Marta Raquel Anágua Bexiga

Bachelor in Chemical and Biochemical Engineering

Analytical Method Transfer of an Active Substance for the Prevention of Nausea and Vomiting Associated with Chemotherapy Sessions

Dissertation submitted to fulfill Master's degree in

Chemical and Biochemical Engineering

Adviser: Eng.ª Clarisse Penedo
Analytical Laboratory Manager
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Co-advisers: Dr.º Mário Eusébio, Associated Professor
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Abstract

This project aimed to transfer an analytical procedure for physical and chemical analysis of an active substance that will be used in a drug product used to prevent vomiting and nausea associated with chemotherapy sessions. Therefore, this crucial step aims to verify that the analytical method developed and validated by the active substance manufacturer provides the same results when performed in different laboratories. This allowed an analytical qualification to confirm the results described by the manufacturer and to ensure that the active substance used in the drug product has the required qualities.

All analytical methods considered critical for proving proper use were transferred under the conditions defined by the European Pharmacopoeia and have been validated according to the International Conference on Harmonization (ICH) guidelines. These include: Identification and Assay by HPLC; Related Substances (Tests 1 and 2) by HPLC; Stereochemical Purity by HPLC; Content of acetic acid by HPLC; Residual Solvents by GC; Content of N-methyl D-glucamine by potentiometry and Water content by Karl-Fisher method.

The results demonstrated that in the Identification and Assay method, the retention time of the main peak matches to the active substance peak and presented a content of 100.7% w/w. In the Related Substances and Stereochemical Purity tests, all impurities and other isomer were present at levels below 0.15%. All Residual Solvents conferred limits according to specifications. The N-methyl D-glucamine content on anhydrous basis was 37.4% and Water Content 2.09%.

The parameters evaluated in the analytical transfer, such as specificity, linearity, precision (system and method repeatability), accuracy and quantitation limit met the defined acceptance criteria and, therefore, the analytical method developed by the API manufacturer was considered successfully transferred.

Keyword: method transfer, qualification, active substance, HPLC

Resumo

Este projeto teve como objetivo a transferência de um método analítico para análise física e química de uma substância ativa que será utilizada num produto farmacêutico utilizado na prevenção de vómitos e náuseas associados a sessões de quimioterapia. Para tal, este passo crucial tem como propósito verificar que o método analítico desenvolvido e validado pelo fabricante da substância ativa providencia os mesmos resultados quando executado em diferentes laboratórios. Assim é permitido fazer uma qualificação analítica de modo a confirmar os resultados descritos pelo fabricante e garantir que a substância ativa utilizada no produto farmacêutico pretendido apresenta as qualidades necessárias exigidas.

Todos os métodos analíticos considerados críticos para a comprovação de uma adequada utilização foram transferidos segundo as condições definidas pela farmacopeia europeia de acordo com as diretrizes da *International Conference on Harmonization (ICH)*. Entre os quais: Identificação e Doseamento do ativo por *HPLC*; Substâncias Relacionados (Testes 1 e 2) por *HPLC*; Pureza Estereoquímica por *HPLC*; Teor de ácido acético por *HPLC*; Solventes Residuais por *GC*; Teor de Dimeglumina por potenciometria; Teor de água, pelo método de Karl-Fisher.

Os resultados mostram que no método de Identificação e Doseamento, o tempo de retenção do pico principal corresponde ao pico da substância ativa e apresentou um teor de 100.7% p/p. Nos testes Substâncias Relacionadas e Pureza Estereoquímica, todas as impurezas e isómero apresentaram um teor inferior a 0.15%. Todos os solventes residuais apresentaram limites de acordo com as especificações. O teor de Dimeglumina em base anidra foi de 37.4% e o teor em água 2.09%.

Os parâmetros avaliados na transferência analítica, como especificidade, linearidade, precisão (repetibilidade do sistema e método), exatidão e limite de quantificação obedeceram aos critérios de aceitação definidos e, portanto, o método analítico desenvolvido pelo fabricante da substância ativa foi considerado transferido com sucesso.

