month)	11	
Product Type	Antifungal / Others	1	The severity of the occurrence of an error in a method depends on the intervention in which it will be used.
	Anti-inflammatory	3	
	Antibiotic	5	
	Corticoid	7	
	Glaucoma	9	
	Anaesthetic / Surgical	11	

The severity classification corresponds to the arithmetic mean of the three parameters considered: type of analyte, frequency of use of the product and product type.

3.1.3. Detectability

Table 9. *Risk level for detectability, taken from company internal documents.*

Detectability	Risk Level
Certain detectability	1
Very high detectability	3
High detectability	5
Average detectability	7
Low detectability	9
Null detectability	11

The detectability of an unsuitable method is achieved through full revalidation of the analytical method. Choosing to perform a partial revalidation of the method there is still a risk of not detecting the error. The non-validation of the method has two possible scenarios, one in which there was no update of the state of the art of the method and another in which there was. In the case that there was no update, since the methods studied here have a long history of results, there is the possibility of detecting any errors in the results. On the other hand, if there has been an update, it is not possible to guarantee that any error will be detected, since the method is outdated.

As a first approach, for all methods the non-revalidation of the method will be considered, since in a risk analysis the worst-case scenario is always studied.

Table 10. *Detectability rational.*

Detectability				
Criteria	**Specification**		**Classification**	**Rational**
Revalidation of the analytical method	Total		1	During the total revalidation of an analytical method, any error in the results will certainly be detected. The partial revalidation of the method allows a high detectability in any error in the results. In the case of non-revalidation of the method, there are two cases to take into account: if there is no update of the state of the art of the method, the highest level is not assigned since all the methods analysed here have a history of results, thus existing a possibility (even if small) to detect any errors in the results; in the case where there was an update of the state of the art of the method, the highest level is assigned since the method is outdated.
	Partial		5	
	No revalidation	There wasn't an update of the method state of the art	9	
		There was an update of the method state of the art	11	

6.1.4. Risk Assessment

Finally, the risk is calculated by multiplying the factors Probability x Severity x Detectability. Depending on the value obtained, it is defined whether the risk is acceptable or not, according to the matrix below. Acceptable risk levels are shown in white, those of unacceptable risk are shown in red and yellow are the levels that require a decision.

Table 11. *Risk Matrix, taken from Edol internal documents. Part 1 of 2.*

Severity		Probability									Detectability	
		Remote	Very low	Low	Average low	Average	Average high	High	Very high	Almost Certain		
		1	2	3	4	5	6	7	9	11		
Critical	11	121	242	363	484	605	726	847	1089	1331	11	Null
		99	198	297	396	495	594	693	891	1089	9	Low
		77	154	231	308	385	462	539	693	847	7	Average low
		55	110	165	220	275	330	385	495	605	5	Average high
		33	66	99	132	165	198	231	297	363	3	Very high
		11	22	33	44	55	66	77	99	121	1	Certain
Almost critical	10	110	220	330	440	550	660	770	990	1210	11	Null
		90	180	270	360	450	540	630	810	990	9	Low
		70	140	210	280	350	420	490	630	770	7	Average low
		50	100	150	200	250	300	350	450	550	5	Average high
		30	60	90	120	150	180	210	270	330	3	Very high
		10	20	30	40	50	60	70	90	110	1	Certain
Very high	9	99	198	297	396	495	594	693	891	1089	11	Null
		81	162	243	324	405	486	567	729	891	9	Low
		63	126	189	252	315	378	441	567	693	7	Average low
		45	90	135	180	225	270	315	405	495	5	Average high
		27	54	81	108	135	162	189	243	297	3	Very high
		9	18	27	36	45	54	63	81	99	1	Certain
High	8	88	176	264	352	440	528	616	792	968	11	Null
		72	144	216	288	360	432	504	648	792	9	Low
		56	112	168	224	280	336	392	504	616	7	Average low
		40	80	120	160	200	240	280	360	440	5	Average high
		24	48	72	96	120	144	168	216	264	3	Very high
		8	16	24	32	40	48	56	72	88	1	Certain
Average	7	77	154	231	308	385	462	539	693	847	11	Null
		63	126	189	252	315	378	441	567	693	9	Low
		49	98	147	196	245	294	343	441	539	7	Average low
		35	70	105	140	175	210	245	315	385	5	Average high
		21	42	63	84	105	126	147	189	231	3	Very high
		7	14	21	28	35	42	49	63	77	1	Certain
Low	6	66	132	198	264	330	396	462	594	726	11	Null
		54	108	162	216	270	324	378	486	594	9	Low
		42	84	126	168	210	252	294	378	462	7	Average low
		30	60	90	120	150	180	210	270	330	5	Average high
		18	36	54	72	90	108	126	162	198	3	Very high
		6	12	18	24	30	36	42	54	66	1	Certain
Very Low	5	55	110	165	220	275	330	385	495	605	11	Null
		45	90	135	180	225	270	315	405	495	9	Low
		35	70	105	140	175	210	245	315	385	7	Average low
		25	50	75	100	125	150	175	225	275	5	Average high
		15	30	45	60	75	90	105	135	165	3	Very high
		5	10	15	20	25	30	35	45	55	1	Certain
Minor	4	44	88	132	176	220	264	308	396	484	11	Null
		36	72	108	144	180	216	252	324	396	9	Low
		28	56	84	112	140	168	196	252	308	7	Average low
		20	40	60	80	100	120	140	180	220	5	Average high
		12	24	36	48	60	72	84	108	132	3	Very high
		4	8	12	16	20	24	28	36	44	1	Certain

Table 12. *Risk Matrix, taken from Edol internal documents. Part 2 of 2.*

Almost insignificant	3	33	66	99	132	165	198	231	297	363	11	Null
		27	54	81	108	135	162	189	243	297	9	Low
		21	42	63	84	105	126	147	189	231	7	Average low
		15	30	45	60	75	90	105	135	165	5	Average high
		9	18	27	36	45	54	63	81	99	3	Very high
		3	6	9	12	15	18	21	27	33	1	Certain
Insignificant	1	11	22	33	44	55	66	77	99	121	11	Null
		9	18	27	36	45	54	63	81	99	9	Low
		7	14	21	28	35	42	49	63	77	7	Average low
		5	10	15	20	25	30	35	45	55	5	Average high
		3	6	9	12	15	18	21	27	33	3	Very high
		1	2	3	4	5	6	7	9	11	1	Certain

3.2. General Analytical Validation Protocol

After carrying out the risk analysis and subsequent creation of a mitigation plan, a method is chosen as an example to be validated / revalidated. Therefore, a general validation protocol for analytical methods is followed, based on the ICH and the company's internal procedures.

3.2.1. Selectivity

Selectivity is evaluated by the analysis of the chromatograms of solvent, placebo, reference solution, sample and solutions of drug substance or drug product spiked with appropriate levels of impurities and/or excipients.

In the case where impurities are not available, or even to demonstrate selectivity towards unknown impurities, forced degradation tests must be carried out. To this end, samples, placebo, and the drug substance must be subjected to conditions such as: light, heat, humidity, acid/base hydrolysis, and oxidation.

3.2.2. Linearity

Linearity must be evaluated across the range of the method, and for dosing methods the range must be from 80 to 120% of the test concentration and for impurities from the reporting level to 120% of the specification [5,35].

Linearity can be assessed directly on the drug substance, by diluting a standard stock solution, and it is recommended to use at least 5 solutions that cover the working range [5,35].

3.2.3. Accuracy

Accuracy must also be established across the range of the analytical method. For drug product assay, accuracy assessment can be done by applying the analytical method to placebo samples loaded with known amounts of drug substance.

For impurities, accuracy should be evaluated on samples spiked with known amounts of impurities.

A minimum of 9 determinations over a minimum of 3 concentration levels covering the range must be used.

3.2.4. Precision

3.2.4.1. Repeatability

A minimum of 6 determinations at 100% of the test concentration should be used to assess repeatability. For system repeatability, 6 injections of the same sample must be evaluated and for analytical repeatability, 6 samples must be prepared.

3.2.4.2. Intermediate Precision

Intermediate precision is assessed by preparing and quantifying 6 samples, by two different analysts, on different days and on different equipment.

3.2.5. Robustness

Robustness is tested by deliberate variations of analytical procedure parameters. The changes to be made must be selected according to the analytical method in question, its characteristics, and the product under analysis.

Robustness can be assessed by studying one change at a time; however, this methodology becomes impractical when you want to evaluate several changes and does not consider interactions between effects.

The best option is to carry out a complete factorial design, however, this method involves a higher number of experiments (2^k, with k being the number of factors to be evaluated). Therefore, it’s chosen to perform a fractional factorial, which requires a smaller number of tests [48]. internally, the Youden test is performed, keeping the required time and effort to a minimum however interactions between the factors cannot be detected [49].

Table 12 presents the design used in the robustness study.

Table 13. *Robustness Study Design (9 variations - 11 trials) – Youden Test.*

Variations	**(Nominal conditions – 3 replicates)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Variation 1	Nominal	+	+	+	+	-	-	-	-
Variation 2	Nominal	+	+	-	-	+	+	-	-
Variation 3	Nominal	-	+	-	+	-	+	-	+
Variation 4	Nominal	+	-	+	-	+	-	+	-

+ higher variation compared to nominal conditions
- lower variation compared to nominal conditions

3.3. Analytical Method

3.3.1. Atropine Sulfate Assay

Chromatographic Conditions

Column: Aquasil C18, 100 x 4.6 mm, 3 µm

Column oven temperature: 22

Auto sampler temperature: 18

Detection: UV at 210 nm

Flow rate: 1.0 ml/min

Injection volume: 10 µL

Run time: 30 min

Solvent: mobile phase A

Mobile Phase:

A: Dissolve 3.5 g of sodium dodecyl sulphate in 606 mL of 7.0 g/L solution of potassium dihydrogen phosphate previously adjusted to pH 3.3 with a 5.8 g/L solution of phosphoric acid and mix with 320 mL of acetonitrile.

B: Acetonitrile

Gradient:

***Table 14**. Atropine Sulfate assay gradient.*

Time (min)	(A)	(B)
0	97%	3%
2	97%	3%
20	70%	30%
25	97%	3%
30	97%	3%

Standard Assay Solution preparation

Accurately weight 100 mg of atropine sulfate to a 100 ml volumetric flask, dissolve with mobile phase A, ultrasound bath about 5 minutes and complete the volume with same solvent. (1000 µg/ml)

Prepare two replicates of this solution.

Sample Solution preparation

Transfer 2 mL of sample into a 20 mL volumetric flask, dissolve with solvent using the ultrasound bath for about 5 minutes and complete the volume with the same solvent (1000 µg/ml).

Prepare two replicates of this solution.

Procedure

Inject solvent once, standard solution 6 times and samples 3 times.
The Atropine Sulfate retention time is about 11 minutes.
Determine RSD between injections, the peak's efficiency, and tailing factor. The results should comply with the following criteria:

Table 15. *Atropine Sulfate assay system suitability.*

Parameters	Acceptance criteria
Efficiency (No. Theoretical Plates)	≥ 2000
Tailing Factor of analyte peak	0.8 – 1.5
Precision of atropine sulfate peak areas - % RSD (n=6)	≤ 2.0%
Precision of atropine sulfate peak retention times - % RSD (n=6)	≤ 2.0%

Calculate the content of atropine sulfate in % by using the following formula:

$$C = \frac{Aa}{Ap} \times \frac{Pp}{Va} \times \frac{fda}{fdp} \times \frac{P}{Td} \qquad (13)$$

Where:

C- Content of atropine sulphate (%)

A_A – Analytical response of the sample solution

A_P – Analytical response of the standard solution

P_P – Weight of the reference substance (mg)

V_a – Aliquot of sample

Fda – Dilution factor of sample solution

Fdp – Dilution factor of reference solution

P – Purity/Content of the standard expressed in % of atropine sulfate as is

Td – Content of atropine sulfate stated in label expressed as mg/ml

The assay value expressed as atropine sulfate must be between **98%** and **102%**, for product release and shelf life.

3.3.2. Atropine Sulfate Related Substances Determination

Chromatographic conditions

Same as described in atropine sulfate assay.

Standard Impurity Solution preparation

Dilute 1 mL of standard assay solution to 100 mL with mobile phase A. Dilute 1 mL of this solution to 10 mL with mobile phase A. (1 µg/ml)

Prepare two replicates of this solution.

Resolution Solution

Dissolve 5 mg of atropine impurity B CRS in the standard assay solution and dilute to 20 mL with the same solution. Dilute 5 mL of this solution to 25 mL with mobile phase A. (Imp. B: 50 µg/ml; Atropine Sulfate: 200 µg/ml)

Reference Solution A (Peak ID)

Dissolve the content of a vial of atropine for peak identification CRS (containing impurities A, D, E, F, G and H) in 1 mL of mobile phase A.

Reference Solution B (Impurity C)

Dissolve 5 mg of tropic acid R (impurity C) in mobile phase A and dilute to 10 mL with mobile phase A. Dilute 1 mL of the solution to 100 mL with mobile phase A. Dilute 1 mL of this solution to 10 mL with mobile phase A. (0.5 µg/ml)

Sample Solution Preparation

Transfer 2 mL of sample into a 20 mL volumetric flask, dissolve with solvent using the ultrasound bath for about 5 minutes and complete the volume with the same solvent (1000 µg/ml).

Prepare two replicates of this solution.

Procedure

Inject solvent, reference solution A, reference solution B, resolution solution, standard impurity solution (6 times) and samples (3 times).

The Atropine Sulfate retention time is about 10 minutes.

Determine RSD between injections, the efficiency peak and tailing factor. The results should comply with the following criteria:

Table 16. *Atropine Sulfate related substances system suitability.*

Parameters	Acceptance criteria
Efficiency (No. Theoretical Plates)	≥ 2000
Tailing Factor of analyte peak	0.8 – 1.5
Precision of atropine Sulphate peak areas - % RSD (n=6)	≤ 5.0%
Precision of atropine Sulphate peak retention times - % RSD (n=6)	≤ 2.0%
Resolution Between Imp B and Atropine Sulfate	≥ 2.5

Correction Factors

For the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor:

Impurity A: 0.6 Impurity C: 0.6

For any other impurities, the correction factor is 1.0.

Relative Retention with Reference to Atropine

Table 17. *Relative retention times of impurities with reference to atropine.*

Impurity	C	E	D	F	B	H	G	A
TRR	0.2	0.67	0.73	0.8	0.89	0.93	1.1	1.7

Determine the individual related compound content, expressed in %, by the following expression:

$$C = \frac{Rs}{Rr} \times \frac{Ar \times Pr}{Dr} \times \frac{100}{Ad} \times RRF \qquad (14)$$

C – Content of individual related compound (%)

Rs – Analytical response of the individual impurity in the chromatogram of the sample solution

Rr - Analytical response of the main peak in the chromatogram of the reference solution

Ar – Weight of the Standard (mg)

Pr – Assay of the reference standard (decimals), as is

Dr – Dilution factor of the reference solution

Ad – Amount of the active substance per dosage

RRF - Relative response factor.

4

PRESENTATION AND DISCUSSION OF THE RESULTS

4.1. Risk Analysis

In order to carry out the risk analysis, information was collected regarding all the analytical methods currently in use in the company and the respective validation dates. In Figure 8 it is possible to observe the number of validation reports carried out in each year, from 2001 to 2022. Of the 121 methods gathered, 64 have a validation date that exceeds 10 years (represented in red in the graph), these being the methods that will be submitted to risk analysis.

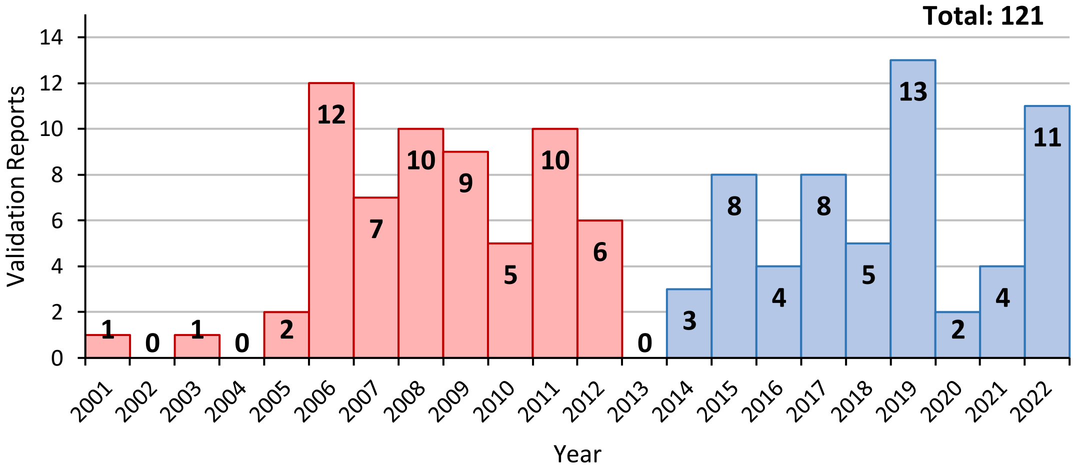

Figure 8. *Number of methods validated each year.*

It is also interesting to note that of the 64 methods whose validation date exceeds 10 years, 47% correspond to API assay methods, 33% to preservative assay methods and the remaining 20% to impurity assay methods.

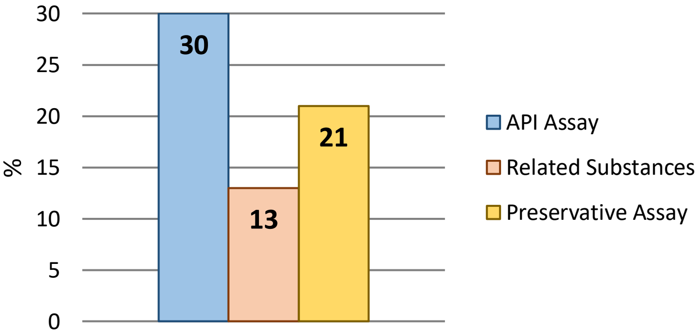

Figure 9. *Distribution of methods whose validation date exceeds 10 years, by type of method.*

4.1.1. Probability / Occurrence

As explained earlier, in chapter 3.1 (Risk Analysis Rational), the probability/occurrence assessment takes into account three main parameters, namely the method performance, the state of the art of method validation and the state of the art of the method.

- **Method Performance**

  Studying the performance of the methods consisted of collecting and analysing historical data for each method.

  The collected data were represented in the form of box plot graphs that allow the visual evaluation of data distribution and the existence of outliers [50].

  Figures 10, 11, 12 and 13, show the boxplots referring to the number of theoretical plates for each type of method.

  In general, most of the methods present a theoretical plate number greater than that considered to be the minimum acceptable in this study (2000). Regarding the API dosage methods, there are 3 methods that present all the values collected below 2000 dishes, these being *1*, *16* and *17*. There are also methods whose average results are greater than 2000, however they present some lower values, such as *4*, *5*, *19*, *21*, and *26*. Furthermore, it is also possible to notice that some methods present a large dispersion, for example *25* which presents values ranging from 4900 to 20000. As mentioned in point 2.1.2., the efficiency of the column depends greatly on the type of column used and on the condition of the column. And considering that these values correspond to analysis between 2018 and 2023, it is natural that there have been column changes that could be the cause of this variability.

  Regarding impurities, there are only two methods that have a theoretical plate number of less than 2000, namely *32* and *33*. All other methods are above the minimum acceptable value.

  Finally, regarding preservative dosage methods, there are also two methods, *57* and *59*, with some values below 2000.