Palavras-chave: transferência de métodos, qualificação, substância ativa, HPLC

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Acronyms

AGP	Alpha1-acid-glycoprotein
API	Active Pharmaceutical Ingredient
ASMF	Active Substance Master File
CINV	Chemotherapy-Induced Nausea and Vomiting
CoA	Certificate of Analysis
DAD	Diode Array Detector
DMF	Drug Master File
ESMO	European Society for Medical Oncology
FID	Flame Ionization Detector
GC	Gas Chromatography
GMP	Good Manufacturing Practice
HPLC	High Performance Liquid Chromatography
ICH	*International Conference on Harmonization*
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
KF	Karl Fisher
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
MASCC	Multinational Association of Supportive Care in Cancer
NCCN	Nacional Comprehensive Cancer Network
NK_1	Neurokinin-1
ODS	Octadecylsilyl
PDA	Photodiode Array Detector

PDE	Permissible Daily Exposure
Ph. Eur	European Pharmacopeia
QL	Quantitation Limit
RRF	Relative Response Factor
TAP	Transfer of Analytical Procedures
UPLC	Ultra Performance Liquid Chromatography
UPLC	Ultra Performance Liquid Chromatography
USP	United States Pharmacopeia
UV/VIS	Ultraviolet/Visible

1. Introduction

1.1 Framework and Objectives

Over the last few years, quality control has shown great progress in the pharmaceutical industry, partly because of customer demand, which leads to increasing competition between companies. Nowadays, drugs are developed more efficiently and rapidly under an inspection and control system coupled with demanding and sophisticated analytical methods, resulting in an high level of confidence and security. To guarantee this issue, guidelines have established to eradicate unforeseen events that may appear during the manufacturing process, mainly the development of validation of analytical procedures to satisfy the technical and legal requirements established by the regulatory authorities [1].

To ensure that the products are controlled according to quality standards, a quality system has been created designated by Good Manufacturing Practise (GMP). This system covers all aspects and procedures of manufacturing since the operations of receipt of materials until drug distribution. All the pharmaceutical facilities must be certified with GMP principles, as well as the qualification of the employees to ensure that all systems provide irrefutable data of the correct procedure for each stage of manufacture. This provides quality data contributing to human and environmental protection [2, 3].

The Tecnimede Group is a private group of pharmaceutical companies, in which it started its activities in 1980. Its activity is focused on the development, production, promotion, and commercialization of medicinal products for human use, to improve health and preserve human life with a strong quality concern and technological innovation. The Labor Qualitas is the Research & Development center where pharmaceutical products are introduced to treat the most relevant diseases from the civilization perspective, such as cardiovascular and metabolic diseases, degenerative diseases of the nervous system and others. Tecnimede Test Laboratory has been certified since 2005 by INFARMED for GMP and by IPAC for NP EN / IEC ISO 17025 since 1994 [4, 5].

Since the last few years, the development of cancer has increased dramatically all over the world. Statistics describe how this disease has spread among all groups of people, by all ages, sex, and ethnicity. There are currently around 14 million new cases annually only in United States and it is expected that by 2030 this number will increase to approximately 24 million. As the disease develops and increases, science has also made significant progress at this level with the improvement of more effective and less toxic treatments [6].

One of the problems in the treatment of patients with cancer is the CINV (Chemotherapy-Induced Nausea and Vomiting). It can occur in two different ways: acute onset, in which the effect appears after 24h of administration of chemotherapy agent or delayed onset, which occurs more than 24h after the treatment, causing some suffering and distress [7].

Several therapies have been developed, including the use of serotonin 5-HT3 (5-hydroxytryptamine-3) receptor antagonists, to block the effects in the central nervous system, but these compounds have a great effect in acute, but not in delayed CINV [8].

ESMO (European Society for Medical Oncology), MASCC (Multinational Association of Supportive Care in Cancer) and NCCN (Nacional Comprehensive Cancer Network) update the guideline of conventional antiemetic agents as 5-HT3 receptor antagonist, corticosteroids and D2 dopamine receptor antagonist replacing them by a neurokinin-1 (NK_1) receptor antagonists, possessing antiemetic properties [8, 9].

The intimate ligand of the NK_1 receptor is substance P, an 11-amino acid neuropeptide, widely distributed in the central nervous system, which sends impulses and messages from the brain. For instance, NK_1 receptors are located in crucial regions of the brain, responsible for the regulation of the vomiting reflex [10]. A drug that antagonizes the effect of human substance P on the NK1 receptor is *substance x*. It crosses the blood-brain barrier and occupies brain NK_1 receptors, blocking the signals. It is indicated for the prevention of acute and delayed CINV, in contrast to 5-HT3 antagonists [11].

On the other hand, *substance x* is an insoluble drug in water and the hard capsule form of administration causes a difficult administration in patients. Alternatively, a water-soluble pro-drug can be intravenously administered [10].

This project is intended to transfer analytical procedures of an Active Pharmaceutical Ingredient supplied by a new manufacturer, in which analytical methods are validated in order to ensure that the tests performed and validated by a particular laboratory are transferred by a second one which must have the ability to perform the entire procedure properly, according to the established criteria. The approach adopted for the Transfer of Analytical Procedures was a partial validation of the analytical procedures by the receiving unit (Labor Qualitas' facilities). The tests to be transferred were chosen based on a risk analysis that considers the previous experience and knowledge of the receiving unit, the complexity, and specifications of the active substance and the procedure.

Chromatographic and non-chromatographic procedures will be transferred, including the Drug substance identification and Assay by HPLC, Content of N-methyl D-glucamine by potentiometry, Water content by Karl-Fisher method, Related substances by HPLC, Stereochemical purity by HPLC,

Content of acetic acid by HPLC and Residual solvents by GC were subjected to the validation procedure.

Besides other tests will also be carried out, but since it will be performed as described in Pharmacopeia, these do not require additional validation. The following tests will be considered transferred without further work: Description, Solubility, Identification by IR, Specific Optical Rotation and, Heavy metals.

All the tests will be performed following the procedures defined by the European Pharmacopeia (Ph. Eur.) and the United States Pharmacopeia (USP) and will be validated according to the International Conference on Harmonization (ICH) guidelines.

2. State of the Art

In this chapter, the relevant topics related to the chromatographic and non-chromatographic techniques, as well as the drug substance general information will be overviewed. In section 2.1, general information relative to the drug substance will be described (*Restricted Part*). In section 2.2, the chromatographic techniques used in this work will be explained, followed by section 2.3, where it will be reviewed the non-chromatographic techniques. In the last section 2.4, it will be presented the concepts and theoretical foundations of the process of validation of analytical methods.

2.2 Chromatographic Techniques

Chromatography is an important technique frequently used that enables a relative identification by checking the analytical response match of the analyte solution versus a standard solution. It enables the identification of the components present in a mixture and the quality analysis of raw materials, drug substance, drug product, and other compounds. The analytes present in a sample are dispersed between the stationary and the mobile phases. The mobile phase can be a liquid, a gas or it can be a supercritical fluid that transports the analytes. The stationary phase can be a solid or a liquid braced on a solid or a gel interacting with each component based on its chemical structure and polarity [22].

The separation can be divided into gas, liquid, and supercritical fluid chromatography, *Figure 2.1*. Techniques based on molecular characteristics, such as size, mass and volume use mechanisms of partition and size exclusion. To analyse interactions between molecules it can be used processes such as affinity chromatography (i.e. ion exchange) and surface adsorption. Other chromatographic techniques are sustained on the stationary bed and they are used to qualify and quantify analytes. They are including a thin layer (TLC), paper, gas (GC), column and high-performance liquid chromatography (HPLC) [23].