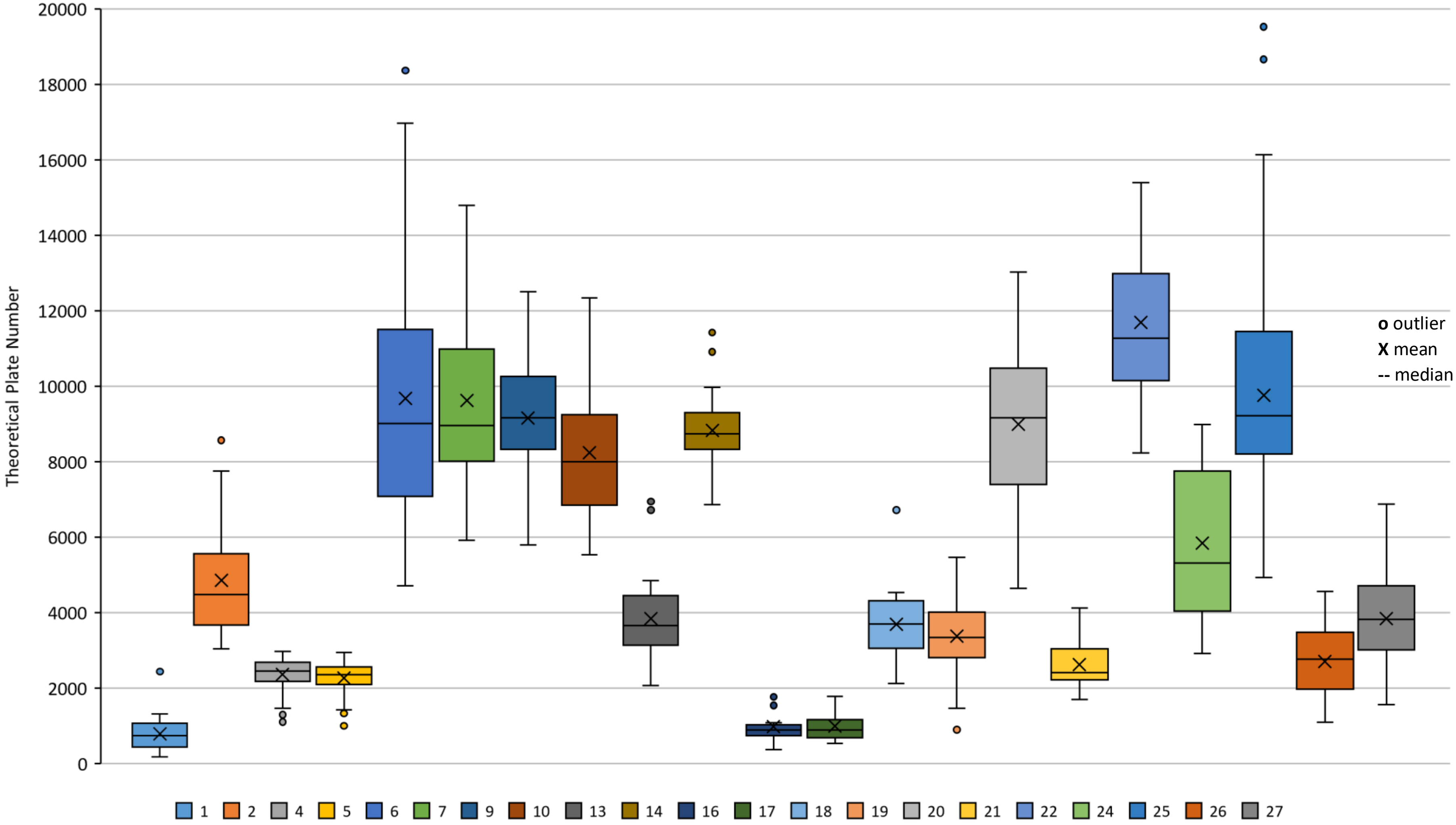

Figure 10. *Box Plot theoretical plate number - API assay.*

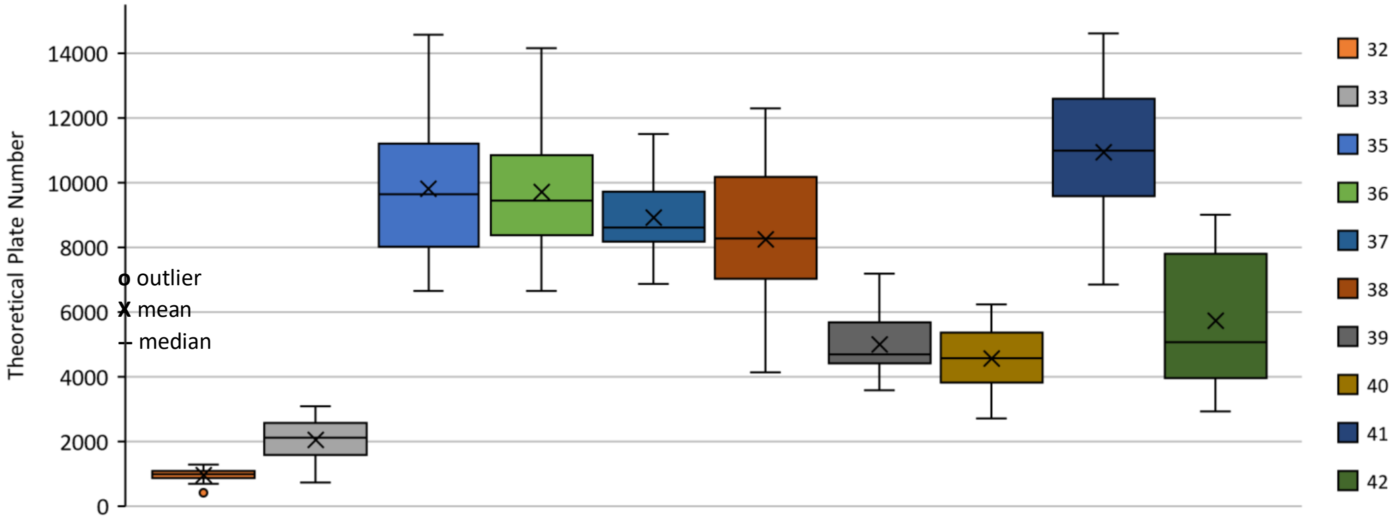

Figure 11. *Box Plot theoretical plate number - related substances.*

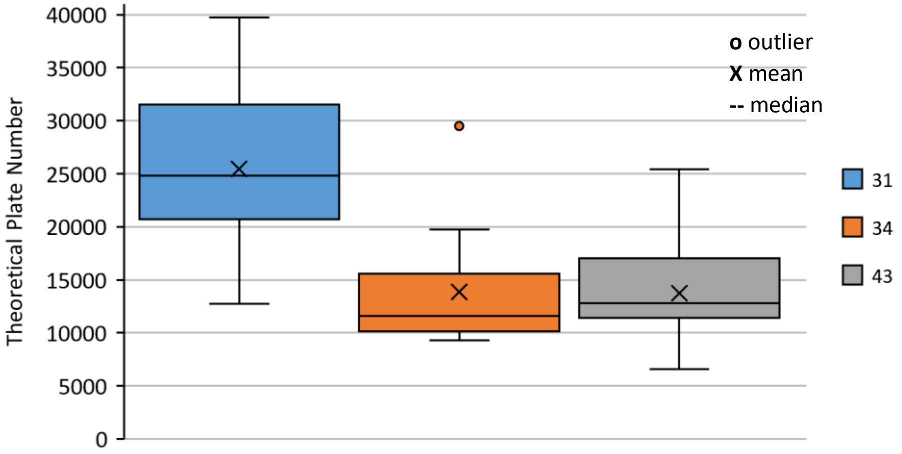

Figure 12. *Box Plot theoretical plate number - related substances (1).*

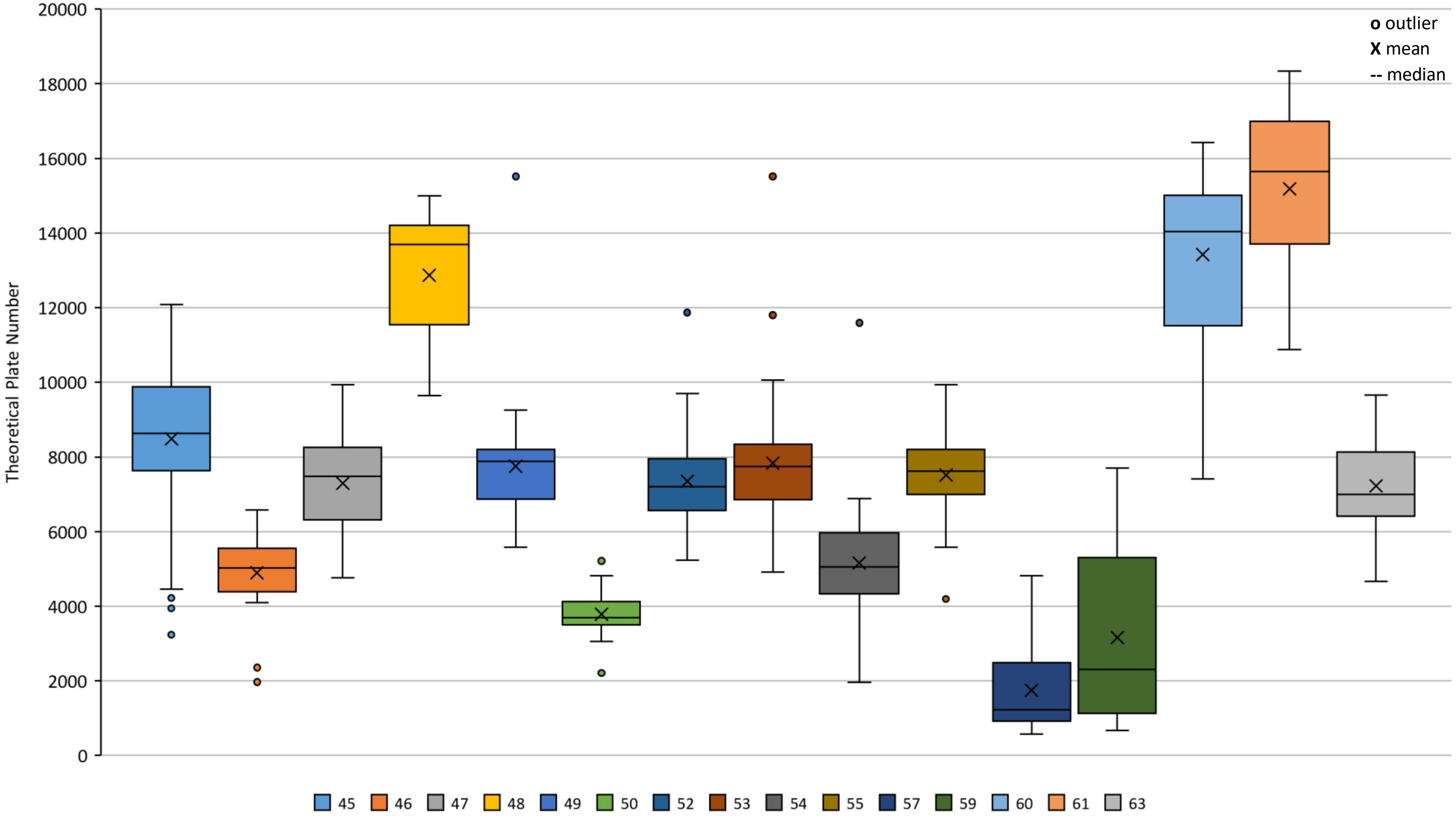

Figure 13*. Box Plot theoretical plate number - preservative assay.*

In Figures 14, 15 and 16 are presented the data collected regarding the symmetry factor. The first graph corresponds to the API dosing methods where it is possible to see that there are some methods whose symmetry factors are not within the values defined as ideal in this study (between 0.8 and 1.5).

Regarding impurities, there are two methods that differ from the others because they present symmetry factors above the ideal, these being *32* and *33*.

Finally, in preservative dosing methods, all methods present values within the established range, and it is also possible to notice the existence of some outliers.

As mentioned in chapter 2.1.2, the condition of the chromatographic column greatly influences the shape of the peaks, so columns with the same characteristics will give different symmetry results depending on their “age” (number of uses).

The Figures from 17 to 24 correspond to the data collected regarding the RSD of the peak areas, retention times and between samples. The RSD values obtained for peak areas and retention times are, for the most part, below the established limit (2.0% for dosages and 5.0% for impurities). Regarding the RSD between samples, there are several values above 2.0%. It is important to highlight that all analyses that presented an RSD value greater than 2.0%, or 5.0% in the case of impurities, were repeated and their results were not accepted

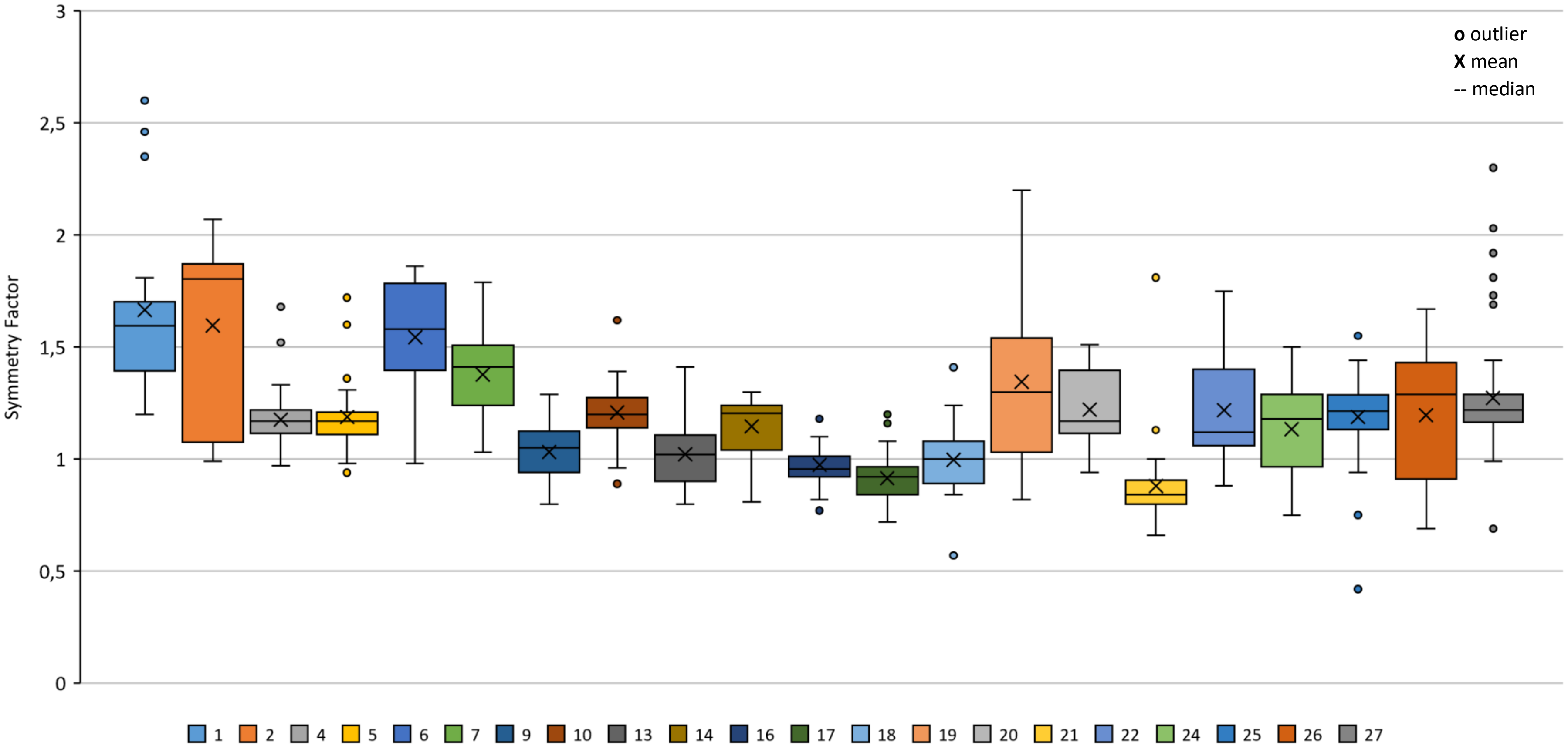

Figure 14. *Box Plot symmetry factor - API assay.*

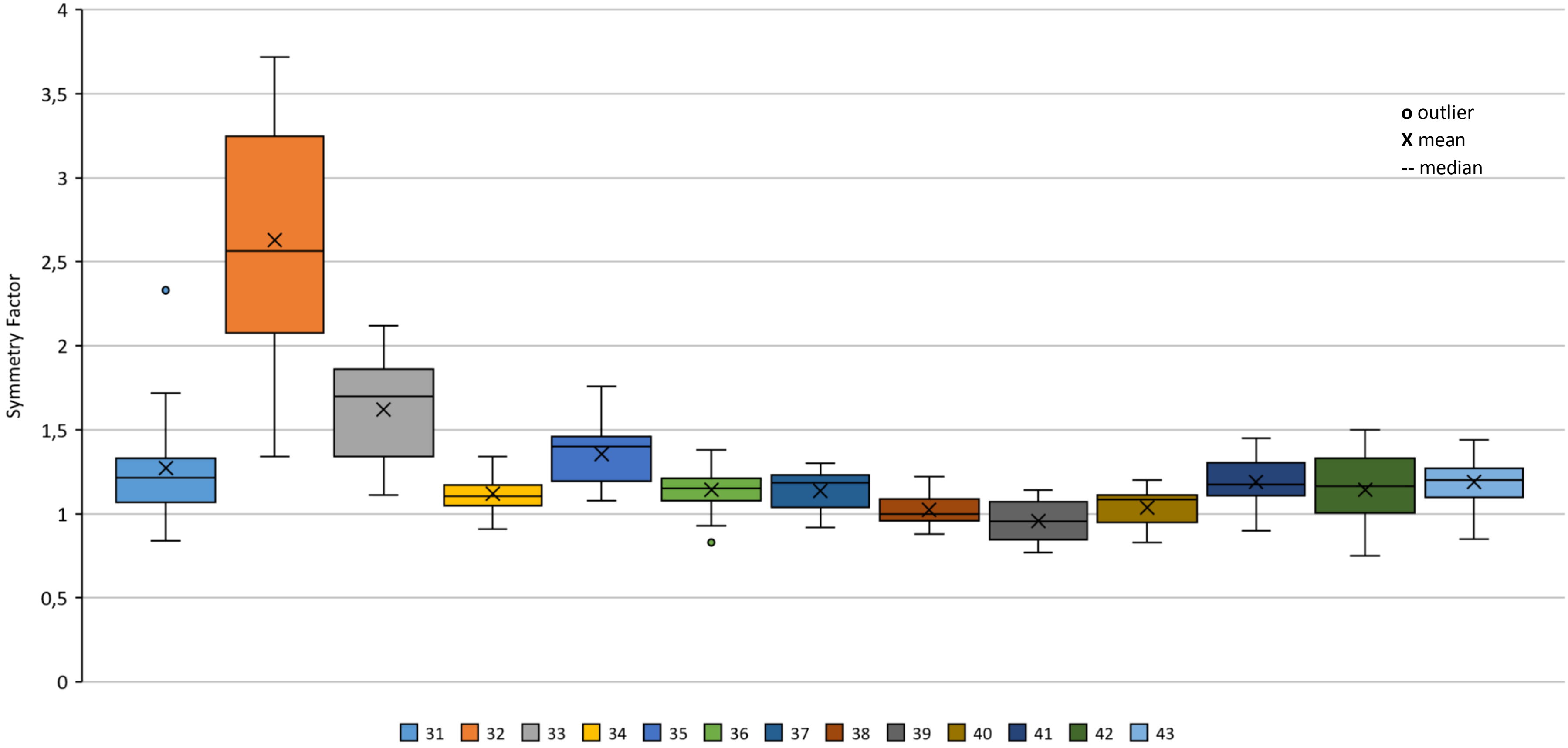

Figure 15. *Box Plot symmetry factor - related substances.*

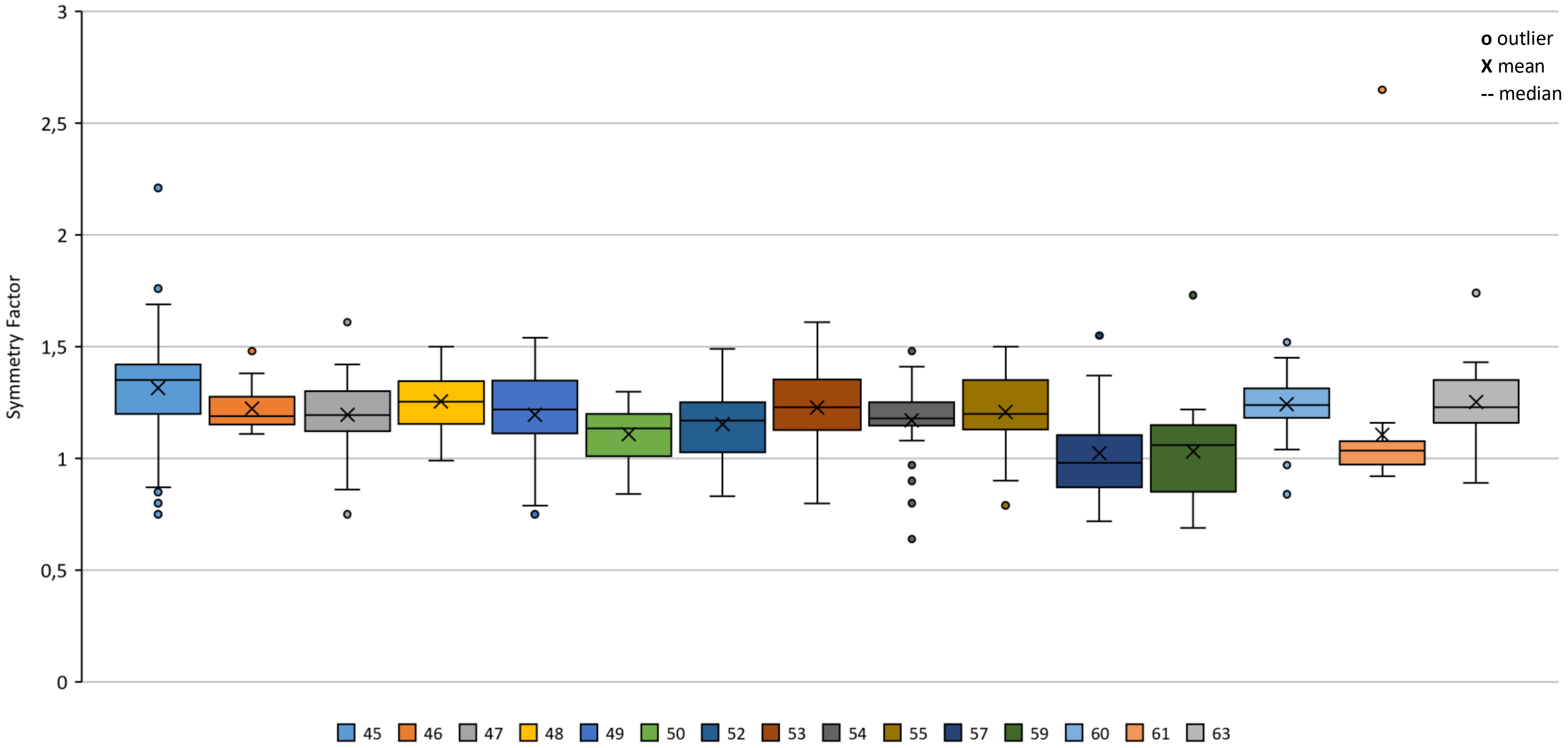

Figure 16. *Box Plot symmetry factor - preservative assay.*

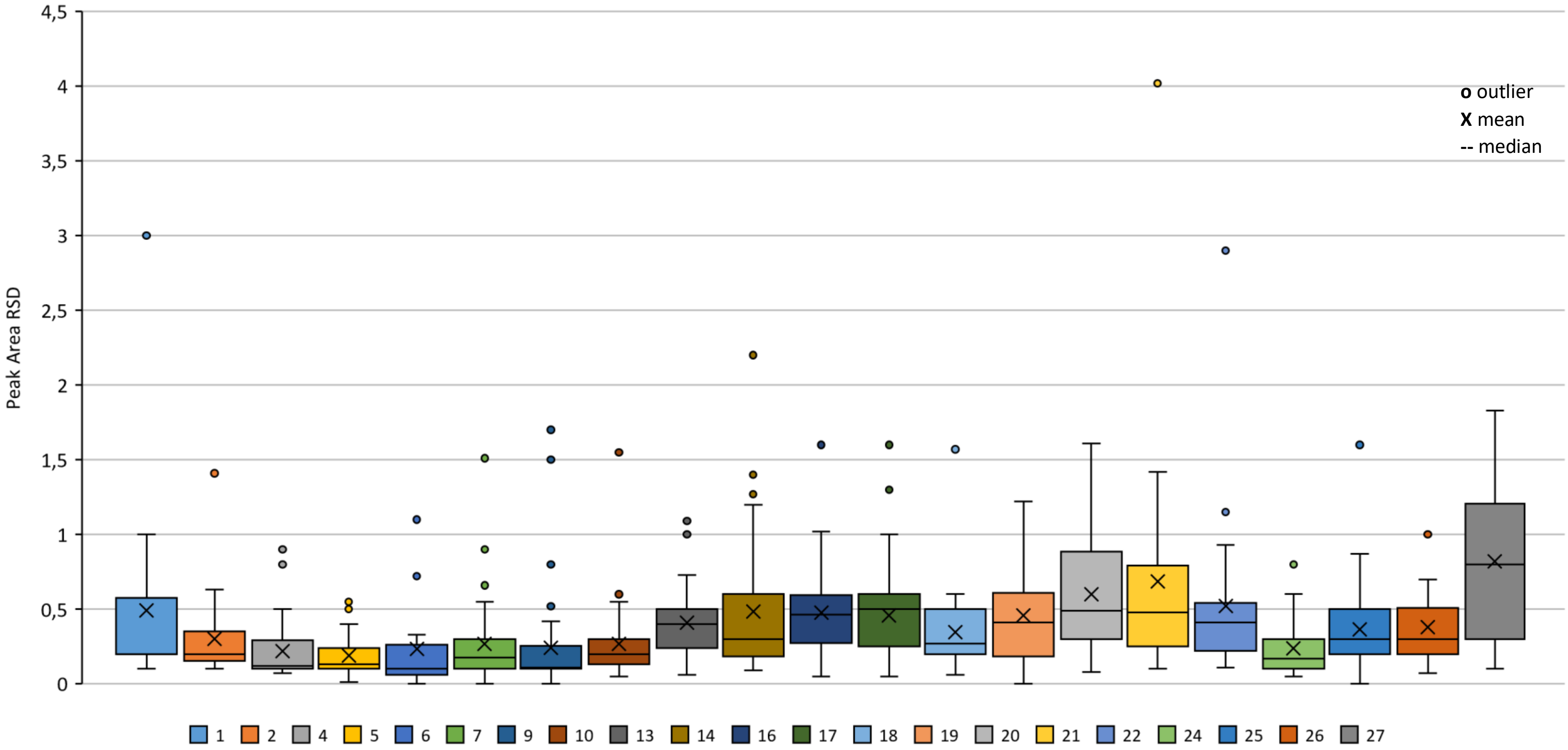

Figure 17. *Box Plot peak area RSD - API assay.*

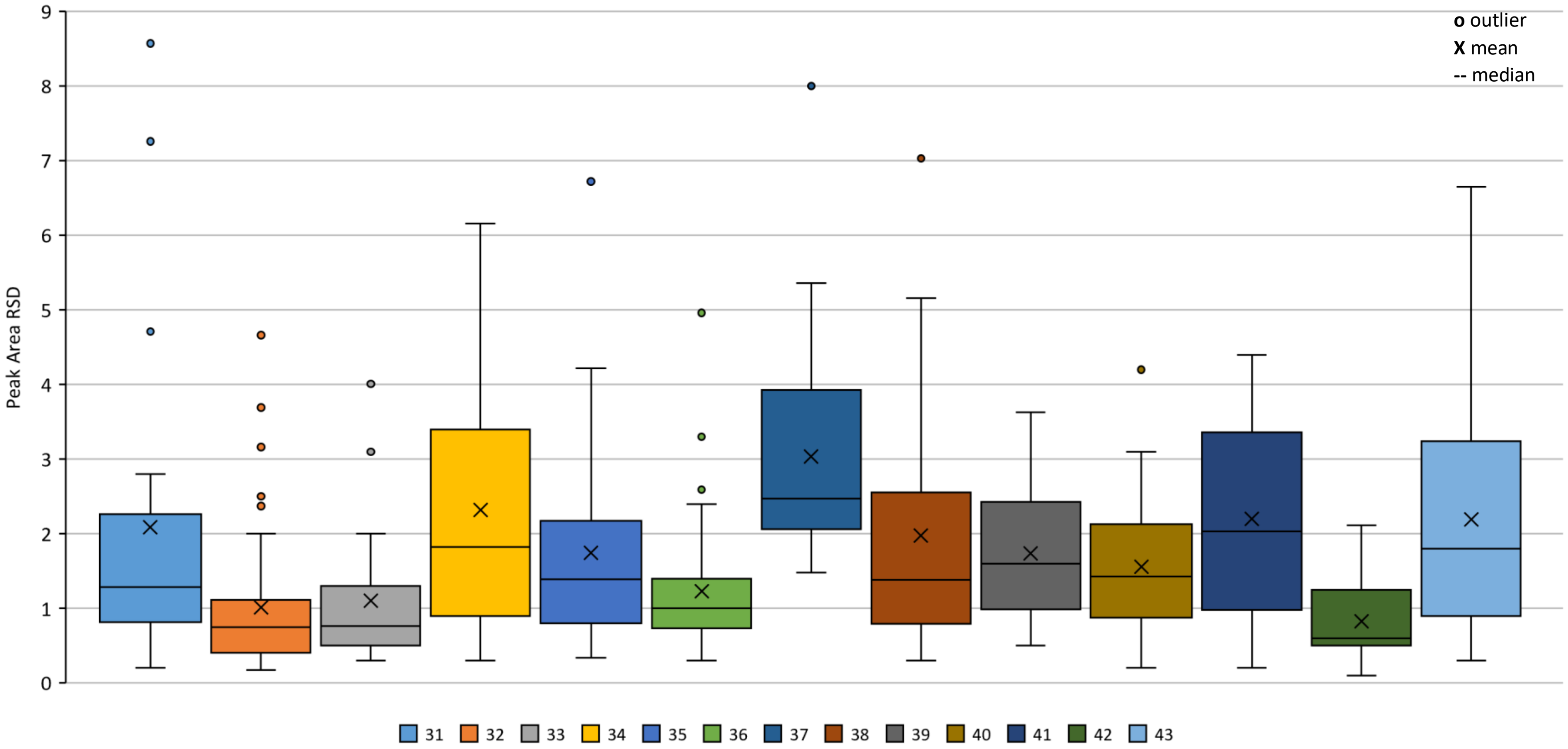

__Figure 18.__ Box Plot peak area RSD - related substances.

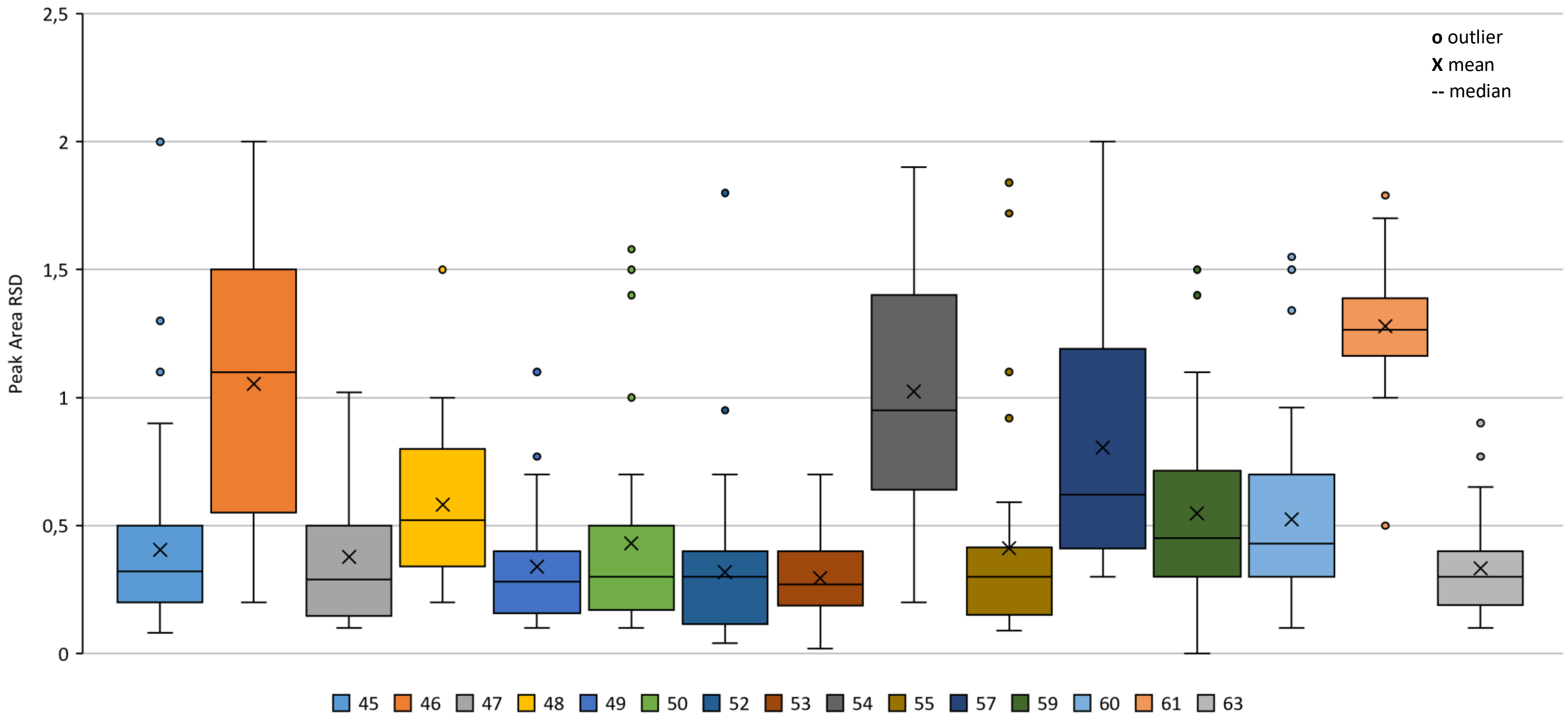

Figure 19. *Box Plot peak area RSD - preservative assay.*

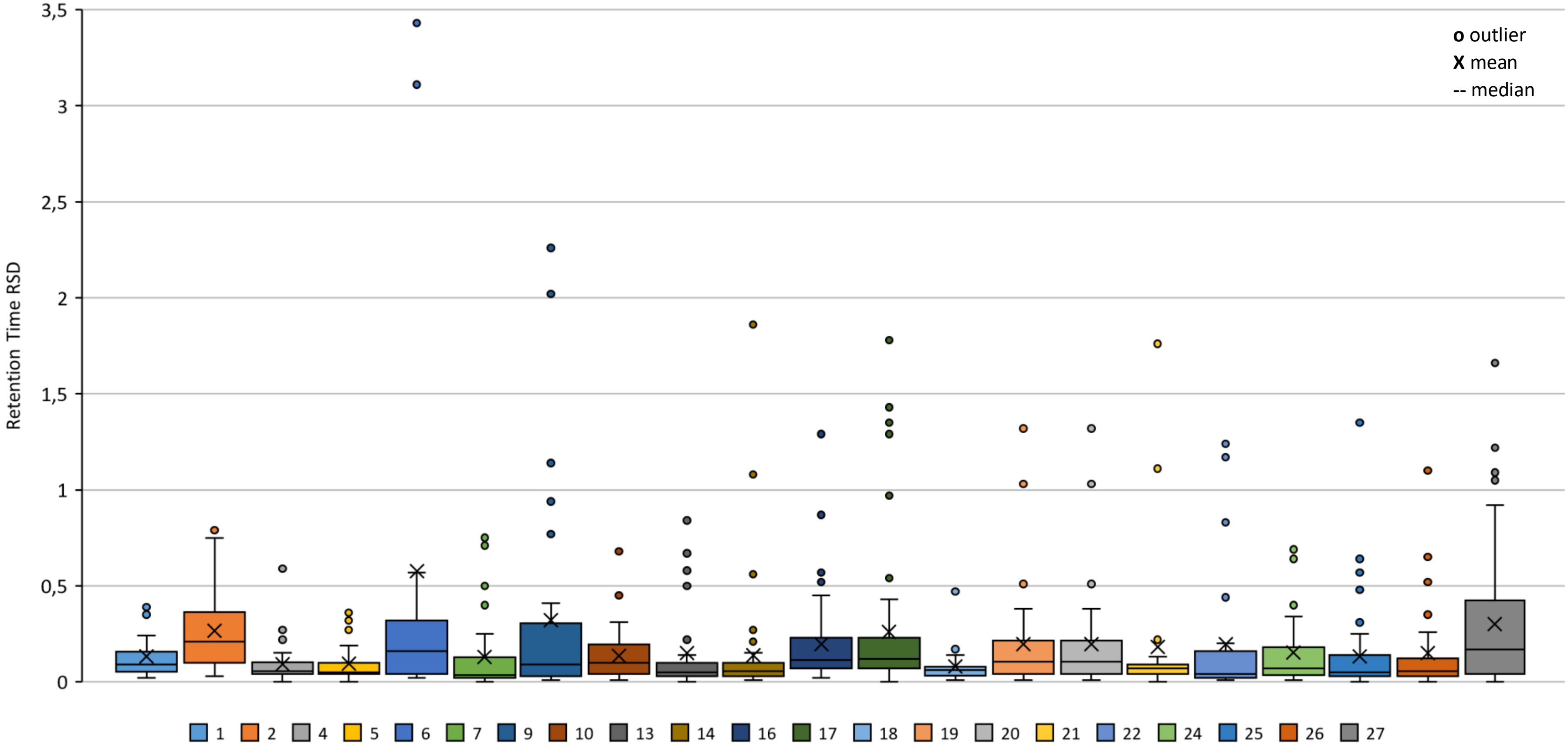

Figure 20. *Box Plot retention time RSD - API assay.*

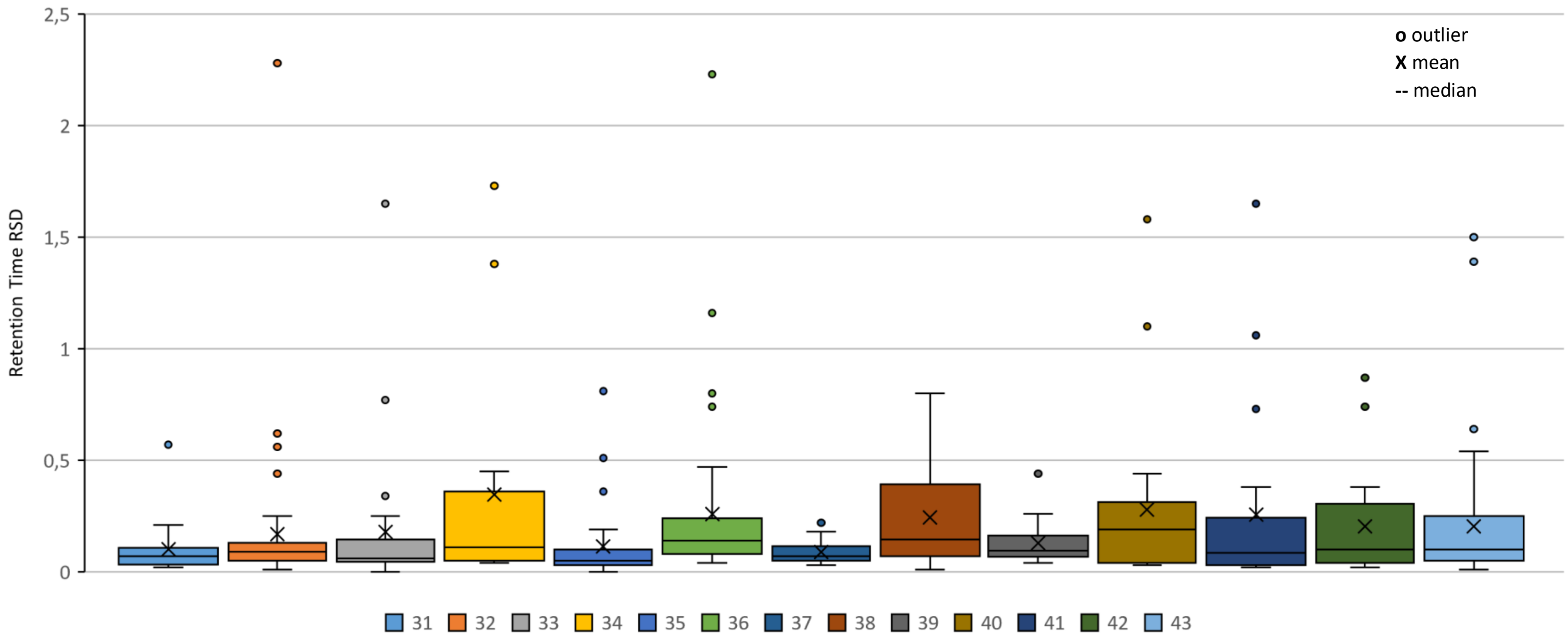

Figure 21. *Box Plot retention time RSD - related substances.*

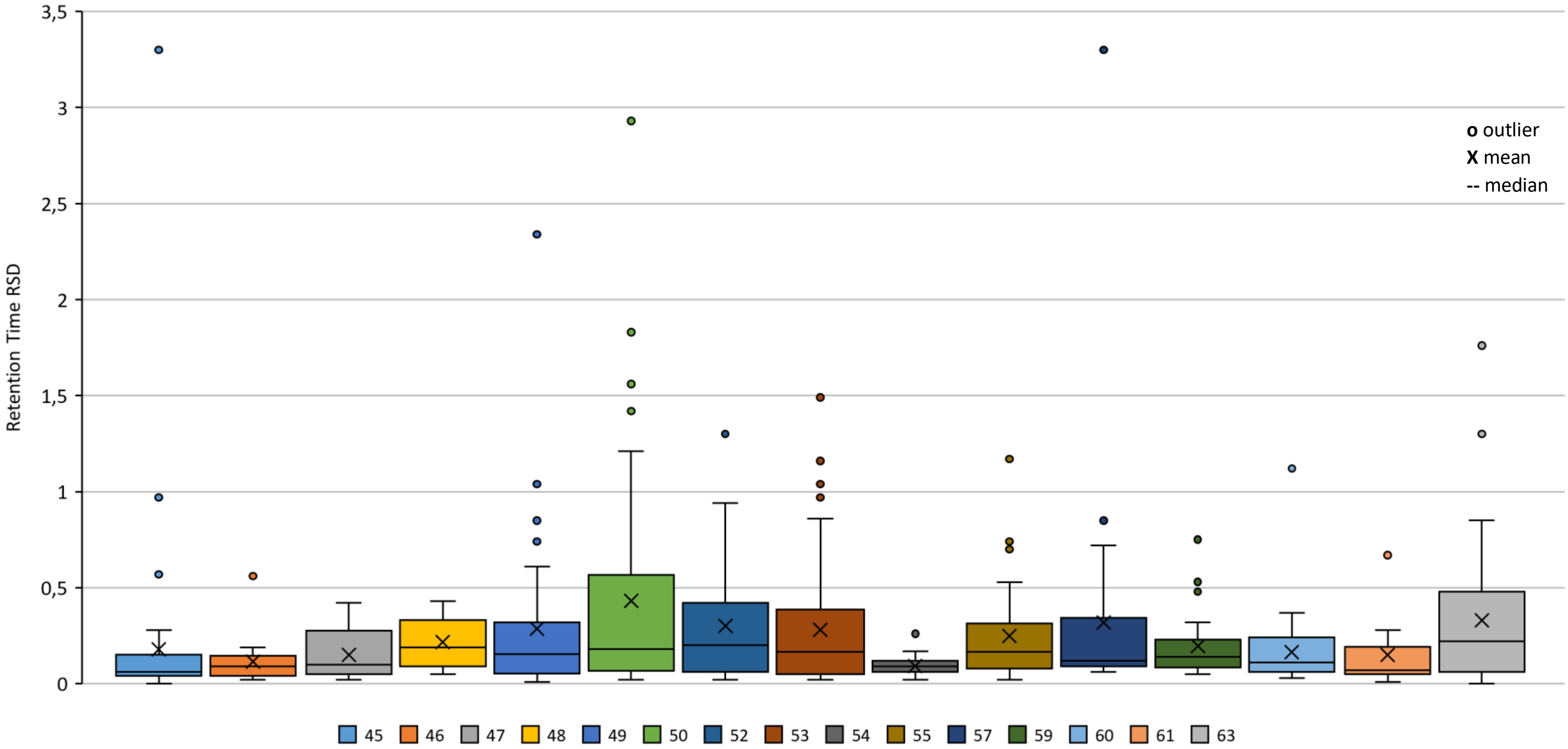

Figure 22. *Box Plot retention time RSD - preservative assay.*

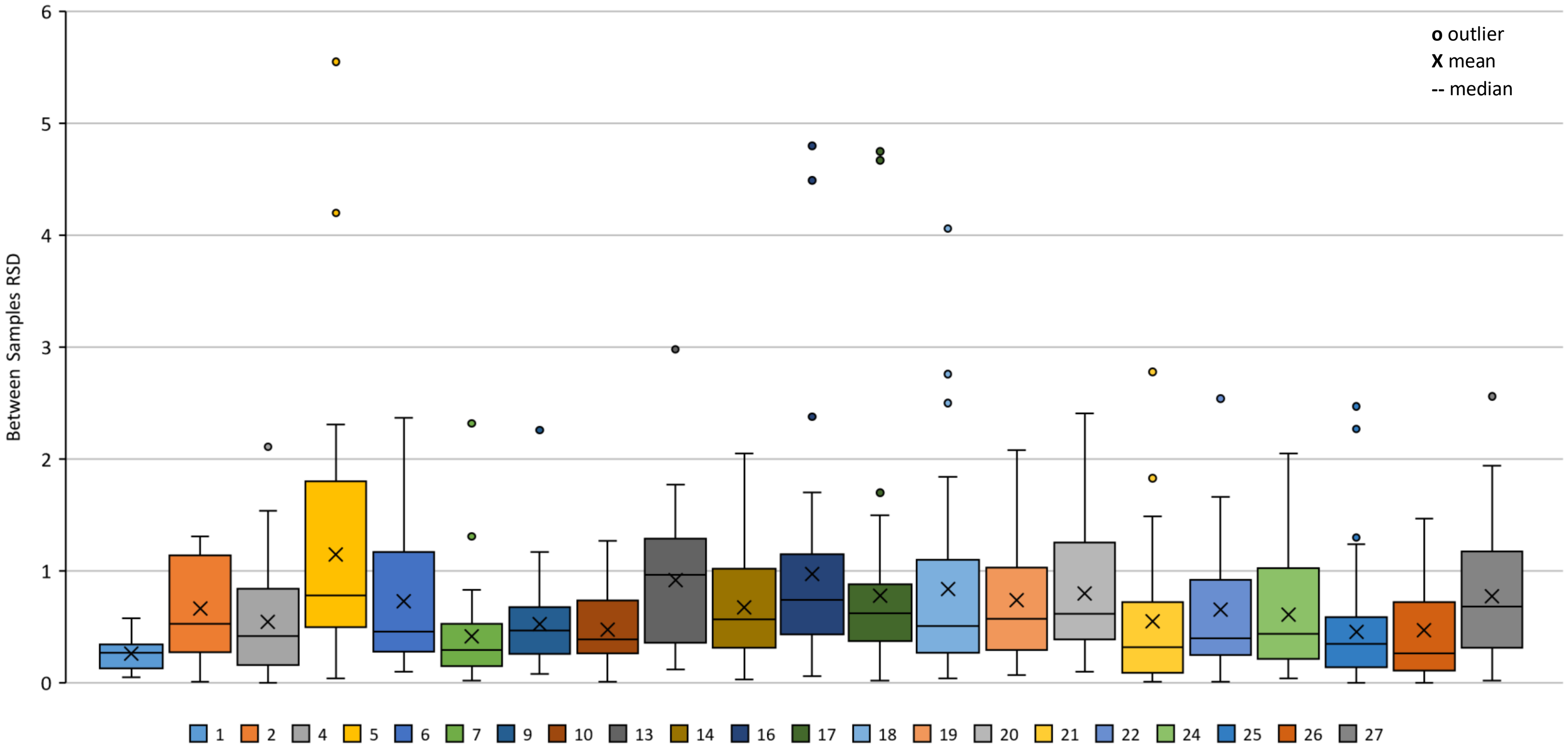

Figure 23. *Box Plot between samples RSD - API assay.*

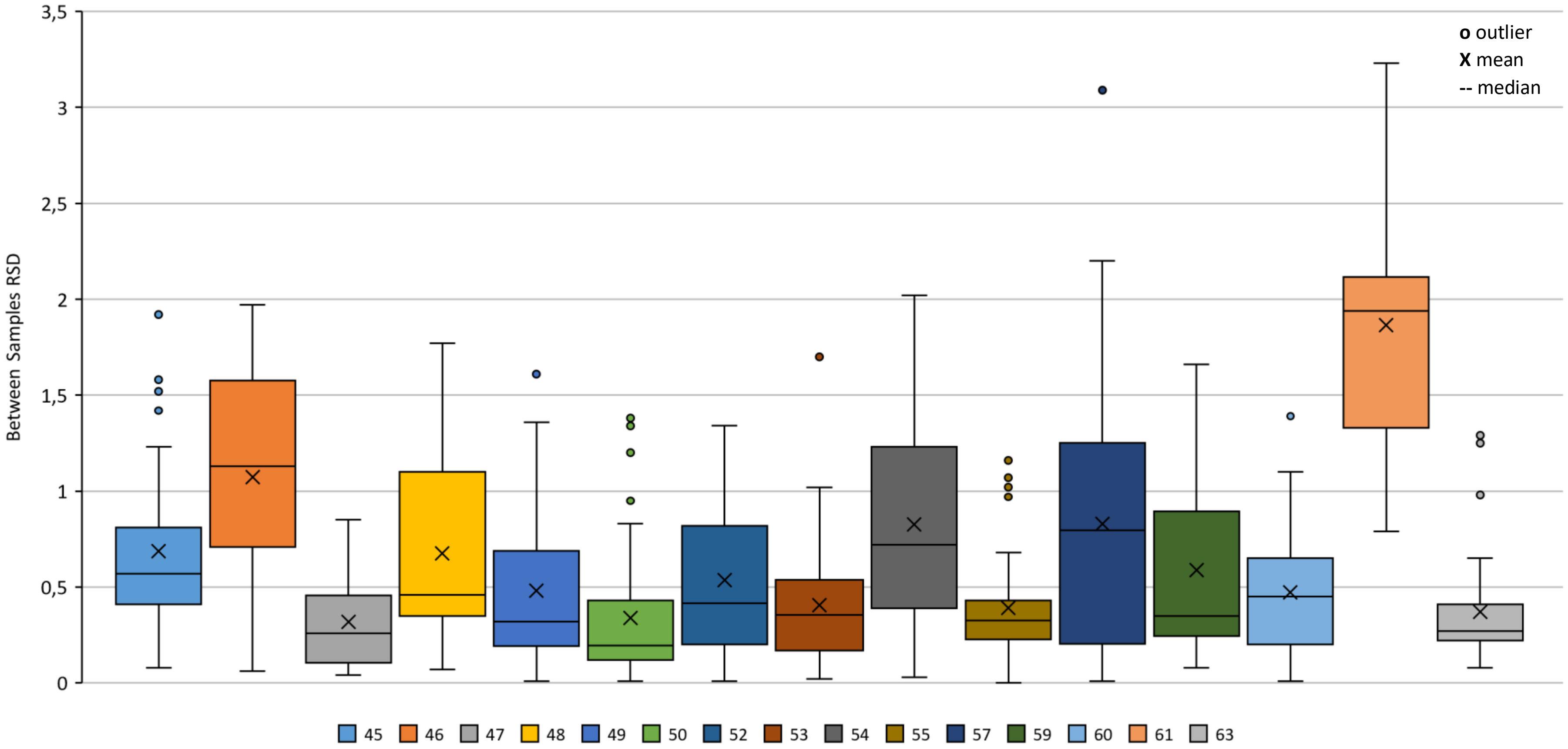

Figure 24. *Box Plot RSD between samples - preservative assay.*

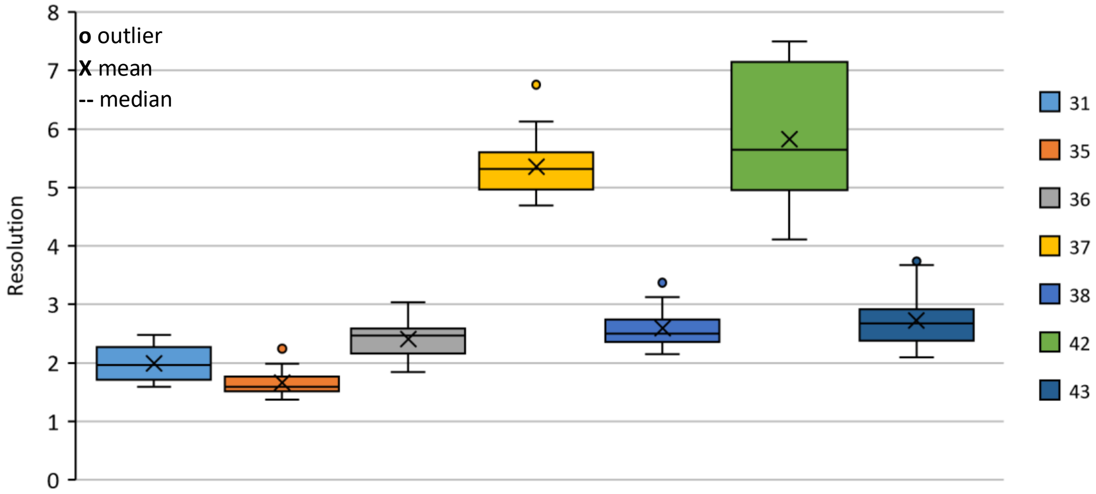

Figure 25. *Box Plot resolution - related substances.*

Figure 25 contains data relating to the resolution of impurity methods. Only method 35 presents resolution values below 1.5 (a value considered acceptable in this study), with all other methods presenting higher values.