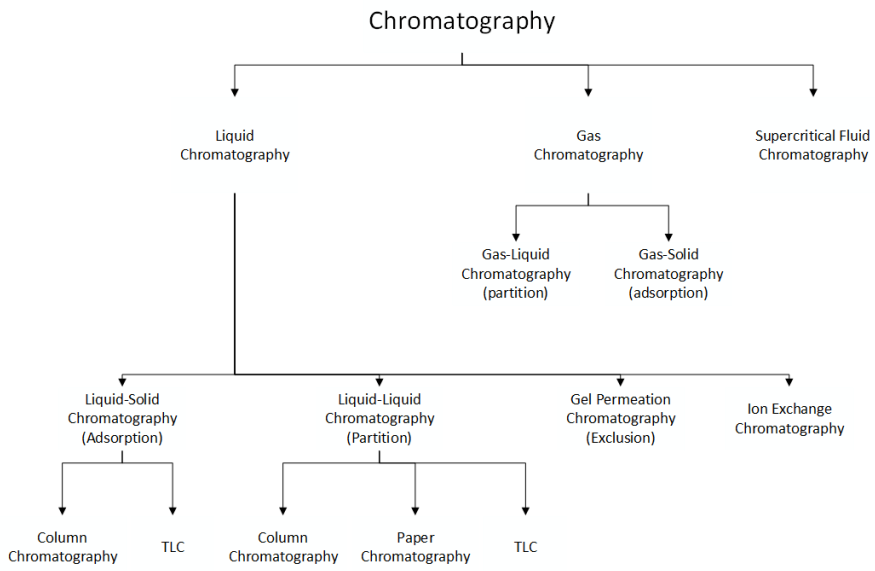


Figure 2.1 - Types of Chromatography and their relationship. Adapted from *[22]*.

2.2.1 Liquid Chromatography

On liquid chromatography, the mobile phase is a liquid. It can be carried out in a column or on a plate (TLC or paper chromatography). Nowadays, LC is characterized by higher pressures to separate smaller particles and it is known as high performance liquid chromatography (HPLC). Recently, the ultra-performance liquid chromatography (UPLC) raised fiercely the performance of this technique to a new level, maintaining the resolution and increasing the speed of run times [25].

HPLC (High performance Liquid Chromatography)

High performance liquid chromatography has become essential to perform the separation of different compounds, under high pressures. The samples are injected into the flow through the mobile phase, a liquid that passes through the packed column, which is forced by high pressure delivered by a pump. On the other hand, the stationary phase refers to the adsorbent in which the column is packed. HPLC separation is based on interaction and differential partition of the sample between both mobile and stationary phases. The components are identified at the exit of the column by a detector.

HPLC can be classified into three types of separation based on the nature of the stationary phase and the retention mechanisms: adsorption, partition, ion-exchange and size exclusion chromatography. In adsorption chromatography, the stationary phase is an adsorbent and the separation is based on repeated adsorption and desorption stages. Besides that, on this separation, it can be used two types of elution procedures: a normal phase, in which the stationary phase is more polar than the mobile phase and a reversed-phase, in which the stationary phase is non-polar relative

to the mobile phase. The most used stationary phases are silica or polymeric beads that are adjusted with the addition of long-chain hydrocarbons [23, 26].

Partition chromatography is the basic principle of high performance liquid chromatography and is a polarity separation mechanism based mainly on differences between the solubilities of the analytes of mobile and stationary phases, due to differences in partition coefficients [58].

The ion-exchange chromatography is used to separate analytes with different ionic charges. The mobile phase is an aqueous buffer with controlled pH and the stationary phase is loaded with the opposite charge of the sample [22].

The size-exclusion chromatography is used to separate molecules through their size. The molecules are separated over the stationary phase, which through the infiltration of smaller molecules in the pores of the packing material, these are retained while the larger ones are eluted, allowing a separation. This technique is also designated as gel-permeation chromatography if the mobile phase is organic. In the case of the aqueous mobile phase, it is characterized as gel-filtration chromatography [27].