With regard to the performance of the method, tables 17, 18 and 19 summarize the results obtained.

In table 17, there are 6 methods that only have assigned the value corresponding to the existence of OOS's. This is because these are not HPLC methods, so the parameters evaluated here do not apply. In this case, the classification of the performance of the method takes into account only the out-of-specification results.

The methods that have the highest rating, which is equal to 9, are methods that do not have sufficient historical data to carry out the analysis, which is why, as explained in the rationale for risk analysis, the worst rating is assigned taking into account an approach of worst-case.

Method *32* has a high rating as column efficiency, symmetry factor, and resolution were assigned the highest level of risk. Considering the rational presented in chapter 3.1, this risk level is only assigned when the cpk value is less than 0, indicating that the collected points are mostly out of specification. However, it is important to point out that these negative values were obtained considering the ideal specification criteria that we want to implement (efficiency > 2000, 0.8 < symmetry factor < 1.5, resolution > 1.5). Calculating these cpk values taking into account the specification criteria found in the method validation report and official internal analytical procedure, these are in fact positive. The decision to consider the lower cpk's is linked to two factors: first, in the case of a risk analysis, the worst-case scenario must be chosen; and secondly, the methods that are being analysed here were in fact developed and validated a long time ago, so the specification limits presented in these are not ideal at all.

This method was not the only one to present negative cpk's, and in all the other methods in which this happened, the reason and approach were the same as those explained above.

Table 18. *Method performance classification - API assay.*

	Pharmaceutical Form	**Active Substance**	**Method**	**Column Efficiency**	**Tailing Factor**	**Peak Area RSD**	**Retention Time RSD**	**Samples RSD**	**OOS**	**Method Performance Classification**
1	Eye Drops, Solution	Atropine Sulfate	API Assay	11	11	7	1	1	1	**5**
2	Eye Drops, Solution	Betaxolol hydrochloride	API Assay	11	11	11	11	11	1	**9**
3	Cutaneous Solution	Erythromycin	API Assay	-	-	-	-	-	1	**1**
4	Eye Drops, Solution	Chloramphenicol	API Assay	9	3	1	1	3	1	**3**
5	Eye Ointment	Chloramphenicol	API Assay	9	7	1	1	9	1	**5**
6	Eye Drops, Solution	Dorzolamide hydrochloride	API Assay	11	11	11	11	11	1	**9**
7	Eye Drops, Solution	Flurbiprofen sodium	API Assay	7	9	1	1	1	1	**3**
8	Eye Drops, Solution	Clonidine hydrochloride	API Assay	11	11	11	11	11	1	**9**
9	Eye Drops, Solution	Ketorolac tromethamine	API Assay	3	9	1	3	1	1	**3**
10	Eye Gel	Ofloxacin	API Assay	1	3	1	1	3	1	**2**
11	Eye Drops, Solution	Gentamicin sulfate	API Assay	-	-	-	-	-	1	**1**
12	Ointment	Zinc oxide	API Assay	-	-	-	-	-	1	**1**
13	Eye Ointment	Prednisolone acetate	API Assay	9	9	1	1	9	1	**5**
14	Cutaneous Solution	Minoxidil	API Assay	1	1	3	1	3	1	**2**
15	Cutaneous Suspension	Zinc oxide	API Assay	-	-	-	-	-	1	**1**
16	Ointment	Hydrocortisone	API Assay	11	7	1	1	9	1	**5**
17	Cream	Hydrocortisone	API Assay	11	9	1	1	3	1	**4**
18	Eye Ointment	Prednisolone acetate	API Assay	7	9	1	1	9	1	**5**
19	Cutaneous Solution	Salicylic acid	API Assay	9	9	3	1	9	1	**5**
20	Cutaneous Solution	Betamethasone dipropionate	API Assay	3	9	3	1	9	1	**4**
21	Ointment	Salicylic acid	API Assay	9	9	9	1	9	1	**6**
22	Ointment	Betamethasone dipropionate	API Assay	3	9	3	1	9	1	**4**
23	Ointment	Salicylic acid + Clioquinol	API Assay	11	11	11	11	11	1	**9**
24	Cutaneous Emulsion	Metronidazole	API Assay	9	9	1	1	9	1	**5**
25	Eye Drops, Solution	Dexamethasone sodium phosphate	API Assay	3	7	1	1	3	1	**3**
26	Cutaneous Liquid	Ketoconazole	API Assay	9	9	1	1	7	1	**5**
27	Shampoo	Ketoconazole	API Assay	9	9	9	3	9	1	**7**
28	Eye Ointment	Oxytetracycline hydrochloride	API Assay	11	11	11	11	11	1	**9**
29	Cream	Pyrithione zinc	API Assay	-	-	-	-	-	1	**1**
30	Cutaneous Solution	Pyrithione zinc	API Assay	-	-	-	-	-	1	**1**

Table 19. *Method performance classification - related substances.*

	Pharmaceutical Form	Active Substance	Method	Column Efficiency	Tailing Factor	Peak Area RSD	Retention Time RSD	Resolution	OOS	Method Performance Classification
31	Eye Drops, Solution	Atropine sulfate	Rel. Subst.	3	9	9	1	9	1	**5**
32	Eye Drops, Solution	Chloramphenicol	Rel. Subst.	11	11	3	1	11	1	**6**
33	Eye Ointment	Chloramphenicol	Rel. Subst.	9	11	3	1	11	1	**6**
34	Eye Drops, Solution	Dorzolamide hydrochloride	Rel. Subst.	11	11	11	11	11	1	**9**
35	Eye Drops, Solution	Flurbiprofen sodium	Rel. Subst.	3	9	7	1	9	1	**5**
36	Eye Gel	Ofloxacin	Rel. Subst.	1	1	3	1	7	1	**2**
37	Cutaneous Solution	Minoxidil	Rel. Subst.	1	1	9	1	1	11	**4**
38	Eye Ointment	Prednisolone acetate	Rel. Subst.	7	7	9	1	7	1	**5**
39	Cutaneous Solution	Salicylic acid	Rel. Subst.	3	9	7	1	11	1	**5**
40	Ointment	Salicylic acid	Rel. Subst.	9	9	3	9	11	1	**7**
41	Ointment	Betamethasone dipropionate	Rel. Subst.	3	9	9	3	11	1	**6**
42	Cutaneous Emulsion	Metronidazole	Rel. Subst.	9	9	1	1	3	1	**4**
43	Eye Drops, Solution	Dexamethasone sodium phosphate	Rel. Subst.	7	7	9	7	9	1	**7**

Table 20. *Method performance classification - preservative assay.*

	Pharmaceutical Form	Preservative	Method	Column Efficiency	Symmetry Factor	Peak Area RSD	Retention Time RSD	Samples RSD	OOS	Method Performance Classification
44	Shampoo	Piroctone Olamine	Preserv. Assay	11	11	11	11	11	1	**9**
45	Comfort Ophthalmic Solution	Chlorhexidine Digluconate	Preserv. Assay	1	9	1	1	7	1	**3**
46	Comfort Ophthalmic Solution	Benzalkonium Chloride	Preserv. Assay	3	3	9	1	9	1	**4**
47	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	9	1	1	1	1	**3**
48	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	11	11	11	11	1	**9**
49	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	1	9	1	3	3	1	**3**
50	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	9	3	3	3	1	**4**
51	Eye Drops, Solution	Benzethonium Chloride	Preserv. Assay	11	11	11	11	11	1	**9**
52	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	1	9	1	3	3	11	**5**
53	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	1	9	1	1	1	1	**2**
54	Eye Gel	Benzalkonium Chloride	Preserv. Assay	3	9	9	9	1	1	**5**
55	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	9	3	1	3	1	**3**
56	Ointment	Parabens	Preserv. Assay	11	11	11	11	11	1	**9**
57	Cutaneous Solution	Parabens	Preserv. Assay	11	9	9	1	9	1	**7**
58	Ointment	Parabens	Preserv. Assay	11	11	11	11	11	1	**9**
59	Cutaneous Emulsion	Parabens	Preserv. Assay	9	9	7	1	9	1	**6**
60	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	1	3	3	1	3	1	**2**
61	Cream	Parabens	Preserv. Assay	1	7	9	1	9	1	**5**
62	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	11	11	11	11	1	**9**
63	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	1	7	1	3	1	1	**2**
64	Cream	Parabens	Preserv. Assay	11	11	11	11	11	1	**9**

- **State of the Art – Method Validation**

Regarding the method validation state of the art, Figure 26 shows, for each validation parameter, the percentage of reports in which the parameter in question is validated. It should be noted that both the relative response factor and the detection / quantification limits are parameters applicable only to related substances methods and, therefore, the percentage presented in these parameters concerns only these methods.

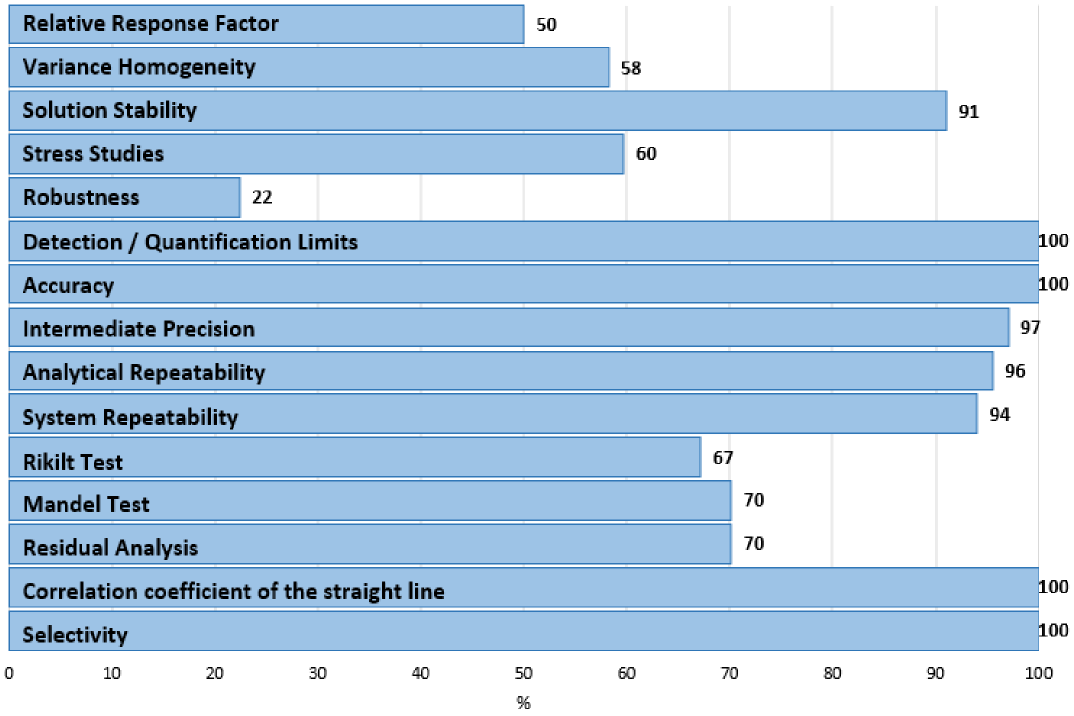

Figure 26. *Percentage of reports that have each parameter validated.*

Analysing the graph, it is possible to notice that the most important parameters, according to the ICH table (table 1), are validated in practically 100% of the reports analysed here. On the other hand, it is interesting to point out that robustness has the lowest percentage in this study, this is due to the fact that at the time these reports were produced, this parameter was not mandatory. However, it is currently one of the most important validation parameters and is increasingly requested by customers.

- **Probability / Occurrence Results**

Tables 20, 21 and 22 contain the values attributed to each parameter and the result attributed to probability/occurrence, which is an average of the three parameters.

Regarding the method validation parameter, there is one method that stands out from the rest, presenting the highest value, 8, as it is extremely incomplete. This is due to the fact that this validation report is the oldest of all those under study here.

It is also possible to observe that there are methods that present a very complete validation report (value of 1) but which, however, do not have the best performance, highlighting that a method is not necessarily good because it presents a complete validation report.

Regarding the risk values obtained for probability/occurrence, they are all within the same register, with the impurity methods presenting the highest risk values. This is due to the method state-of-the-art parameter, which takes into account the existence of more advanced technology in the company and the level of knowledge of the substance. In this case, all methods classified as 6 are out of date in relation to the existence of known impurities.

Table 21. *Probability / occurrence classification - API assay.*

	Pharmaceutical Form	Active Substance	Method	Method Performance	Method Validation	Method State of the Art	(O)
1	Eye Drops, Solution	Atropine Sulfate	API Assay	5	3	1	**3**
2	Eye Drops, Solution	Betaxolol hydrochloride	API Assay	9	6	1	**5**
3	Cutaneous Solution	Erythromycin	API Assay	1	8	9	**6**
4	Eye Drops, Solution	Chloramphenicol	API Assay	3	3	1	**2**
5	Eye Ointment	Chloramphenicol	API Assay	5	2	1	**3**
6	Eye Drops, Solution	Dorzolamide hydrochloride	API Assay	9	6	1	**5**
7	Eye Drops, Solution	Flurbiprofen sodium	API Assay	3	2	1	**2**
8	Eye Drops, Solution	Clonidine hydrochloride	API Assay	9	2	1	**4**
9	Eye Drops, Solution	Ketorolac tromethamine	API Assay	3	3	1	**2**
10	Eye Gel	Ofloxacin	API Assay	2	3	1	**2**
11	Eye Drops, Solution	Gentamicin sulfate	API Assay	1	3	9	**4**
12	Ointment	Zinc oxide	API Assay	1	2	1	**1**
13	Eye Ointment	Prednisolone acetate	API Assay	5	1	1	**2**
14	Cutaneous Solution	Minoxidil	API Assay	2	6	1	**3**
15	Cutaneous Suspension	Zinc oxide	API Assay	1	2	1	**1**
16	Ointment	Hydrocortisone	API Assay	5	6	1	**4**
17	Cream	Hydrocortisone	API Assay	4	6	1	**4**
18	Eye Ointment	Prednisolone acetate	API Assay	5	6	1	**4**
19	Cutaneous Solution	Salicylic acid	API Assay	5	1	1	**2**
20	Cutaneous Solution	Betamethasone dipropionate	API Assay	4	1	1	**2**
21	Ointment	Salicylic acid	API Assay	6	2	1	**3**
22	Ointment	Betamethasone dipropionate	API Assay	4	2	1	**2**
23	Ointment	Salicylic acid + Clioquinol	API Assay	9	1	1	**4**
24	Cutaneous Emulsion	Metronidazole	API Assay	5	1	1	**2**
25	Eye Drops, Solution	Dexamethasone sodium phosphate	API Assay	3	6	1	**3**
26	Cutaneous Liquid	Ketoconazole	API Assay	5	6	1	**4**
27	Shampoo	Ketoconazole	API Assay	7	6	1	**5**
28	Eye Ointment	Oxytetracycline hydrochloride	API Assay	9	1	1	**4**
29	Cream	Pyrithione zinc	API Assay	1	3	1	**2**
30	Cutaneous Solution	Pyrithione zinc	API Assay	1	3	1	**2**

Table 22. *Probability / occurrence classification - related substances.*

	Pharmaceutical Form	Active Substance	Method	Method Performance	Method Validation	Method State of the Art	(O)
31	Eye Drops, Solution	Atropine sulfate	Rel. Subst.	5	4	6	**5**
32	Eye Drops, Solution	Chloramphenicol	Rel. Subst.	6	4	1	**4**
33	Eye Ointment	Chloramphenicol	Rel. Subst.	6	3	1	**3**
34	Eye Drops, Solution	Dorzolamide hydrochloride	Rel. Subst.	9	6	6	**7**
35	Eye Drops, Solution	Flurbiprofen sodium	Rel. Subst.	5	3	6	**5**
36	Eye Gel	Ofloxacin	Rel. Subst.	2	2	6	**3**
37	Cutaneous Solution	Minoxidil	Rel. Subst.	4	4	6	**5**
38	Eye Ointment	Prednisolone acetate	Rel. Subst.	5	3	6	**5**
39	Cutaneous Solution	Salicylic acid	Rel. Subst.	5	2	1	**3**
40	Ointment	Salicylic acid	Rel. Subst.	7	2	1	**3**
41	Ointment	Betamethasone dipropionate	Rel. Subst.	6	2	6	**5**
42	Cutaneous Emulsion	Metronidazole	Rel. Subst.	4	2	1	**2**
43	Eye Drops, Solution	Dexamethasone sodium phosphate	Rel. Subst.	7	5	6	**6**

Table 23. *Probability / occurrence classification - preservative assay.*

	Pharmaceutical Form	**Preservative**	**Method**	**Method Performance**	**Method Validation**	**Method State of the Art**	**(O)**
44	Shampoo	Piroctone Olamine	Preserv. Assay	9	1	1	**4**
45	Comfort Ophthalmic Solution	Chlorhexidine Digluconate	Preserv. Assay	3	3	1	**2**
46	Comfort Ophthalmic Solution	Benzalkonium Chloride	Preserv. Assay	4	3	1	**3**
47	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	6	1	**3**
48	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	9	6	1	**5**
49	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	3	1	**2**
50	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	4	3	1	**3**
51	Eye Drops, Solution	Benzethonium Chloride	Preserv. Assay	9	3	1	**4**
52	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	5	3	1	**3**
53	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	2	3	1	**2**
54	Eye Gel	Benzalkonium Chloride	Preserv. Assay	5	3	1	**3**
55	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	3	1	**2**
56	Ointment	Parabens	Preserv. Assay	9	1	1	**4**
57	Cutaneous Solution	Parabens	Preserv. Assay	7	1	1	**3**
58	Ointment	Parabens	Preserv. Assay	9	1	1	**4**
59	Cutaneous Emulsion	Parabens	Preserv. Assay	6	1	1	**3**
60	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	2	6	1	**3**
61	Cream	Parabens	Preserv. Assay	5	6	1	**4**
62	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	9	3	1	**4**
63	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	2	3	1	**2**
64	Cream	Parabens	Preserv. Assay	9	1	1	**4**

4.1.2. Severity

As mentioned in chapter 3.1, severity was evaluated taking into account three parameters: the type of analyte, the frequency of use of the product and the type of product.

For APIs, the assessment was made considering the respective PDE. The PDE values for each of the products were taken from the company's internal documents and in cases where the product can be used in both humans and animals, the corresponding veterinary PDE value was considered, as it is always lower.

Both the frequency of use and the type of medication were taken from the company's website.

Regarding the classification obtained for severity, the results are found in tables 23, 24 and 25.