HPLC has some main components described in *Figure 2.2* - Injectors, pumps, column, detector [26]:

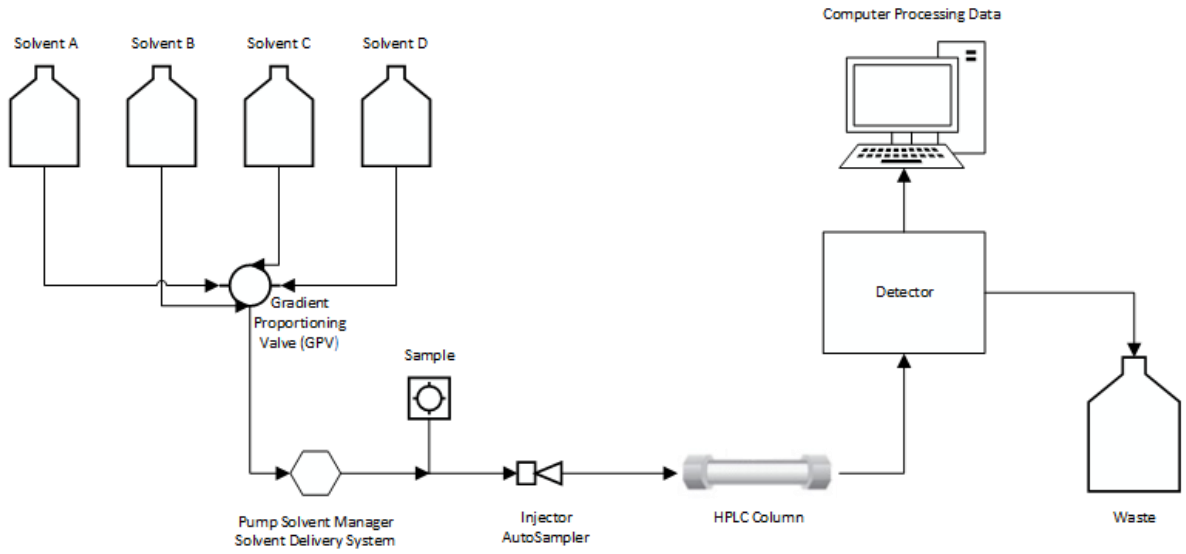


Figure 2.2 - High-Performance Liquid Chromatography System. Adapted from [26].

HPLC Pumps

The pump must provide a mobile phase flow to ensure the correct retention time and peak area, a pulse-free output through the system and a low dead volume. To regulate the flow precisely, current pumps integrate piston with valves, divided by pneumatic or mechanical pumps, which can be single or dual piston, syringe, or diaphragm pump designs.

The elution of the mobile phase can be isocratic if the composition of mobile phase is constant during the entire run and isocratic pumps are used or in the case of the composition of mobile phase is variable during the run, the elution is by gradient and are used binary or quaternary pumps.

Usually, pumps can support flow rates from 0,1 to 10 mL/min, and pressures up to 415 bar. After that there is a huge risk of losing liquid and can cause noisy baselines, spikes in chromatogram or erratic retention time.

HPLC Injector

HPLC sampler injector has the function of introducing the liquid sample into the system effectively into the flowing mobile phase stream, maintaining constant flow and pressure.

The injector can be manually operated, or an autosampler that is programmed for unattended injections of a sample sequence. Besides that, in these systems, the temperature of sample compartments can be controlled to maintain sample integrity over many hours.

Current systems use fixed or variable loop or syringe-type injectors, that can be switched on or off. When the system is in load position (a), the syringe cleans and fills the loop with the sample at atmospheric pressure, while the mobile phase flows directly into the column. When the valve rotates to the injection position, the sample contained in the loop is introduced into the mobile phase at high pressure (b), *Figure 2.3*.

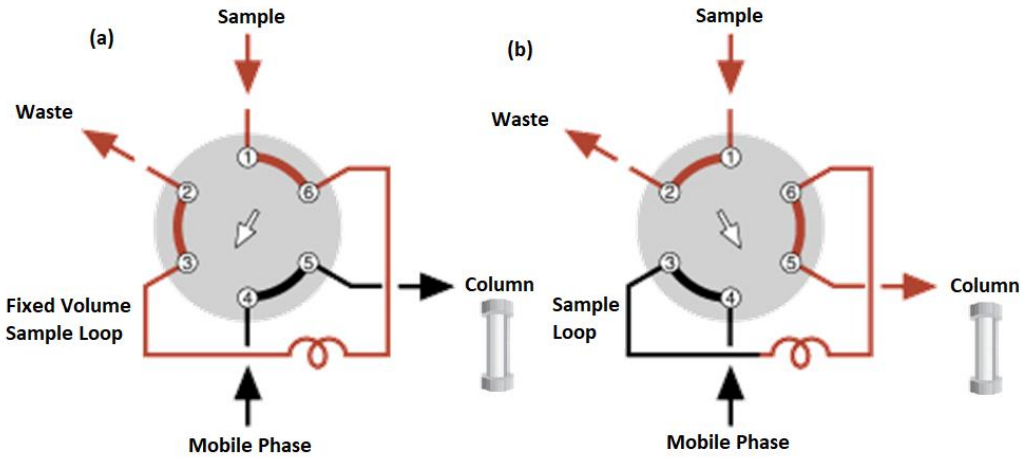


Figure 2.3 - Injector on load (a) and inject (b) positions. Adapted from [25].

Inside the syringe, there is a system to prevent the spreading of the samples band situated close to the column inlet and between sample injections, a wash solvent, similar to the solvent used in samples preparation, cleans the syringe to prevent the contamination of the following sample by the previous one (carryover) [25].

The most useful it's a six-port valves that are overfilled by syringe, responsible for precision and accuracy. Normally, volumes are 5 to 500 µL but can be larger [26].

HPLC Columns

HPLC columns are one of the most important parts of the system. Primarily, it is where the stationary phase takes place and the separation of the compounds occurs, by means of the phenomena discussed previously.

Even now, most of the columns are packed in stainless steel cilindrical tubes, because this material offers greater advantages, in particular, corrosion resistance, productivity, and affordable costs. On the inside, there are a huge range of different packing material that can be used according to the polarity of the mobile phase, *Figure 2.4* [28].

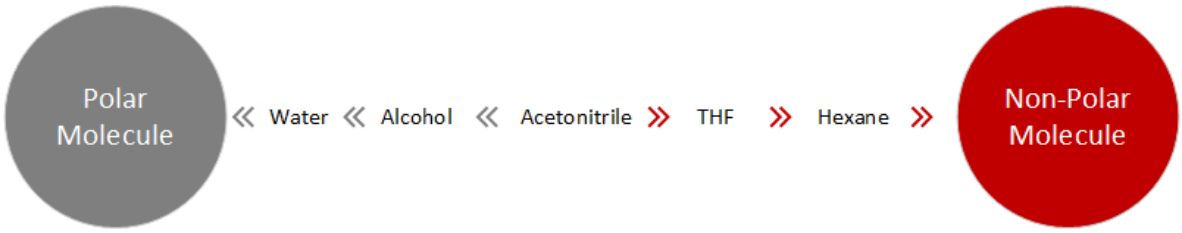


Figure 2.4 - Polarity Scale - Mobile Phases. Adapted from *[29].*

The polarity antagonism of both stationary and mobile phases for different analytes creates a separation. It means that the molecules move according to their own characteristics and electron charge distribution, causing different speed rates between them. It occurs when the mobile phase wets the chromatographic surface of the particle (stationary phase) [29]. So, it is important that the stationary phase has intermolecular forces similar to the characteristics of the analyte, allowing better interaction and separation [30]. *Figure 2.5* shows different stationary phases and their polarities.