Table 24. *Severity classification - API assay.*

	Pharmaceutical Form	Active Substance	Method	Analyte Type	Use Frequency	Product Type	(S)
1	Eye Drops, Solution	Atropine Sulfate	API Assay	11	3	11	**8**
2	Eye Drops, Solution	Betaxolol hydrochloride	API Assay	11	11	9	**10**
3	Cutaneous Solution	Erythromycin	API Assay	1	7	5	**4**
4	Eye Drops, Solution	Chloramphenicol	API Assay	7	3	5	**5**
5	Eye Ointment	Chloramphenicol	API Assay	7	3	5	**5**
6	Eye Drops, Solution	Dorzolamide hydrochloride	API Assay	11	11	9	**10**
7	Eye Drops, Solution	Flurbiprofen sodium	API Assay	7	3	3	**4**
8	Eye Drops, Solution	Clonidine hydrochloride	API Assay	9	11	9	**10**
9	Eye Drops, Solution	Ketorolac tromethamine	API Assay	9	7	3	**6**
10	Eye Gel	Ofloxacin	API Assay	1	3	5	**3**
11	Eye Drops, Solution	Gentamicin sulfate	API Assay	7	3	5	**5**
12	Ointment	Zinc oxide	API Assay	1	3	1	**2**
13	Eye Ointment	Prednisolone acetate	API Assay	9	3	7	**6**
14	Cutaneous Solution	Minoxidil	API Assay	3	11	1	**5**
15	Cutaneous Suspension	Zinc oxide	API Assay	1	3	1	**2**
16	Ointment	Hydrocortisone	API Assay	7	3	7	**6**
17	Cream	Hydrocortisone	API Assay	7	3	7	**6**
18	Eye Ointment	Prednisolone acetate	API Assay	9	3	7	**6**
19	Cutaneous Solution	Salicylic acid	API Assay	1	11	7	**6**
20	Cutaneous Solution	Betamethasone dipropionate	API Assay	11	11	7	**10**
21	Ointment	Salicylic acid	API Assay	1	11	7	**6**
22	Ointment	Betamethasone dipropionate	API Assay	11	11	7	**10**
23	Ointment	Salicylic acid + Clioquinol	API Assay	1	7	1	**3**
24	Cutaneous Emulsion	Metronidazole	API Assay	9	11	5	**8**
25	Eye Drops, Solution	Dexamethasone sodium phosphate	API Assay	11	7	7	**8**
26	Cutaneous Liquid	Ketoconazole	API Assay	5	7	1	**4**
27	Shampoo	Ketoconazole	API Assay	5	7	1	**4**
28	Eye Ointment	Oxytetracycline hydrochloride	API Assay	7	3	5	**5**
29	Cream	Pyrithione zinc	API Assay	7	7	5	**6**
30	Cutaneous Solution	Pyrithione zinc	API Assay	7	7	5	**6**

***Table 25**. Severity classification - related substances.*

	Pharmaceutical Form	Active Substance	Method	Analyte Type	Use Frequency	Product Type	(S)
31	Eye Drops, Solution	Atropine sulfate	Rel. Subst.	11	3	11	**8**
32	Eye Drops, Solution	Chloramphenicol	Rel. Subst.	11	3	5	**6**
33	Eye Ointment	Chloramphenicol	Rel. Subst.	11	3	5	**6**
34	Eye Drops, Solution	Dorzolamide hydrochloride	Rel. Subst.	11	11	9	**10**
35	Eye Drops, Solution	Flurbiprofen sodium	Rel. Subst.	11	3	3	**6**
36	Eye Gel	Ofloxacin	Rel. Subst.	11	3	5	**6**
37	Cutaneous Solution	Minoxidil	Rel. Subst.	11	11	1	**8**
38	Eye Ointment	Prednisolone acetate	Rel. Subst.	11	3	7	**7**
39	Cutaneous Solution	Salicylic acid	Rel. Subst.	11	11	7	**10**
40	Ointment	Salicylic acid	Rel. Subst.	11	11	7	**10**
41	Ointment	Betamethasone dipropionate	Rel. Subst.	11	11	7	**10**
42	Cutaneous Emulsion	Metronidazole	Rel. Subst.	11	11	5	**9**
43	Eye Drops, Solution	Dexamethasone sodium phosphate	Rel. Subst.	11	7	7	**8**

***Table 26.** Severity classification - preservative assay.*

	Pharmaceutical Form	Preservative	Method	Analyte Type	Use Frequency	Product Type	(S)
44	Shampoo	Piroctone Olamine	Preserv. Assay	7	11	1	**6**
45	Comfort Ophthalmic Solution	Chlorhexidine Digluconate	Preserv. Assay	11	11	1	**8**
46	Comfort Ophthalmic Solution	Benzalkonium Chloride	Preserv. Assay	11	11	1	**8**
47	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	3	11	**8**
48	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	11	9	**10**
49	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	3	11	**8**
50	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	3	5	**6**
51	Eye Drops, Solution	Benzethonium Chloride	Preserv. Assay	11	11	9	**10**
52	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	7	3	**7**
53	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	3	5	**6**
54	Eye Gel	Benzalkonium Chloride	Preserv. Assay	11	3	5	**6**
55	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	3	5	**6**
56	Ointment	Parabens	Preserv. Assay	7	3	1	**4**
57	Cutaneous Solution	Parabens	Preserv. Assay	7	3	1	**4**
58	Ointment	Parabens	Preserv. Assay	7	7	1	**5**
59	Cutaneous Emulsion	Parabens	Preserv. Assay	7	11	5	**8**
60	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	7	7	**8**
61	Cream	Parabens	Preserv. Assay	7	7	1	**5**
62	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	11	9	10
63	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	11	11	9	10
64	Cream	Parabens	Preserv. Assay	7	7	5	6

4.1.3. Risk Analysis Results

The final risk classification was obtained by multiplying the probability/occurrence x severity x detectability.

Regarding detectability, for this first phase of the risk analysis, non-revalidation was considered, as it is the worst-case scenario. In this way, the methods were classified as 11 or 9 depending on whether the state of the art of the method was updated.

The results of the risk analysis are found in tables 26, 27 and 28. Of the 30 API assay methods considered here, half present an acceptable risk, 5 present an unacceptable risk and the remaining 10 require a decision. For related substances methods, only one method presents an acceptable risk, there are 3 that require a decision and the remaining 9 are unacceptable. Finally, the preservative assay methods have only 3 unacceptable, 12 that require decision and 6 acceptable.

Thus, of the 64 methods considered for this risk analysis, around a third obtained an acceptable risk, around 40% require a decision and the remaining present an unacceptable risk.

Table 27. *Final risk classification - API assay.*

	Pharmaceutical Form	Active Substance	Method	(O)	(S)	(D)	Risk	Score
1	Eye Drops, Solution	Atropine Sulfate	API Assay	3	8	9	**216**	**Decision Required**
2	Eye Drops, Solution	Betaxolol hydrochloride	API Assay	5	10	9	**450**	**Not Acceptable**
3	Cutaneous Solution	Erythromycin	API Assay	6	4	11	**264**	**Not Acceptable**
4	Eye Drops, Solution	Chloramphenicol	API Assay	2	5	9	**90**	**Acceptable**
5	Eye Ointment	Chloramphenicol	API Assay	3	5	9	**135**	**Acceptable**
6	Eye Drops, Solution	Dorzolamide hydrochloride	API Assay	5	10	9	**450**	**Not Acceptable**
7	Eye Drops, Solution	Flurbiprofen sodium	API Assay	2	4	9	**72**	**Acceptable**
8	Eye Drops, Solution	Clonidine hydrochloride	API Assay	4	10	9	**360**	**Not Acceptable**
9	Eye Drops, Solution	Ketorolac tromethamine	API Assay	2	6	9	**108**	**Acceptable**
10	Eye Gel	Ofloxacin	API Assay	2	3	9	**54**	**Acceptable**
11	Eye Drops, Solution	Gentamicin sulfate	API Assay	4	5	11	**220**	**Not Acceptable**
12	Ointment	Zinc oxide	API Assay	1	2	9	**18**	**Acceptable**
13	Eye Ointment	Prednisolone acetate	API Assay	2	6	9	**108**	**Acceptable**
14	Cutaneous Solution	Minoxidil	API Assay	3	5	9	**135**	**Acceptable**
15	Cutaneous Suspension	Zinc oxide	API Assay	1	2	9	**18**	**Acceptable**
16	Ointment	Hydrocortisone	API Assay	4	6	9	**216**	**Decision Required**
17	Cream	Hydrocortisone	API Assay	4	6	9	**216**	**Decision Required**
18	Eye Ointment	Prednisolone acetate	API Assay	4	6	9	**216**	**Decision Required**
19	Cutaneous Solution	Salicylic acid	API Assay	2	6	9	**108**	**Acceptable**
20	Cutaneous Solution	Betamethasone dipropionate	API Assay	2	10	9	**180**	**Decision Required**
21	Ointment	Salicylic acid	API Assay	3	6	9	**162**	**Acceptable**
22	Ointment	Betamethasone dipropionate	API Assay	2	10	9	**180**	**Decision Required**
23	Ointment	Salicylic acid + Clioquinol	API Assay	4	3	9	**108**	**Acceptable**
24	Cutaneous Emulsion	Metronidazole	API Assay	2	8	9	**144**	**Decision Required**
25	Eye Drops, Solution	Dexamethasone sodium phosphate	API Assay	3	8	9	**216**	**Decision Required**
26	Cutaneous Liquid	Ketoconazole	API Assay	4	4	9	**144**	**Acceptable**
27	Shampoo	Ketoconazole	API Assay	5	4	9	**180**	**Decision Required**
28	Eye Ointment	Oxytetracycline hydrochloride	API Assay	4	5	9	**180**	**Decision Required**
29	Cream	Pyrithione zinc	API Assay	2	6	9	**108**	**Acceptable**
30	Cutaneous Solution	Pyrithione zinc	API Assay	2	6	9	**108**	**Acceptable**

Table 28. *Final risk classification - related substances.*

	Pharmaceutical Form	Active Substance	Method	(O)	(S)	(D)	Risk	Score
31	Eye Drops, Solution	Atropine sulfate	Rel. Subst.	5	8	11	440	Not Acceptable
32	Eye Drops, Solution	Chloramphenicol	Rel. Subst.	4	6	9	216	Not Acceptable
33	Eye Ointment	Chloramphenicol	Rel. Subst.	3	6	9	162	Acceptable
34	Eye Drops, Solution	Dorzolamide hydrochloride	Rel. Subst.	7	10	11	770	Not Acceptable
35	Eye Drops, Solution	Flurbiprofen sodium	Rel. Subst.	5	6	11	330	Not Acceptable
36	Eye Gel	Ofloxacin	Rel. Subst.	3	6	11	198	Not Acceptable
37	Cutaneous Solution	Minoxidil	Rel. Subst.	5	8	11	440	Not Acceptable
38	Eye Ointment	Prednisolone acetate	Rel. Subst.	5	7	11	385	Not Acceptable
39	Cutaneous Solution	Salicylic acid	Rel. Subst.	3	10	9	270	Decision Required
40	Ointment	Salicylic acid	Rel. Subst.	3	10	9	270	Decision Required
41	Ointment	Betamethasone dipropionate	Rel. Subst.	5	10	11	550	Not Acceptable
42	Cutaneous Emulsion	Metronidazole	Rel. Subst.	2	9	9	162	Decision Required
43	Eye Drops, Solution	Dexamethasone sodium phosphate	Rel. Subst.	6	8	11	528	Not Acceptable

Table 29. *Final risk classification - preservative assay.*

	Pharmaceutical Form	Preservative	Method	(O)	(S)	(D)	Risk	Score
44	Shampoo	Piroctone Olamine	Preserv. Assay	4	6	9	216	Decision Required
45	Comfort Ophthalmic Solution	Chlorhexidine Digluconate	Preserv. Assay	2	8	9	144	Decision Required
46	Comfort Ophthalmic Solution	Benzalkonium Chloride	Preserv. Assay	3	8	9	216	Decision Required
47	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	8	9	216	Decision Required
48	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	5	10	9	450	Not Acceptable
49	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	2	8	9	144	Decision Required
50	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	6	9	162	Acceptable
51	Eye Drops, Solution	Benzethonium Chloride	Preserv. Assay	4	10	9	360	Not Acceptable
52	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	7	9	189	Decision Required
53	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	2	6	9	108	Acceptable
54	Eye Gel	Benzalkonium Chloride	Preserv. Assay	3	6	9	162	Acceptable
55	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	2	6	9	108	Acceptable
56	Ointment	Parabens	Preserv. Assay	4	4	9	144	Acceptable
57	Cutaneous Solution	Parabens	Preserv. Assay	3	4	9	108	Acceptable
58	Ointment	Parabens	Preserv. Assay	4	5	9	180	Decision Required
59	Cutaneous Emulsion	Parabens	Preserv. Assay	3	8	9	216	Decision Required
60	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	8	9	216	Decision Required
61	Cream	Parabens	Preserv. Assay	4	5	9	180	Decision Required
62	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	4	10	9	360	Not Acceptable
63	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	2	10	9	180	Decision Required
64	Cream	Parabens	Preserv. Assay	4	6	9	216	Decision Required

4.1.4. Risk Mitigation

After carrying out the risk analysis, risk mitigation follows, in order to understand what actions must be taken to turn unacceptable risks into acceptable ones. In this case, since we cannot act on the severity parameter, we acted on the probability/occurrence, more specifically on the state of the art of the method validation and, consequently, on detectability.

Regarding API assay methods, the 15 methods that have an unacceptable risk or that require a decision were analysed.

Firstly, method *1* was classified as "decision required" and looking at the performance of the method it is clear that both the column efficiency and the peak symmetry factor present a very poor performance. In this case, a partial revalidation of the method would not act on these factors, which is why it was decided to carry out a total revalidation of the method.

In the methods *2*, *6,* and *25*, it was decided to update the validation reports, adding parameters that are not described in them, such as residue analysis, Mandel and Rikilt tests and homogeneity of variances. Robustness will also be carried out, once that it is a very important parameter. The performance of robustness alone also transforms the method *8* from unacceptable to acceptable.

The update of the method, by adding residue analysis, Mandel and Rikilt tests and homogeneity of variances, will also be carried out in the methods *14*, *16*, *17* and *18*.

In the method *11*, it is sufficient to carry out a partial revalidation, by carrying out method robustness and also forced degradation tests, as these are not found in the method validation report.

Regarding method *3*, this is one of the methods presented here that is not a HPLC method, existing the possibility to update the respective technology, and which presents a validation report that is quite old and incomplete. Therefore, it was chosen to develop and validate a new method.

For methods *20* and *22* the classification of "decision required" is due to the high severity they present, since the state of the art of validation is already the lowest possible and the classification of the occurrence has a very low value. Therefore, it was chosen not to revalidate any of these methods and evaluate the case later.

For method 28, since the state-of-the-art of method validation presents the lowest risk value and the severity is also not very high, the final classification of 'requires decision' is due to the fact that it does not have enough points for performance analysis. Therefore, it is decided not to revalidate the method and reevaluate the case later.

Finally, in the method *24* method, by looking at the performance of the method it is possible to see that the Cpk values obtained below 1 are due to the high variability of the data and not to the existence of values out of specification. Therefore, it was chosen not to revalidate the method and evaluate the case later.

Table 29 summarizes the mitigation actions to be implemented in API assay methods.

*Ta**ble 30**. Decisions matrix – API assay.*

Method	Active Substance	Method	Risk Score	Decision
1	Atropine sulphate	API Assay	16	Poor performance of the method - **total revalidation**.
2	Betaxolol hydrochloride	API Assay	200	Update the method. Perform **robustness**.
6	Dorzolamide hydrochloride	API Assay		
25	Dexamethasone sodium phosphate	API Assay	80	
8	Clonidine hydrochloride	API Assay	4	Perform **robustness**.
3	Erythromycin	API Assay	264	Update method technology for HPLC - **development and validation of a new method**.
11	Gentamicin sulphate	API Assay	100	Perform **robustness** and **stress studies**.
20	Betamethasone dipropionate	API Assay	180	Do not revalidate the method and **reevaluate the case later**.
22	Betamethasone dipropionate	API Assay		
24	Metronidazole	API Assay	144	
28	Oxytetracycline hydrochloride	API Assay	180	
16	Hydrocortisone	API Assay	162	Update the method.
17	Hydrocortisone	API Assay		
18	Prednisolone acetate	API Assay		
27	Ketoconazole	API Assay	144	

Table 30 presents the classifications obtained for each parameter after risk mitigation, with the classifications that were changed with the decisions taken being in blue.

***Table 31.** Final risk classification after mitigation - API assay.*

	Pharmaceutical Form	Active Substance	Method	(O)	(S)	(D)	Risk	Score
1	Eye Drops, Solution	Atropine Sulfate	API Assay	2	8	1	16	Acceptable
2	Eye Drops, Solution	Betaxolol hydrochloride	API Assay	4	10	5	200	Acceptable
3	Cutaneous Solution	Erythromycin	API Assay	1	4	1	4	Acceptable
4	Eye Drops, Solution	Chloramphenicol	API Assay	3	5	9	135	Acceptable
5	Eye Ointment	Chloramphenicol	API Assay	3	5	9	135	Acceptable
6	Eye Drops, Solution	Dorzolamide hydrochloride	API Assay	4	10	5	200	Acceptable
7	Eye Drops, Solution	Flurbiprofen sodium	API Assay	3	4	9	108	Acceptable
8	Eye Drops, Solution	Clonidine hydrochloride	API Assay	4	10	5	200	Acceptable
9	Eye Drops, Solution	Ketorolac tromethamine	API Assay	3	6	9	162	Acceptable
10	Eye Gel	Ofloxacin	API Assay	3	3	9	81	Acceptable
11	Eye Drops, Solution	Gentamicin sulfate	API Assay	4	5	5	100	Acceptable
12	Ointment	Zinc oxide	API Assay	1	2	9	18	Acceptable
13	Eye Ointment	Prednisolone acetate	API Assay	2	6	9	108	Acceptable
14	Cutaneous Solution	Minoxidil	API Assay	3	5	9	135	Acceptable
15	Cutaneous Suspension	Zinc oxide	API Assay	1	2	9	18	Acceptable
16	Ointment	Hydrocortisone	API Assay	3	6	9	162	Acceptable
17	Cream	Hydrocortisone	API Assay	3	6	9	162	Acceptable
18	Eye Ointment	Prednisolone acetate	API Assay	3	6	9	162	Acceptable
19	Cutaneous Solution	Salicylic acid	API Assay	3	6	9	162	Acceptable
20	Cutaneous Solution	Betamethasone dipropionate	API Assay	2	10	9	180	Acceptable
21	Ointment	Salicylic acid	API Assay	3	6	9	162	Acceptable
22	Ointment	Betamethasone dipropionate	API Assay	2	10	9	180	Acceptable
23	Ointment	Salicylic acid + Clioquinol	API Assay	4	3	9	108	Acceptable
24	Cutaneous Emulsion	Metronidazole	API Assay	2	8	9	144	Acceptable
25	Eye Drops, Solution	Dexamethasone sodium phosphate	API Assay	2	8	5	80	Acceptable
26	Cutaneous Liquid	Ketoconazole	API Assay	4	4	9	144	Acceptable
27	Shampoo	Ketoconazole	API Assay	4	4	9	144	Acceptable
28	Eye Ointment	Oxytetracycline hydrochloride	API Assay	4	5	9	180	Acceptable
29	Cream	Pyrithione zinc	API Assay	2	6	9	108	Acceptable
30	Cutaneous Solution	Pyrithione zinc	API Assay	2	6	9	108	Acceptable

Regarding related substances methods, methods *31*, *34*, *35*, *36* and *38* have several parameters missing from their validation reports. Therefore, instead of revalidating a series of different parameters, it was opted for a total revalidation of the methods.

In the case of method *32*, the concentration range used in the validation report is not adequate, so both linearity and accuracy must be revalidated. The partial revalidation of this method transforms the risk into 'decision required', however, considering the poor performance of the method in relation to column efficiency and symmetry factor, it is decided to carry out a total revalidation.

Also in method 37, a partial revalidation of the method leads to a risk of 'decision required', however this method presents an OOS and therefore a total revalidation of the method is chosen.

For the remaining methods, due to the fact that they are outdated in relation to known impurities, it is necessary to carry out a new selectivity study to prove that the methods identify these impurities. In addition, a robustness study must also be carried out.

Tables 31 and 32 contain the matrix of decisions taken and the results of the final risk classification, respectively.

Table 32. *Decisions matrix - related substances.*

Method	Active Substance	Method	Risk Score	Decision
31	Atropine sulphate	Rel. Subst.	24	**Total revalidation of the method**
34	Dorzolamide hydrochloride	Rel. Subst.	40	
35	Flurbiprofen sodium	Rel. Subst.	18	
36	Ofloxacin	Rel. Subst.	12	
38	Prednisolone acetate	Rel. Subst.	21	
32	Chloramphenicol	Rel. Subst.	24	
37	Minoxidil	Rel. Subst.	16	
39	Salicylic Acid	Rel. Subst.	150	Perform **robustness**.
40	Salicylic Acid	Rel. Subst.		
42	Metronidazole	Rel. Subst.	90	
41	Betamethasone dipropionate	Rel. Subst.	150	Perform **robustness and selectivity.**
43	Dexamethasone sodium phosphate	Rel. Subst.	120	Update the method. Perform **robustness and selectivity.**

Table 33. *Final risk classification after mitigation - related substances.*

	Pharmaceutical Form	Active Substance	Method	(O)	(S)	(D)	Risk	Score
31	Eye Drops, Solution	Atropine sulfate	Rel. Subst.	3	8	1	24	Acceptable
32	Eye Drops, Solution	Chloramphenicol	Rel. Subst.	4	6	1	24	Acceptable
33	Eye Ointment	Chloramphenicol	Rel. Subst.	3	6	1	18	Acceptable
34	Eye Drops, Solution	Dorzolamide hydrochloride	Rel. Subst.	4	10	1	40	Acceptable
35	Eye Drops, Solution	Flurbiprofen sodium	Rel. Subst.	3	6	1	18	Acceptable
36	Eye Gel	Ofloxacin	Rel. Subst.	2	6	1	12	Acceptable
37	Cutaneous Solution	Minoxidil	Rel. Subst.	2	8	1	16	Acceptable
38	Eye Ointment	Prednisolone acetate	Rel. Subst.	3	7	1	21	Acceptable
39	Cutaneous Solution	Salicylic acid	Rel. Subst.	3	10	5	150	Acceptable
40	Ointment	Salicylic acid	Rel. Subst.	3	10	5	150	Acceptable
41	Ointment	Betamethasone dipropionate	Rel. Subst.	3	10	5	150	Acceptable
42	Cutaneous Emulsion	Metronidazole	Rel. Subst.	2	9	5	90	Acceptable
43	Eye Drops, Solution	Dexamethasone sodium phosphate	Rel. Subst.	3	8	5	120	Acceptable

Finally, the 15 preservative assay methods that did not achieve an acceptable risk were evaluated. The summary table of the risk assessment after mitigation is below.

The methods *44* and *64* present the lowest risk value for the state-of-the-art method validation parameter and do not have a very high severity. Therefore, the fine classification of “decision required” is since they do not have sufficient historical data, which is why it was decided not to revalidate these methods and reevaluate these two cases later.

Similarly, to what was previously decided for other methods, for methods *61*, *48* and *60* it was decided to update them, by carrying out residual analysis, Mandel and Rikilt tests and homogeneity of variances. In addition, a robustness study will also be carried out, which will also be carried out for the *45*, *46* and *51* methods.

Regarding method *59,* looking at the performance of the method, it is observed that in terms of column efficiency and peak symmetry factor, it presents a poor performance, presenting several values below the specification value. Therefore, it was opted for a total revalidation of the method.

Finally, the last methods under analysis were evaluated as a group, since they are practically identical analytical methods, these being methods *47*, *49*, *52*, *62* and *63*. Since one of these methods has an OOS due to its performance, it was considered a worrying factor that must be considered in all methods that belong to this group. Therefore, it was decided to fully revalidate these methods.

Table 34. *Decisions matrix - preservative assay.*

Method	Preservative	Method	Risk Score	Decision
44	Piroctone Olamine	Preserv. Assay	216	Do not revalidate the method and **reevaluate the case later**.
64	Parabens	Preserv. Assay		
45	Chlorhexidine Digluconate	Preserv. Assay	120	Perform **robustness**.
46	Benzalkonium Chloride	Preserv. Assay		
51	Benzethonium Chloride	Preserv. Assay	200	
48	Benzalkonium Chloride	Preserv. Assay	200	Update the method. Perform **robustness**.
60	Benzalkonium Chloride	Preserv. Assay	80	
47	Benzalkonium Chloride	Preserv. Assay	16	**Total revalidation of the method.**
49	Benzalkonium Chloride	Preserv. Assay		
52	Benzalkonium Chloride	Preserv. Assay	21	
62	Benzalkonium Chloride	Preserv. Assay	40	
63	Benzalkonium Chloride	Preserv. Assay	30	
59	Parabens	Preserv. Assay	24	Poor performance of the method - **total revalidation**.
61	Parabens	Preserv. Assay	135	Update the method.

Table 35. *Final risk classification after mitigation - preservative assay.*

	Pharmaceutical Form	Preservative	Method	(O)	(S)	(D)	Risk	Score
44	Shampoo	Piroctone Olamine	Preserv. Assay	4	6	9	216	Acceptable
45	Comfort Ophthalmic Solution	Chlorhexidine Digluconate	Preserv. Assay	3	8	5	120	Acceptable
46	Comfort Ophthalmic Solution	Benzalkonium Chloride	Preserv. Assay	3	8	5	120	Acceptable
47	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	2	8	1	16	Acceptable
48	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	4	10	5	200	Acceptable
49	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	2	8	1	16	Acceptable
50	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	6	9	162	Acceptable
51	Eye Drops, Solution	Benzethonium Chloride	Preserv. Assay	4	10	5	200	Acceptable
52	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	7	1	21	Acceptable
53	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	6	9	162	Acceptable
54	Eye Gel	Benzalkonium Chloride	Preserv. Assay	3	6	9	162	Acceptable
55	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	6	9	162	Acceptable
56	Ointment	Parabens	Preserv. Assay	4	4	9	144	Acceptable
57	Cutaneous Solution	Parabens	Preserv. Assay	3	4	9	108	Acceptable
58	Ointment	Parabens	Preserv. Assay	4	5	9	180	Acceptable
59	Cutaneous Emulsion	Parabens	Preserv. Assay	3	8	1	24	Acceptable
60	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	2	8	5	80	Acceptable
61	Cream	Parabens	Preserv. Assay	3	5	9	135	Acceptable
62	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	4	10	1	40	Acceptable
63	Eye Drops, Solution	Benzalkonium Chloride	Preserv. Assay	3	10	1	30	Acceptable
64	Cream	Parabens	Preserv. Assay	4	6	9	216	Acceptable

4.2. Analytical Method Validation

Considering the risk analysis and respective mitigation plan carried out two methods were chosen that require total revalidation, one for API assay and the other for related substances, both relating to the same finished product. It was decided to validate method *1* (assay) and *31* (related substances). Since these methods present poor performance, it was decided to use a raw material pharmacopoeia method adapted to the finished product.

The analytical methods are described in chapter 3.3.

4.2.1. Selectivity

Selectivity was evaluated by the analysis of the solvent, a placebo solution at a concentration equivalent to the sample solution, a standard assay solution containing Atropine Sulfate at about 1000 µg/mL, a standard impurity solution containing Atropine Sulfate at about 1 µg/mL, a sample solution containing Atropine Sulfate at about 1000 µg/mL, a resolution solution containing Atropine Sulfate and Impurity B at about 200 µg/mL and 50 µg/mL, respectively, a standard impurity C solution containing impurity C at about 0.5 µg/mL, and a peak ID solution containing impurities A, D, E, F, G and H.
It was also analysed placebo, sample and Atropine Sulfate solutions submitted to stress conditions, including acid agent (HCl 1 N), basic agent (NaOH 0.01 N), oxidant agent (H_2O_2 30%), UV light and temperature (60ºC).

Chromatographic peaks resulting from the solvent and the placebo should not interfere with the analytes of interest. Any peaks corresponding to the analytes should be separated from any other peaks and from each other. The resolution factor between impurity B and Atropine Sulfate should be at least 2.5.

The analytical method for the determination of Atropine Sulfate in samples of Atropocil Eye Drops has demonstrated to be selective because the solvent and the placebo do not interfere with the analyte, and all the peaks are well separated from each other, as shown in Table 35.

Table 36. *Selectivity results.*

Solution	Retention Time (min)	Peaks	Relative Retention Time Concerning Atropine Sulfate	Resolution Factor
Solvent	-	-	-	-
Placebo	-	-	-	-
Standard Assay Solution	11.088	Atropine Sulfate	1.00	-
Standard Impurity Solution	11.121	Atropine Sulfate	1.00	-
Sample Solution	1.717	Impurity C	0.15	-
	11.087	Atropine Sulfate	1.00	50.51
Resolution Solution	9.995	Impurity B	0.90	-
	11.121	Atropine Sulfate	1.00	4.75
Peak ID Solution	7.277	Impurity E	0.66	-
	7.888	Impurity D	0.71	2.56
	8.845	Impurity F	0.80	3.42
	10.467	Impurity H	0.94	6.50
	11.107	Atropine Sulfate	1.00	2.94
	12.031	Impurity G	1.08	4.05
	18.354	Impurity A	1.65	26.00
Impurity C Solution	1.717	Impurity C	0.15	-

The chromatograms are presented in Annex A.

The results of the stress studies are in compliance with the established acceptance criteria, showing that the analytical method is selective for the determination of related compounds of atropine sulfate in drug product samples. The results also show that a decrease content of atropine sulfate corresponds to an increased content of related substances.

The placebo and solvent chromatograms don't show any other peaks; thus the method has demonstrated to be selective concerning any potential degradation of placebo or solvent.

Table 37. *Stress Studies for drug product.*

Stress Condition	RRT	Related Compounds Individual (%)	Total (%)	Assay (%)	Mass Balance
Drug Product solution + HCl 1N	(Imp. C) 0.16	0.44	0.44	100.27	100.71
Drug Product solution + NaOH 0.01N	(Imp. C) 0.16	0.39	0.39	100.13	100.52
Drug Product solution + H_2O_2 30%	(Imp. C) 0.16 0.47 0.55 (Imp. E) 0.66 0.77 (Imp. H) 0.94	0.60 0.40 0.73 0.71 0.07 0.24	2.75	95.19	97.94
Drug Product solution at 60ºC	(Imp. C) 0.16 (Imp. A) 1.67	9.92 0.25	10.12	89.24	99.36
Drug Product Solution under UV light	(Imp. C) 0.16	0.46	0.46	96.62	97.08

Table 38. *Stress studies for drug substance (atropine sulfate).*

Stress Condition	RRT	Related Compounds Individual (%)	Total (%)	Assay (%)	Mass Balance
Drug Substance solution + HCl 1N	(Imp. C) 0.16	0.35	0.35	99.83	100.18
Drug Substance solution + NaOH 0.01N	(Imp. C) 0.16	0.59	0.59	99.08	99.67
Drug Substance solution + H_2O_2 30%	0.55	0.05	0.05	100.20	100.25
Drug Substance solution at 60ºC	(Imp. C) 0.16	0.05	0.05	100.04	100.09
Drug Substance solution under UV light	1.55	0.06	0.06	100.74	100.80

4.2.2. Linearity

The linearity of the API assay analytical method was evaluated under the concentration range equivalent to 50% to 150% of the test concentration (1000 µg/mL Atropine Sulfate), using 5 different solutions.

For the related substances method, the linearity was evaluated under the concentration range equivalent to 0.05% to 2.5% of the test concentration, using 10 different solutions.

The regression line of the analytical response as a function of analyte concentration was calculated by the method of least squares. The correlation coefficient (r^2), y-intercept, slope of the regression line, a plot of the data and an analysis of the deviations (D %) of the actual data points from the regression line is reported. The deviations are expressed by:

$$D\ \% = \frac{C_C}{C_n} \times 100 \qquad (15)$$

C_C – Calculated concentration from the regression line; C_n – Nominal concentration.

The obtained results are presented in Tables 38 and 39 and Figures 27 and 28.

Table 39. *Linearity parameters for Atropine Sulfate - assay.*

Parameters	Results	Acceptance Criteria
Range (µg/mL)	477.2 – 1432.1	---
	Deviations (%)	
Minimum / Maximum (%)	99.53 – 100.52	**98.0 – 102.0**
Average (%)	99.98	---
RSD (%)	0.36	**≤ 2.0%**
r^2	0.99996	**> 0.9950**
	Regression Line	
Slope	11915.16	---
Intercept	287202	---

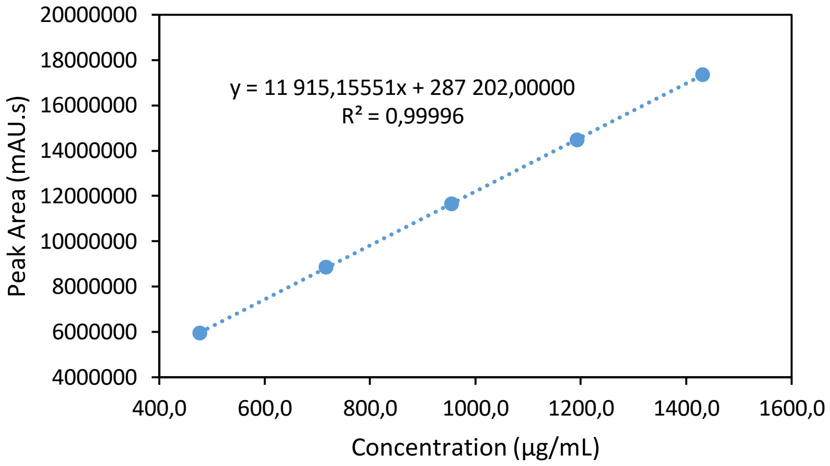

Figure 27. *Regression line plot for Atropine Sulfate - assay.*

Table 40. *Linearity parameters for Atropine Sulfate - related substances.*

Parameters	**Results**	**Acceptance Criteria**
Range (µg/mL)	0.5 – 24.2	---
	Deviations (%)	
Minimum / Maximum (%)	99.52 – 100.54	**98.0 – 102.0**
Average (%)	100.03	---
RSD (%)	0.35	**≤ 2.0%**
r^2	0.99997	**> 0.9950**
	Regression Line	
Slope	12242.8	---
Intercept	-225.7	---

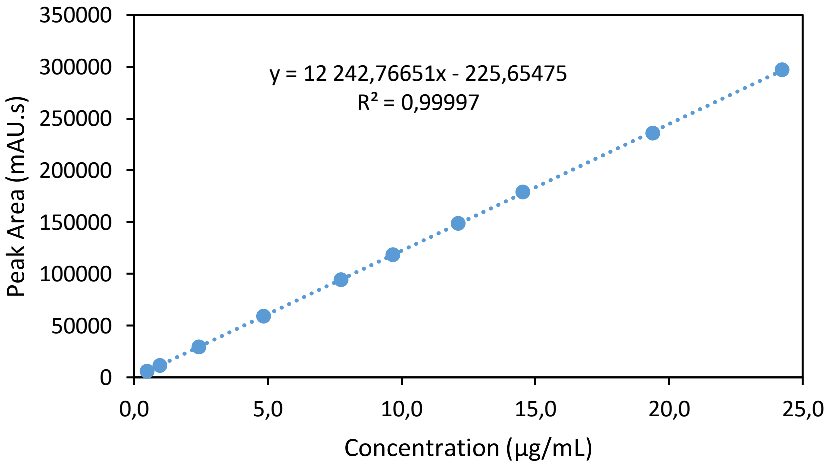

Figure 28*. Regression line plot for Atropine Sulfate - related substances.*

The results are in compliance with the established acceptance criteria, showing that the correlation coefficient is higher than 0.9950 in both methods.

Residual Analysis

The residual analysis shows the residuals of experimental point's distributions. In linear function residuals must be casually distributed. The results of residuals, by the following formula, are presented in Tables 40 and 41.

$$Residuals = \frac{y_i - y_{c_i}}{y_{c_i}} \times 100 \qquad (16)$$

Table 41. *Residual Analysis for Atropine Sulfate - assay.*

(i)	Conc (µg/mL)	Peak Area (mAU.s) y_i	Estimated Peak Area (mAU.s) y_{ci}	Residuals
1	477.2	5946177	5972919	-26742
2	715.8	8860327	8815777	44550
3	954.4	11656066	11658636	-2569
4	1193.0	14479951	14501494	-21542
5	1431.6	17350656	17344352	6304

Slope (b) = 11915.16

Intercept (a) = 287202.00

(r) = 0.99998

(r^2) = 0.99996

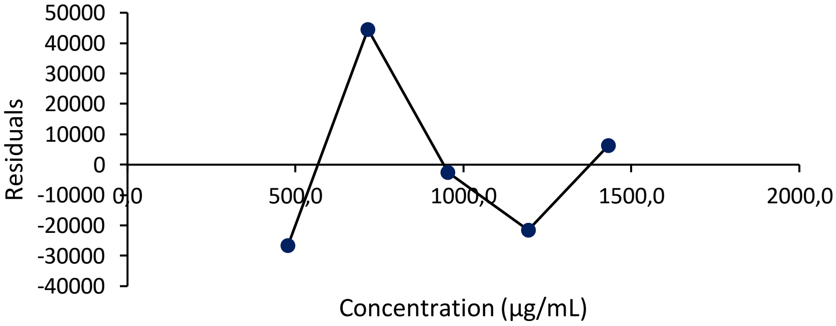

Figure 29. *Residual Analysis for Atropine Sulfate - assay.*

Table 42. *Residual Analysis for Atropine Sulfate - related substances.*

(i)	Conc (µg/mL)	Peak Area (mAU.s) y_i	Estimated Peak Area (mAU.s) y_{ci}	Residuals
1	0.5	5742	5710.2	32
2	1.0	11632	11646.1	-14
3	2.4	29398	29453.8	-56
4	4.8	59264	59133.3	130
5	7.8	94550	94748.7	-198
6	9.7	118124	118492.3	-368
7	12.1	148471	148171.7	300
8	14.5	178761	177851.2	910
9	19.4	236061	237210.2	-1149
10	24.2	296983	296569.1	413

Slope (b) =	12242.8
Intercept (a) =	-225.7
(r) =	0.99998
(r^2) =	0.99997

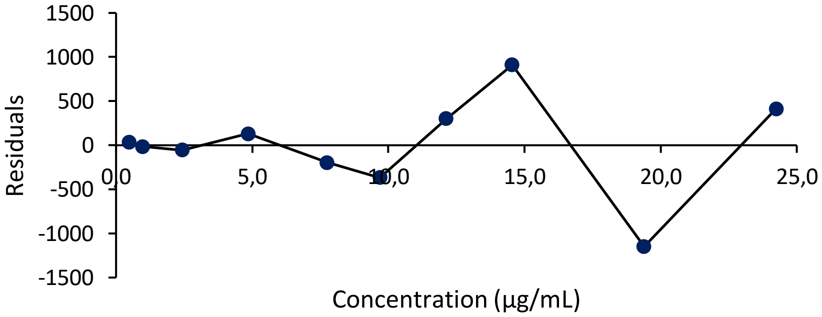

Figure 30. *Residual Analysis for Atropine Sulfate - related substances.*

The results are in compliance with the established acceptance criteria, showing that the analytical methods have a random distribution of residuals.

Correlation coefficient is a good correlation indicator, but not linearity indicator. Mandel Test and Rikilt Test must be used to prove that linearity function can be adapted to experimental representation.

Mandel Test

The results are presented in Tables 42 to 45.

Table 43. *Mandel Test - Results / Calculations for Atropine Sulfate - assay.*

(i)	Conc (µg/ml)	Peak Area (mAU.s) yi	Linear Adjustment Signal y_{Li}	Polynomial Adjustment Signal y_{Pi}
1	477.2	5946177	5972918.8	5964526.6
2	715.8	8860327	8815777.2	8819973.3
3	954.4	11656066	11658635.6	11667027.8
4	1193.0	14479952	14501494.0	14505690.1
5	1431.6	17350656	17344352.4	17335960.2

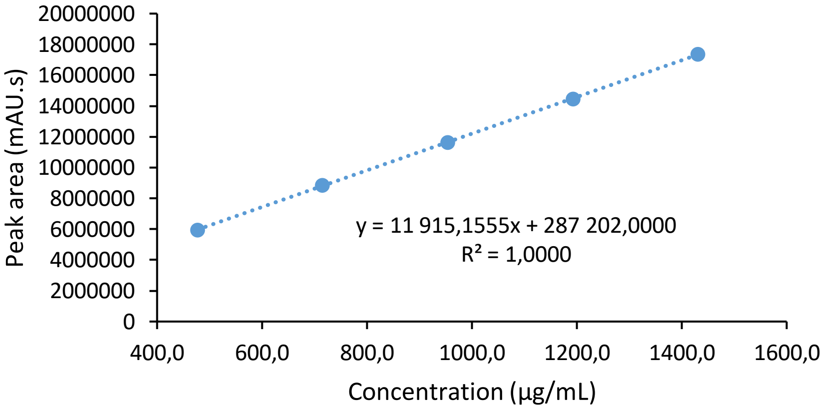

Figure 31. *Linear Adjustment - Mandel Test - assay.*

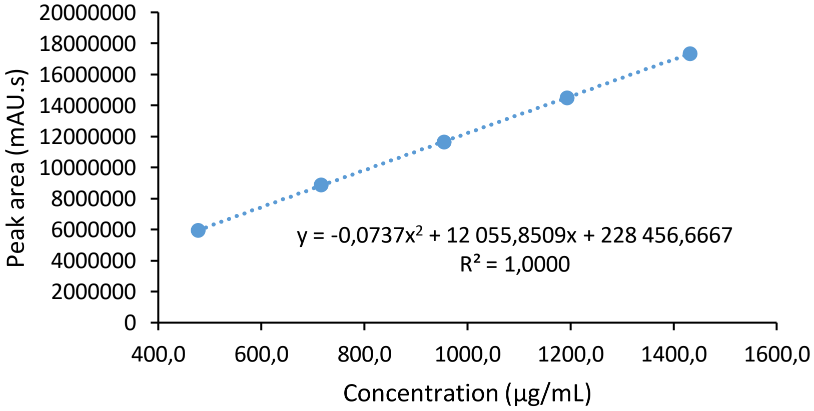

***Figure 32**. Polynomial Adjustment - Mandel Test - assay.*

Table 44. *Linear and Polynomial Adjustments - assay.*

(i)	$(y\text{-}yi)^2$
1	715141695
2	1984684680
3	6601131,204
4	464072125
5	39739575
Sum	3210239207
N-2	3
$S_{y/x}$	32712.1

(i)	$(y\text{-}yi)^2$
1	336720403
2	1628421488
3	120153542
4	662466705
5	215976055
Sum	2963738194
N-3	2
$S_{y/x\,(2^o)}$	38495,1

Regarding the assay method, DS^2 parameter, calculated according to the following formula, was 2.47 x 10^8.

$$S_{y/x} = \sqrt{\frac{\sum_{i=1}^{N}[y_i-(a+b.x_i)]^2}{N-2}} \qquad (17)$$

$$S_{y/x(2^o)} = \sqrt{\frac{\sum_{i=1}^{N}[y_i-(a+b.x_i+c.x_i^2)]^2}{N-3}} \qquad (18)$$

$$DS^2 = (N-2).S_{y/x}^2 - (N-3).S_{y/x(2^o)}^2 \qquad (19)$$

The Test Value (TV), obtained using the equation presented below, was 0.17.

$$TV = \frac{DS^2}{S_{y/x(2^o)}^2} \qquad (20)$$

The tabulated F factor, for a confidence range of 95% and N-3 degrees of freedom, is 18.51. As TV < F, linear function can be used for experimental representation.

Table 45. *Mandel Test - Results / Calculations for Atropine Sulfate - related substances.*

(i)	Conc. (µg/ml)	Peak Area (mAU.s) yi	Linear Adjustment Signal y_{Li}	Polynomial Adjustment Signal y_{Pi}
1	0.5	5742	5710.2	5717.9
2	1.0	11632	11646.1	11652.5
3	2.4	29398	29453.8	29456.6
4	4.8	59264	59133.3	59131.3
5	7.8	94550	94748.7	94742.9
6	9.7	118124	118492.3	118485.1
7	12.1	148471	148171.7	148164.2
8	14.5	178761	177851.2	177844.7
9	19.4	236061	237210.2	237210.1
10	24.2	296983	296569.1	296581.3

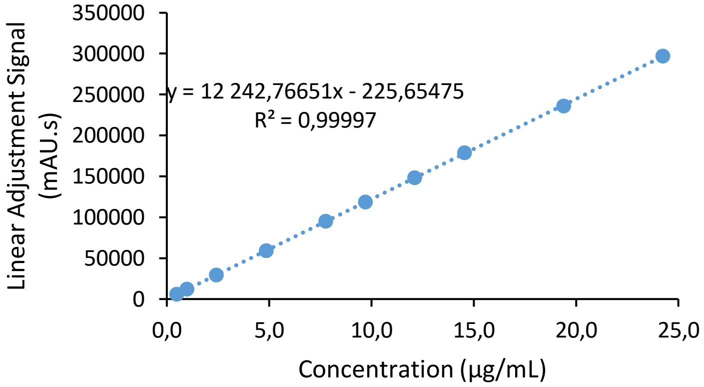

***Figure 33**. Linear Adjustment - Mandel Test - related substances.*

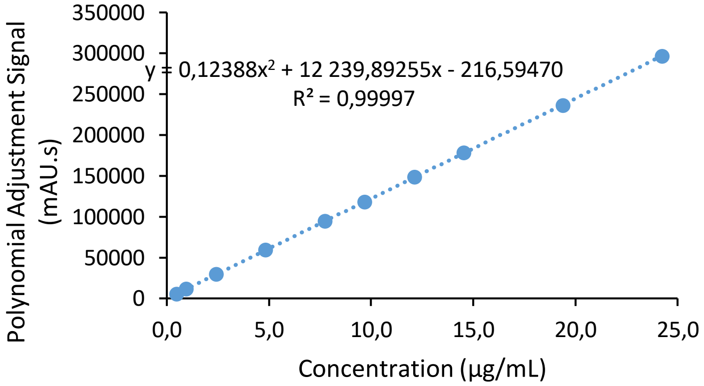

***Figure 34.** Polynomial Adjustment - Mandel Test - related substances.*

***Table 46.** Linear and Polynomial Adjustments - related substances.*

(i)	$(y-y_i)^2$
1	1034
2	209
3	3116
4	16995
5	39339
6	135367
7	89760
8	827715
9	1319819
10	170963
Sum	2604319
N-2	8
$S_{y/x}$	570.6

(i)	$(y-y_i)^2$
1	598
2	435
3	3439
4	17511
5	37080
6	130150
7	94356
8	839647
9	1319626
10	161031
Sum	2603873
N-3	7
$S_{y/x\ (2^{o})}$	609.9

Related substances method presents a DS^2 parameter equal to 4.45×10^2 and a Test Value (TV) of 1.20×10^{-3}. Since the tabulated F value, for a 95% confidence level and N-3 degrees of freedom, is 5.59, TV < F, thus linear function can be used for experimental representation.

Rikilt Test

Rikilt Test is used for evaluation of response factor (RF), to predict if RF can be used in routine laboratorial work, instead of diary calibration curve. The results are presented in Tables 46 and 47.

Table 47. *Rikilt Test - Results / Calculations for Atropine Sulfate - assay.*

(i)	Conc (µg/mL) (x_i)	Peak Area (mAU.s) (y_i)	Response Factor (y_i/x_i)	RFi / average RF x 100	Acceptance Criteria (%)
1	477.2	5946178	12460.98	101.62	**98.0 - 102.0**
2	715.8	8860327	12378.64	100.95	
3	954.4	11656066	12213.40	99.60	
4	1193.0	14479952	12137.85	98.99	
5	1431.6	17350656	12120.18	98.84	
		Average =	12262.21	100.00	**98.0 - 102.0**
		Standard Deviation =	150.90	1.23	-
		RSD (%) =	1.23	1.23	**≤ 2.0**

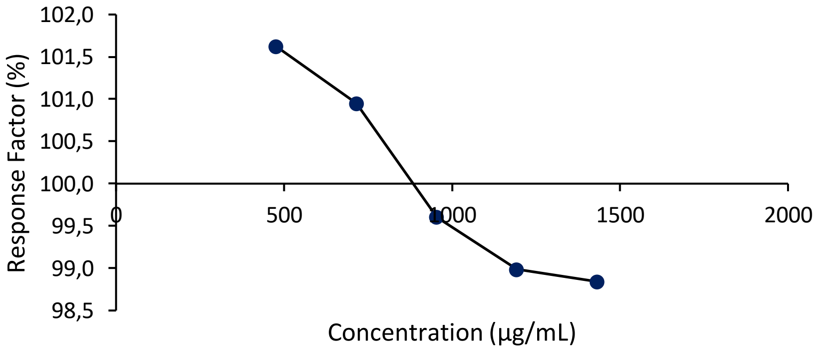

Figure 35. *Response factor analysis for Atropine Sulfate - assay.*

***Table 48**. Rikilt Test - Results / Calculations for Atropine Sulfate - related substances.*

(i)	Conc (µg/mL) (x_i)	Peak Area (mAU.s) (y_i)	Response Factor (y_i/x_i)	RFi / average RF x 100	Acceptance Criteria (%)
1	0.5	5742	11843.9	97.46	**90.0 - 110.0**
2	1.0	11632	11995.1	98.71	
3	2.4	29398	12126.7	99.79	
4	4.8	59264	12223.1	100.59	
5	7.8	94550	12188.1	100.30	
6	9.7	118124	12181.6	100.24	
7	12.1	148471	12248.9	100.80	
8	14.5	178761	12289.8	101.13	
9	19.4	236061	12171.9	100.16	
10	24.2	296983	12250.51	100.81	
		Average =	12151.9	100.00	**90.0 - 110.0**
		Standard Deviation =	135.8	1.12	-
		RSD (%) =	1.12	1.12	**≤ 5.0**

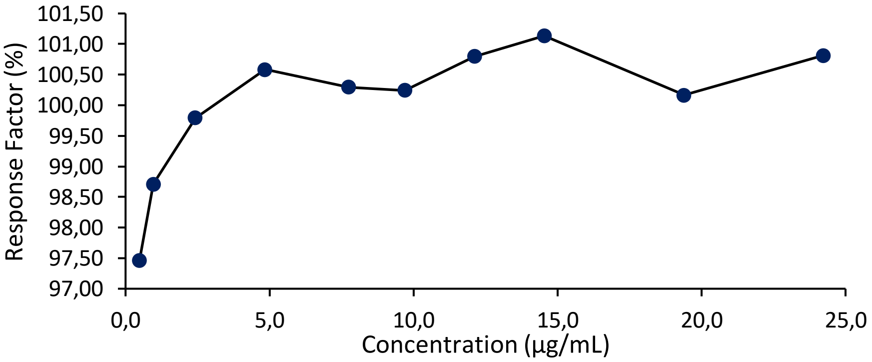

***Figure 36.** Response factor analysis for Atropine Sulfate - related substances.*

The results are in compliance with the established acceptance criteria, showing that the analytical methods are linear in the working range. Response factor can also be used in these linear functions (routine work).

Variance Homogeneity

To evaluate if working range of linearity is well defined, a PG value is calculated by the quotient of higher and lower standard variances (S_l^2 and S_s^2) obtained by the signals of ten replicate injections of reference solutions containing Atropine Sulfate at lower concentration (50% solution for the API assay method and 0.05% for the related substances) and higher concentration (150% solution for the API assay method and 2.50% for the related substances) of the working range. This PG value, calculated as follows, is compared to a tabulated value (F).

$$PG = \frac{S_S^2}{S_I^2} \quad if \quad S_S^2 > S_I^2 \qquad (21)$$

$$PG = \frac{S_I^2}{S_S^2} \quad if \quad S_I^2 > S_S^2 \qquad (22)$$

If PG is less than F, differences are not significant and working range is valid. If PG is higher than F, new work range must be studied (slighter working range). The results are presented in Tables 48 and 49.

Table 49. *Variance Homogeneity - assay.*

Concentration (µg/mL)	**477.2**	**1431.6**
Concentration Level	**50%**	**150%**
Signal	5953097	17349454
	5950477	17348162
	5934956	17354353
	5921409	17292487
	5927399	17326015
	5915347	17328768
	5914624	17335250
	5899705	17326113
	5896505	17332657
	5891073	17318737

Signal average y_I = 5920459

Standard Deviation Signal y_I = 21483

Relative Standard Deviation y_I = 0.36

S_I^2 = 4.62×10^8

Signal average y_S = 17331199

Standard Deviation Signal y_S = 17914

Relative Standard Deviation y_S = 0.10

S_S^2 = 3.21×10^8

PG value obtained for API assay method is 1.44 that is minor than F (5.35 for a confidence range of 99.0% and 9 degrees of freedom).

Table 50. *Variance Homogeneity - related substances.*

Concentration (µg/mL)	0.5	24.2
Concentration Level	**0.05%**	**2.5%**
Signal	5910	296857
	5765	297384
	5346	296045
	5880	296913
	6016	296118
	5656	296662
	5162	297445
	6262	297705
	5791	296696
	5636	298001

Signal average y_I = 5742
Standard Deviation Signal y_I = 318
Relative Standard Deviation y_I = 5.53
S_I^2 = 1.01×10^5

Signal average y_S = 296983
Standard Deviation Signal y_S = 647
Relative Standard Deviation y_S = 0.22
S_S^2 = 4.18×10^5

For the related substances it was obtained a PG value of 4.15, which is also minor than F. Therefore, differences between variances are not significant and working range is well defined for both methods.

Detection / Quantification Limits

The detection and quantification limits were estimated using the formulas found in chapter 2.3.

Table 51. *Detection / Quantification limits results.*

Slope (b) = 12242.8
Intercept (a) = -225.7
(r) = 0.99998
(r^2) = 0.99997
Residual Standard Deviation of the line ($S_{Y/X}$) = 570.56

Detection Limit	Quantification Limit
0.154 µg/ml 0.02%	0.466 µg/ml 0.05 %

The results are in compliance with the established acceptance criteria, showing that it is possible to report related compounds above 0.05%, which is the ICH recommendation for reporting threshold.

4.2.3. Accuracy

The accuracy was evaluated by the assay variability of 9 determinations, prepared of the placebo spiked with known amounts of the analyte over 3 concentration levels, covering the working range (50%, 100% and 150% of the test concentration for API assay and 0.05%, 0.10% and 2.50% for related substances).

There were prepared 3 replicates to each concentration level and the accuracy was reported as recovery percentage (ratio between experimental concentration and nominal concentration). The results are presented in Tables 51 and 52.

Table 52. *Accuracy results for Atropine Sulfate assay.*

Sample Solutions	Nominal Concentration (µg/mL)	Experimental Concentration (µg/mL)	Recovery			Acceptance Criteria	Comment
			Individual (%)	Average (%)	RSD (%)		
50%	480.5 480.5 480.5	493.20 494.30 490.07	102.6 102.9 102.0	102.5	0.44	**Recovery** **97.0 – 103.0%** **RSD** **≤ 2.0%**	Complies
100%	961.1 961.1 961.1	961.65 965.28 962.95	100.1 100.4 100.2	100.2	0.19		Complies
150%	1437.8 1437.8 1437.8	1398.17 1398.50 1394.56	97.2 97.3 97.0	97.2	0.16		Complies

Table 53. *Accuracy results for Atropine Sulfate - related substances.*

Sample Solutions	Nominal Concentration (µg/mL)	Experimental Concentration (µg/mL)	Recovery			Acceptance Criteria	Comment
			Individual (%)	Average (%)	RSD (%)		
0.05%	0.5 0.5 0.5	0.52 0.53 0.53	104.7 106.4 107.5	106.2	1.34	**Recovery** **90.0 – 110.0%** **RSD** **≤ 5.0%**	Complies
0.10%	1.0 1.0 1.0	1.05 1.05 1.03	105.9 105.6 104.5	105.3	0.72		Complies
2.50%	23.9 23.9 23.9	24.50 25.33 24.42	102.4 105.9 102.1	103.5	2.04		Complies

The results are in compliance with the established acceptance criteria, showing that the analytical method is accurate in the working range.

4.2.4. Precision

System Suitability Tests

System suitability tests were established based on the obtained results. There were determined the following parameters for the analyte peak:

- *Efficiency* = $5.54 \times (t_r/W_{0.5})^2$
- *Tailing Factor* = $W_{0.05}/2.f$
- *Precision of replicate injections (RSD%) = Standard deviation* R_a */ Average* R_a *x 100%*

Repeatability was evaluated by the variability of the analytical response of a reference solution containing Atropine Sulfate at 100% of the test concentration (≈ 1000 µg/ml) and a reference solution containing Atropine Sulfate at about 0,10% of the test concentration (≈1 µg/ml). The obtained results are presented in Tables 53 and 54.

Table 54. *System Suitability results for Atropine Sulfate at about 1000 µg/ml - assay.*

Parameters	Reference Solution Results	Acceptance Criteria	Comment
Efficiency of analyte peak	25745	**≥ 2000**	Complies
Tailing factor of analyte peak	1.02	**0.8 – 1.5**	Complies
Precision of replicate injections Response RSD% (n=6)	0.35	**≤ 2.0%**	Complies
Precision of replicate injections RT RSD% (n=6)	0.61	**≤ 2.0%**	Complies

Table 55. *System Suitability results for Atropine Sulfate at about 1 µg/ml (related substances).*

Parameters	Reference Solution Results	Acceptance Criteria	Comment
Efficiency of analyte peak	27551	**≥ 2000**	Complies
Tailing factor of analyte peak	1.42	**0.8 – 1.5**	Complies
Precision of replicate injections Response RSD% (n=6)	4.20	**≤ 5.0%**	Complies
Precision of replicate injections RT RSD% (n=6)	1.48	**≤ 2.0%**	Complies

Analysis Repeatability

Analysis repeatability was evaluated by the variability of the analysis of 6 replicates of drug product samples. The obtained results are presented in Tables 55 and 56.

Table 56. *Analysis repeatability results for Atropine Sulfate - assay.*

Assay (%)	Average (%) n=6	RSD (%) n=6	Acceptance Criteria (RSD %)	Comment
99.62 99.68 99.79 99.31 99.53 99.73	99.61	0.17	≤ 2.0%	Complies

Table 57. *Analysis repeatability results for Atropine Sulfate - related substances.*

	Assay (%)	Average (%) n=6	RSD (%) n=6	Acceptance Criteria (RSD %)	Comment
Impurity C	1.793 1.797 1.799 1.786 1.794 1.798	1.79	0.26	**Concentrations below 0.5%: ≤ 10.0 %** **Concentrations equal or above 0.5%: ≤ 5.0%**	Complies
Impurity A	0.186 0.185 0.186 0.187 0.183 0.183	0.19	0.90		Complies
Unknown impurity	0.089 0.088 0.085 0.084 0.090 0.089	0.09	3.02		Complies

Intermediate Precision

Two analysts prepared individually, on different days and using different equipment systems and different columns, 6 replicates of sample solution and a reference solution. The obtained results are presented in Tables 57 and 58.

Table 58. *Intermediate Precision results for Atropine Sulfate - assay.*

Parameters	Results Test I	Results Test II	Acceptance Criteria
Assay (%)	99.62	100.05	**98.0 – 102.0%**
	99.68	100.29	
	99.79	100.42	
	99.31	101.00	
	99.53	99.97	
	99.73	99.81	
Average (%)	99.61	100.25	**98.0 – 102.0%**
RSD (%)	0.17	0.42	**≤ 2.0%**
Pooled Average (%)	99.93		**98.0 – 102.0%**
Pooled RSD (%)	0.33		**≤ 2.0%**
Relative Diff. Between Tests (%)	0.64		**≤ 2.0%**

Table 59. *Intermediate Precision results for Atropine Sulfate - related substances.*

	Content (%)							
	Impurity C		**Impurity A**		**Unknown Impurity**		**Total impurities**	
Test	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**
Assay	1.793	1.708	0.186	0.175	0.089	0.082	2.068	1.965
	1.797	1.711	0.185	0.175	0.088	0.085	2.070	1.971
	1.799	1.715	0.186	0.179	0.085	0.081	2.070	1.975
	1.786	1.726	0.187	0.177	0.084	0.084	2.056	1.987
	1.794	1.701	0.183	0.175	0.090	0.082	2.068	1.958
	1.798	1.704	0.183	0.176	0.089	0.081	2.069	1.961
Average	1.794	1.711	0.185	0.176	0.087	0.082	2.067	1.969
Pooled Average	1.75		0.18		0.08		2.02	
RSD (%)	0.26	0.50	0.90	0.92	3.02	1.86	0.26	0.53
Pooled RSD (%)	0.39		0.91		2.54		0.41	
Rel diff. Between tests (%)	4.76		5.01		6.02		4.84	
Acceptance Criteria	**Concentrations below 0.5%: ≤ 10.0 %** **Concentrations equal or above 0.5%: ≤ 5.0%**							
Comment	Complies		Complies		Complies		Complies	

The results are in compliance with the established acceptance criteria, showing that the analytical methods are precise in the working range.

4.2.5. Robustness

The study consisted of a total of 11 trials where the analytical method was tested. The variations in analytical conditions are shown in the table below.

Table 60. *Robustness Study Design.*

Variations	1 (3 replicates)	2	3	4	5	6	7	8	9
Flow Rate (mL/min)	**1**	1.2	1.2	1.2	1.2	0.8	0.8	0.8	0.8
% ACN in Gradient	**3**	1	1	5	5	1	1	5	5
ph of Mobile Phase	**3.3**	3	3.6	3	3.6	3	3.6	3	3.6
Column Oven Temperature (°C)	**22**	24	20	24	20	24	20	24	20

The robustness of the method was assessed taking into account two main aspects: method performance (system suitability) for each test; and comparison of the results of each variation with the nominal conditions.

The results can be found in table 56 and the overlaid chromatograms are in the Annex B.

Regarding the system suitability results, it is possible to see that the biggest variations are found in the atropine sulfate retention time and column efficiency. As previously mentioned, retention time varies easily with small changes in the composition of the mobile phase, but also changes in column temperature. This study was carried out solely with the aim of proving the robustness of the method, however it would be interesting to deepen this study from the perspective of optimizing the method. For example, in this case of retention time, there would be every interest in the analyte peak having a shorter retention time, either for reasons of time savings or mobile phase savings. The variation in column efficiency is not a critical factor to consider here, as it is always well above the method prediction.

Regarding the results of the quantification of atropine sulfate, the values obtained are between 97.0% and 100.0%, therefore falling within the acceptance criteria. On the other hand, none of the trials present a significant relative difference against nominal conditions, all being below 2.0%, with the highest value being 1.50% for variation 7. Also, in the quantification of impurities, the results are within specification and very similar results were generated between samples.

Considering the exposed above, it can be concluded that the method is robust.

***Table 61**. Robustness study results.*

	TESTS	Acceptance criteria	Variations								
			1 (Nominal)	2	3	4	5	6	7	8	9
System Suitability Results	**Atropine Sulfate retention time (min)**	N. A. (Informative)	**10.755**	10.548	10.715	8.314	8.443	13.740	13.853	11.433	11.591
	Column Efficiency	≥ 2000	**27184**	31295	32230	17310	17544	44334	44389	25866	26544
	Tailing factor of analyte peak	0.8 – 1.5	**1.1**	1.11	1.11	1.09	1.10	1.07	1.08	1.07	1.08
	Precision of 6 replicate injections - Peak Area (RSD)	≤ 2.0%	**0.298**	0.173	0.168	0.067	0.045	0.239	0.078	0.198	0.069
	Precision of 6 replicate injections - RT (RSD)	≤ 2.0%	**0.17**	0.062	0.100	0.060	0.261	0.111	0.170	0.022	0.022
	Final Evaluation		**Complies**	**Complies**	**Complies**	**Complies**	**Complies**	**Complies**	**Complies**	**Complies**	**Complies**
Analyte Quantification Results	**Atropine Sulfate content (%)**	95 % - 105%	99.14	97.83	98.47	98.41	98.87	98.36	97.65	99.25	99.85
	Impurity C content (%)	≤ 2.0%	1.76	1.762	1.783	1.686	1.708	1.677	1.741	1.689	1.707
	Impurity A content (%)	≤ 0.5%	0.18	0.184	0.175	0.182	0.177	0.165	0.172	0.167	0.175
	Any other impurity content (%)	≤ 0.5%	0.083	0.083	0.087	0.082	0.089	0.077	0.082	0.078	0.077
	Total impurities content (%)	≤ 3.0%	2.02	2.03	2.05	1.95	1.97	1.919	1.995	1.934	1.959
Comparison between quantification results (each variation against nominal conditions)	**Atropine Sulfate content (%)**	Relative difference against nominal conditions: ≤ 2.0%	NA	1.32	0.67	0.73	0.26	0.78	1.50	0.12	0.72
	Impurity C content (%)	Relative difference against nominal conditions: ≤ 10.0% for concentration levels < 0.5% ≤ 5.0% for concentration levels ≥ 0.5%	NA	0.27	1.46	4.06	2.81	4.57	0.93	3.89	2.86
	Impurity A content (%)		NA	2.99	2.05	1.87	0.93	7.65	3.73	6.53	2.05
	Any other impurity content (%)		NA	0.00	4.82	1.20	7.23	7.23	1.20	6.02	7.23
	Total impurities content (%)		NA	0.50	1.29	3.42	2.23	4.95	1.19	4.21	2.97
	Final Evaluation		**Complies**	**Complies**	**Complies**	**Complies**	**Complies**	**Complies**	**Complies**	**Complies**	**Complies**

4.2.6. Conclusion

Overall, validation of the pharmacopeia method adapted to the finished product was successful. In this validation, the stability of the solutions was not tested, however it will be carried out and added to the validation report.

In Table 61 there is a comparison of the state of the art of method validation for the three methods considered here.

Method 31 presents several parameters classified as “+/- “, because the concentration range used to validate the method is not adequate.

As previously mentioned, method 1 presents a very complete validation report, and the reason for its update is simply due to its poor performance.

Table 62. *Comparison of the state of the art of method validation.*

Validation Parameters	Method 1	Method 31	New Method
Selectivity	+	+	+
Correlation Coefficient of the Straight Line	+	+/-	+
Residual Analysis	+	+/-	+
Mandel Test	+	+/-	+
Rikilt Test	-	+/-	+
System Repeatability	+	+	+
Analytical Repeatability	+	+	+
Intermediate Precision	+	+	+
Accuracy	+	+/-	+
Detection / Quantification Limits	NA	+	+
Robustness	+	-	+
Stress Studies	+	-	+
Solution Stability	+	+	-
Variance Homogeneity	-	-	+

+ parameter validated
- parameter not validated
+/- concentration range not adequate

Regarding methods performance, method *1* presents an average column efficiency value of around 1000 and an average symmetry factor of 1.6, method *31* has around 25000 theoretical plates and 1.3 symmetry factor and the new method presents around 25000 theoretical plates as well and a symmetry factor of around 1.05 for the assay standard peak and 1.4 for the related substances standard peak.

The application of the risk analysis developed in this work to this new method would result in an acceptable risk level, since both the state of the art of method validation and the state of the art of the method would be classified with a risk level of 1, the severity would remain unchanged and detectability would also be classified as 1, since the method has been fully revalidated.

In this way, it is proven that the application of the pharmacopoeia method for raw material adapted to the finished product was successful and that the method is suitable for its specific purpose.

5

CONCLUSIONS & FUTURE WORK

The main objective of this work was to carry out a risk analysis of internal analytical methods whose validation date exceeds 10 years. 64 methods were part of this risk analysis, of which only 22 presented a risk level classified as acceptable.

For the remaining methods, a risk mitigation plan was developed, which consisted of understanding what actions would need to be taken so that the risks became acceptable. The risk level classification of these methods was recalculated taking into account changes to the mitigation plan and all risks became acceptable.

Therefore, the next steps consist of putting the mitigation plan into practice in all methods evaluated here. Furthermore, it would be interesting to extend this risk analysis to all current analytical methods in the company, to understand the current performance of these methods.

It would also be interesting to update this risk analysis regularly so that any unusual results are immediately recognized, considering the history of the method. In this way, it would also be possible to evaluate trends in the methods.

Regarding the validated pharmacopeia method, it was proven to be suitable for its purpose through validation of selectivity, linearity, accuracy, precision, and robustness. However, there are several parameters that can be improved.

Firstly, through the attached chromatograms it is possible to see that the method's baseline is not the best. This is due to the purity of the reagents used in the mobile phase. Therefore, the ideal would be to try a reagent with a higher purity level and see if the problem would be resolved.

Next, in Annex A the chromatogram relating to peak ID is presented. In this chromatogram it is possible to see that the H impurity and atropine sulfate peaks are very close. Although the resolution between these two peaks is equal to 2.9 (a value above 1.5 usually used as a reference), the possibility of trying to separate them a little better should be studied. To do this, perhaps one should start by studying the effect of pH and/or changing the organic percentage of the mobile phase.

Finally, a robustness study of the method was carried out, where the flow rate, the percentage of acetonitrile in the mobile phase, the pH of the mobile phase and the temperature of the column oven were varied. It was concluded that the method is robust and that the existence of small changes in the chromatographic conditions of the method does not affect the quantification of the analytes in question.

However, it would also be interesting to carry out a robustness study from the perspective of optimizing the chromatographic conditions of the method, also taking into account what was said previously regarding the resolution between impurity H and atropine sulfate.

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ANNEX A – SELECTIVITY CHROMATOGRAMS

Figures 37 to 43 show the chromatograms obtained in the selectivity test (chapter 4.2.1.).

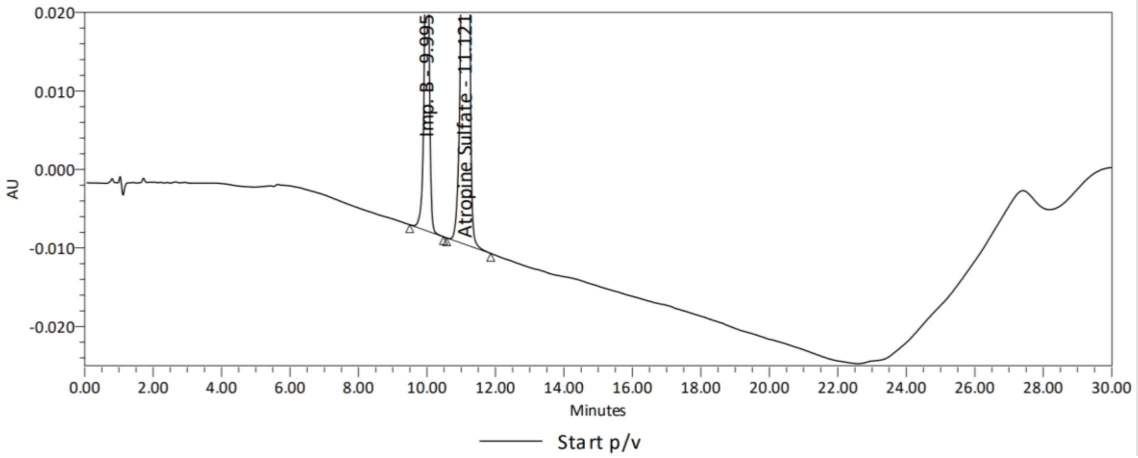

Figure 37. *Chromatogram of the impurity b resolution solution.*

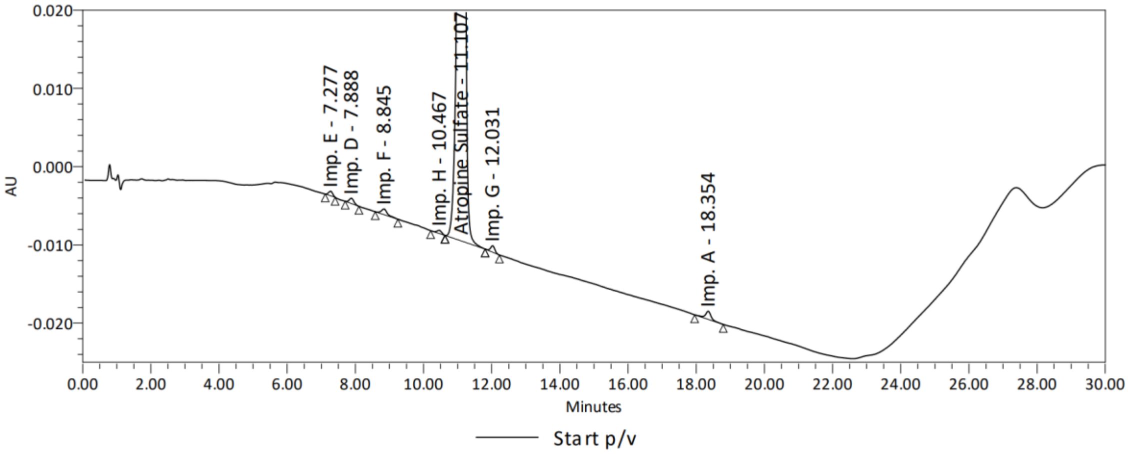

Figure 38. *Peak ID solution chromatogram.*

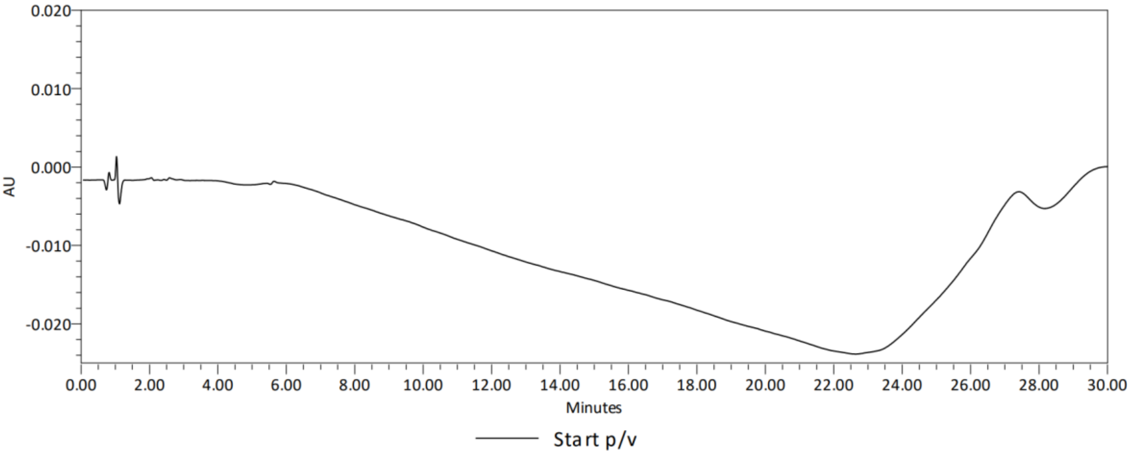

Figure 39. *Placebo chromatogram.*

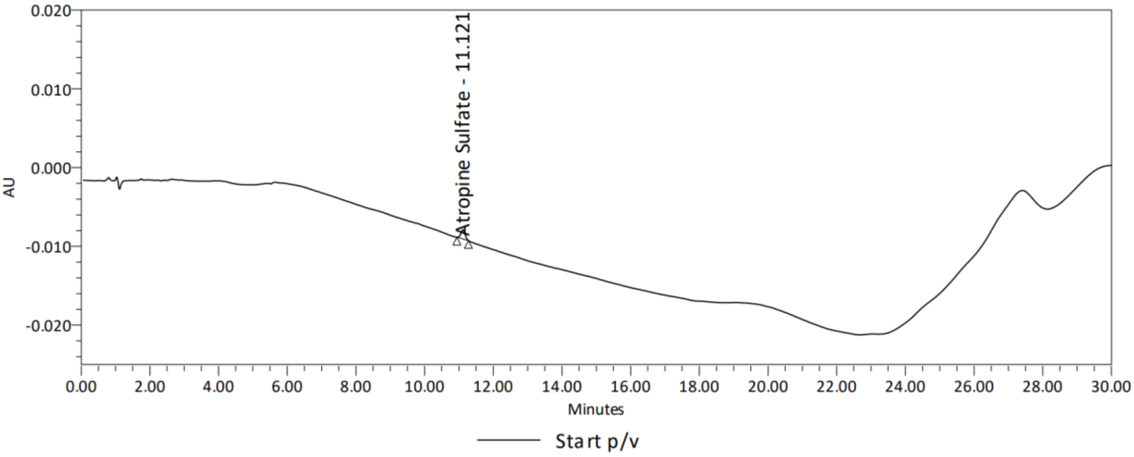

Figure 40*. Related substances standard chromatogram.*

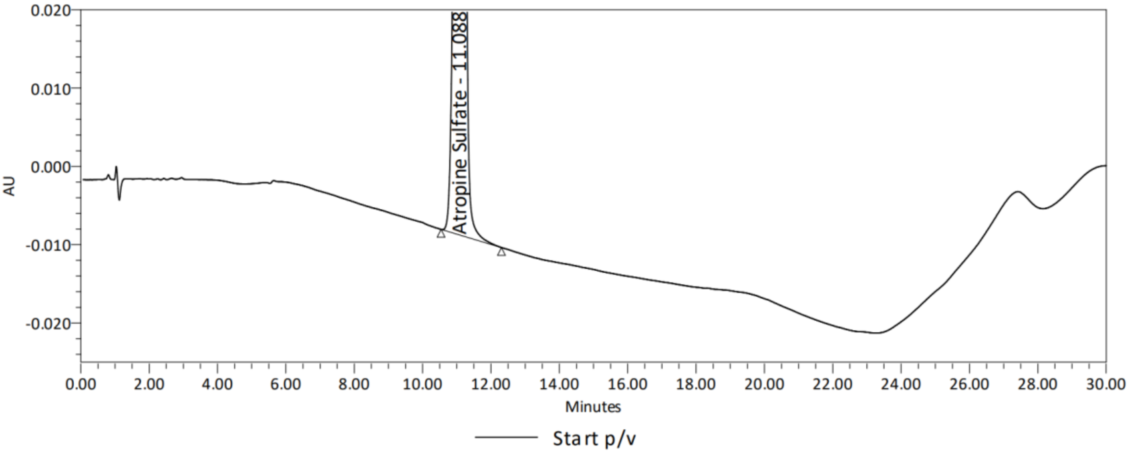

Figure 41. *API assay standard chromatogram.*

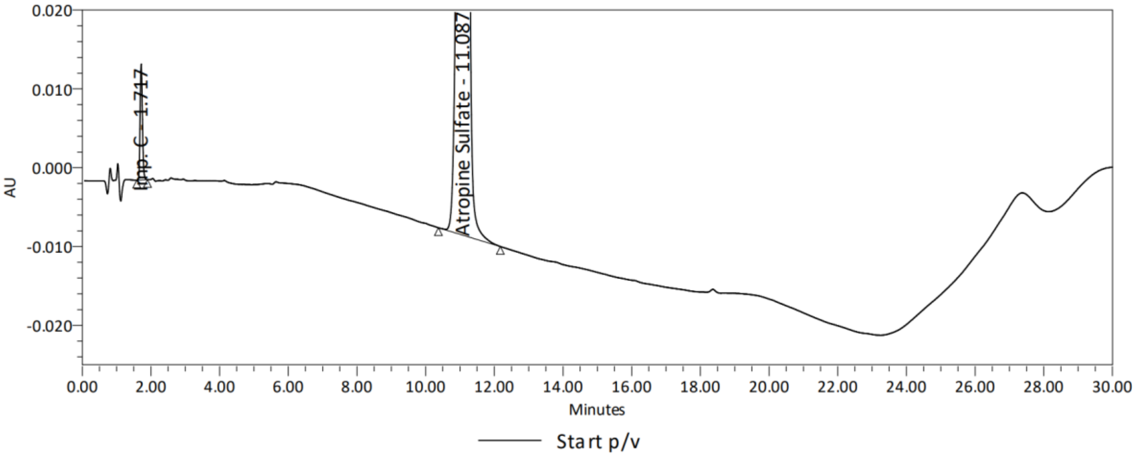

Figure 42. *Drug product chromatogram.*

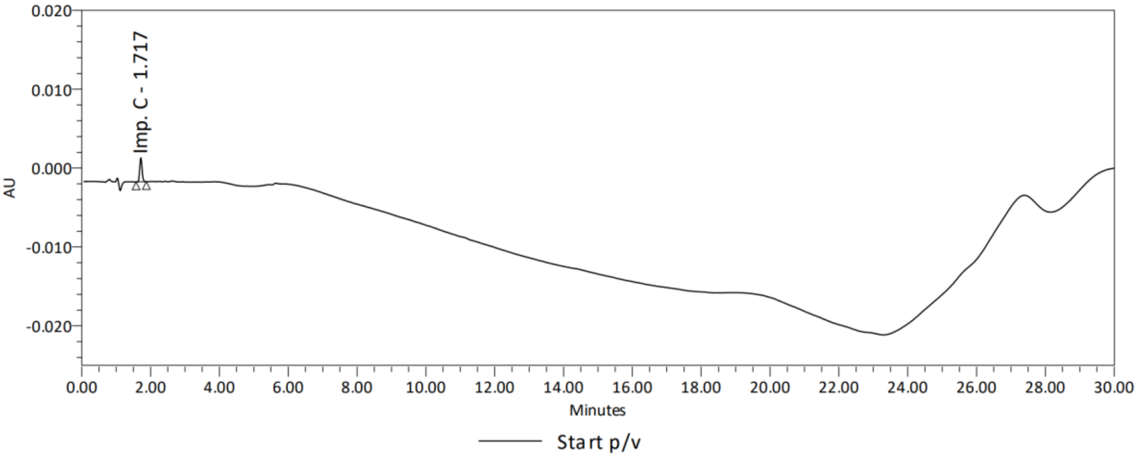

Figure 43. *Impurity C solution chromatogram.*

ANNEX B – ROBUSTNESS STUDY CHROMATOGRAMS

Figures 44 to 51 show the chromatograms obtained in the robustness study (chapter 4.2.5.).

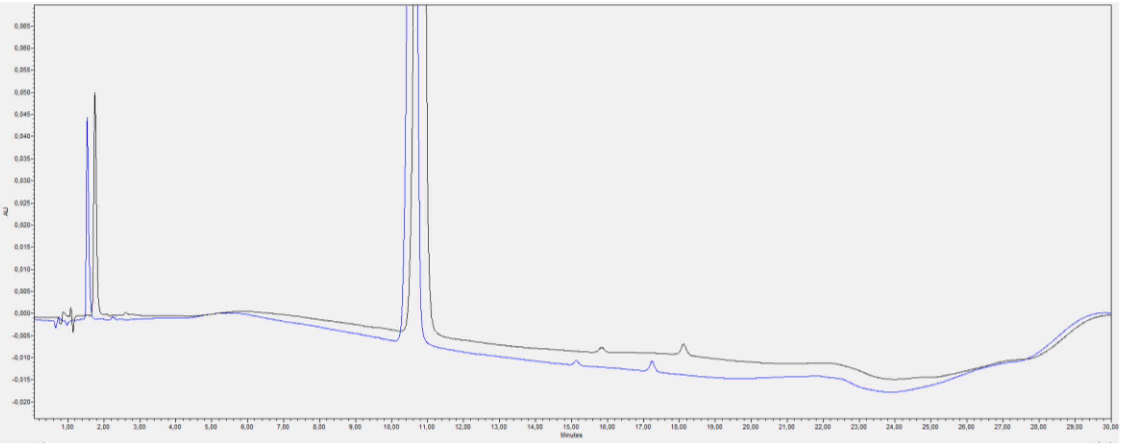

Figure 44. *Overlaid chromatograms - variation 1 (nominal- black) vs. variation 2 (blue).*

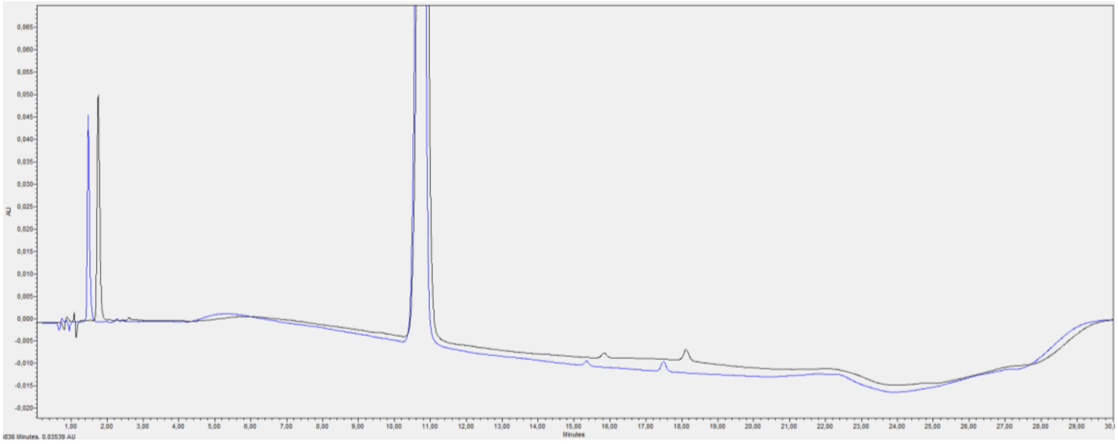

Figure 45. *Overlaid chromatograms - variation 1 (nominal- black) vs. variation 3 (blue).*

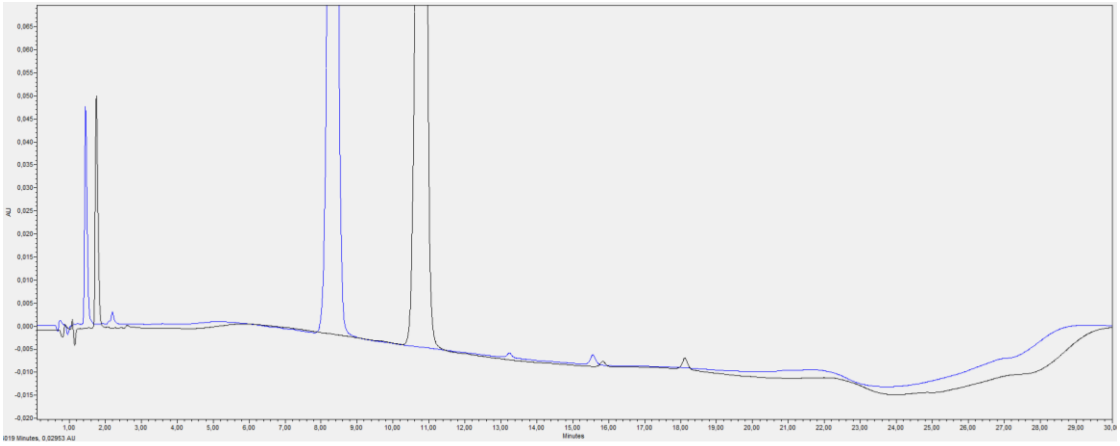

Figure 46. *Overlaid chromatograms - variation 1 (nominal- black) vs. variation 4 (blue).*

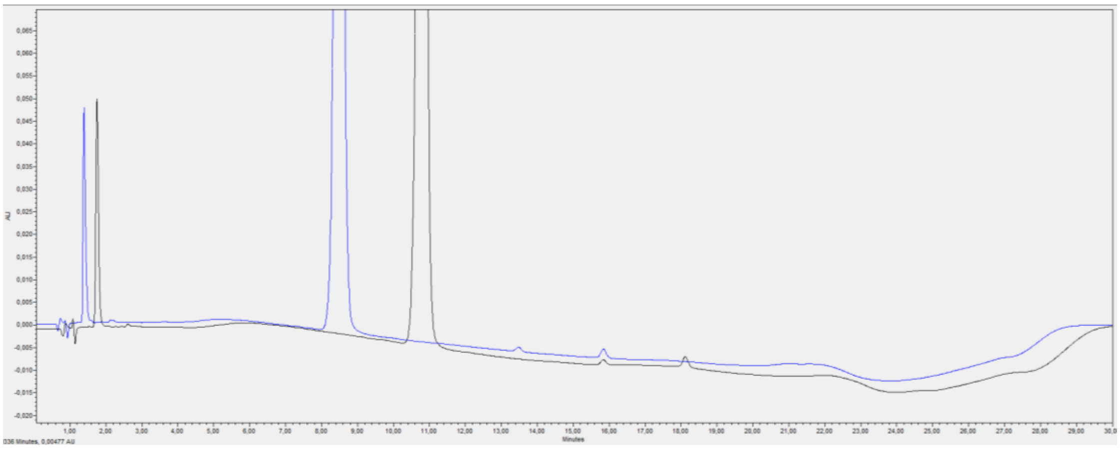

Figure 47. *Overlaid chromatograms - variation 1 (nominal- black) vs. variation 5 (blue).*

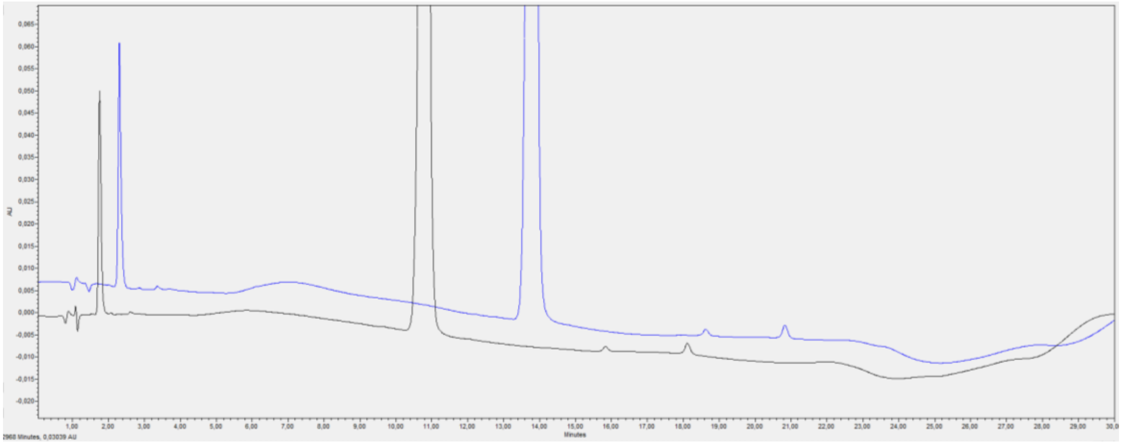

Figure 48. *Overlaid chromatograms - variation 1 (nominal- black) vs. variation 6 (blue).*

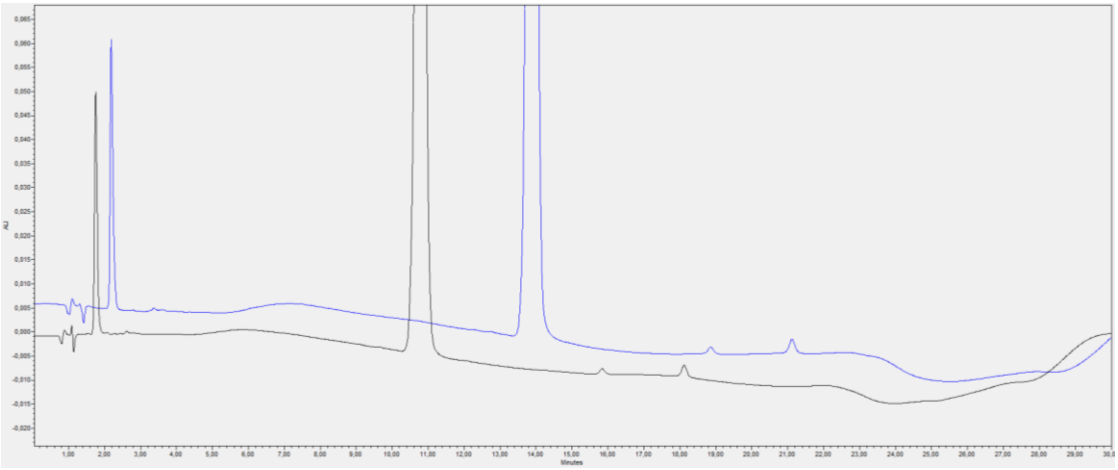

__Figure 49.__ Overlaid chromatograms - variation 1 (nominal- black) vs. variation 7 (blue).

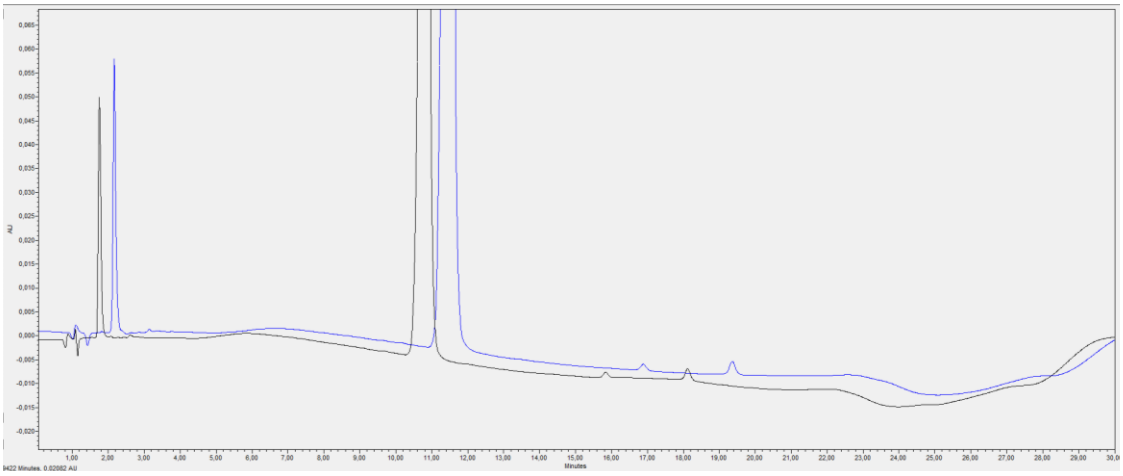

__Figure 50.__ Overlaid chromatograms - variation 1 (nominal- black) vs. variation 8 (blue).

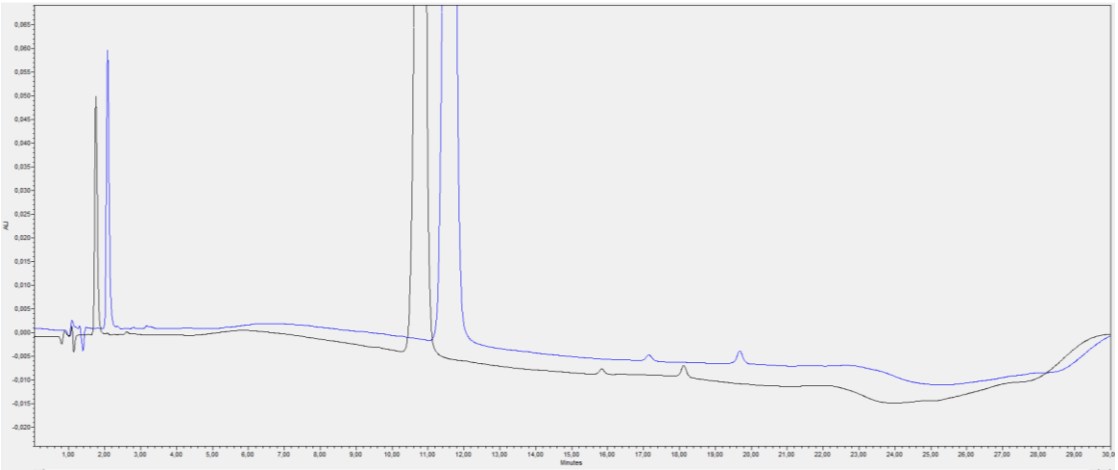

__Figure 51.__ Overlaid chromatograms - variation 1 (nominal- black) vs. variation 9 (blue).

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