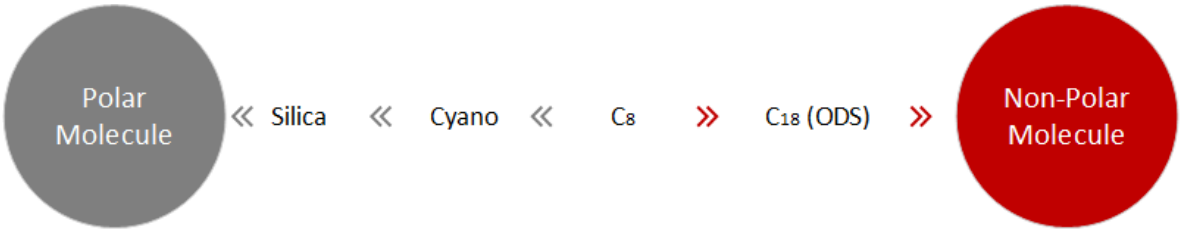


Figure 2.5 - Polarity Scale-Particle/Stationary Phase. Adapted from *[29].*

The most commonly HPLC columns is reversed-phase. This column is packed with the octadecyl group bounded type silica gel (C18, ODS) and therefore, the mobile phase must be non-polar.

Zorbax Eclipse XDB-C18 is the most use column to reduce or eliminate strong adsorption of highly polar compounds by reversed-phase liquid chromatography. It provides excellent separations at low and mid pH values (2-9) and contains an extra dense bonding of organo-silane ligands and double endcapping to protect the silica layer from mobile phases [31]. With this column the following tests are performed: Identification and Assay by HPLC and Related Substances by HPLC – Test 1, in which partial validation is described in the *Chapter 3.2.1, 3.2.2 and 3.2.3*.

To improve peak shape for bases and to increase pH range hybrid columns are used. Xterra RP-18 columns are equipped with hybrid particles. It contains both inorganic (silica) and organic (organosiloxane) components which allow a better peak shape performance and pH stability for basic compounds. This technology integrates a polar group reversed-phase ligand with inert particles allowing mobile phases with a pH range of 1-12, high efficiency, predictable retention, and no ionic interactions [32, 33]. So, it is ideal to perform the method Related Substances by HPLC – Test 2, in which partial validation is described in the *Chapter 3.2.4.*

Isomers have the same physical and chemical properties, but different optical rotation and interactions with other molecules, so they must be separated by chiral columns. One of the columns that separate a wide range of isomers is a Chiral-AGP (alpha1-acid-glycoprotein). Normally proteins have a wide range of chiral centers that facilities the retention process. In this case, this protein gains stability when bonded and supports high concentrations of organic solvents, high temperatures, and pH values from 4 to 7, which is the most import parameter once it affects the ionization of the solutes and the protein stationary phase [34]., With this column, Stereochemical purity by HPLC is performed, in which partial validation is described in the *Chapter 3.2.5.*

Inertsil ODS-3V is a highly efficient column filled with octadecylsilyl groups (bonding endcapping technology that eliminate residual silanol groups, responsible for peak tailing of basic components) bonded to a silica gel. These columns feature a relatively small pore size, a large number of theoretical plates and rapid equilibration and are developed to provide excellent separations for acidic and basic compounds [35]. With this column, Content of Acetic Acid by HPLC is performed, in which partial validation is described in the *Chapter 3.2.6.*

HPLC Detector

After being separated by the column, components pass through the detector equipped with a flow through cell which has the function of detecting the analytes on a mobile phase basis, by measuring physical and chemical properties send a response through an electrical signal to a computer data station. Current LC detectors have a wide range of types including [26]:

- UV/Vis detector
- Fluorescence detector
- Conductivity detector
- Refractive index detector
- Mass spectrometer (MS)

Commonly, the UV-visible detector is the most used to analytes that absorb UV light at a particular wavelength region (from 190-600 nm), normally designated as Diode Array Detector (DAD). UV detectors operate a lower range, typically (<210nm), considering them specific. Besides that, Photodiode Array Detector (PDA) is also used, which detects an entire spectrum. It provides results in two and third dimensions allowing the determination of the most suitable wavelength as well as peak purity [36].

At a low wavelength about every organic compound absorb UV light. Sample concentration is determined by Beer's Law, *Equation 2.1*:

$$A = \log\left(\frac{I_0}{I}\right) = \varepsilon bc \tag{2.1}$$

Where *A* is absorbance, I_0 is the incident light intensity, *I* is the intensity of transmitted light, ε is the molar extinction coefficient of the sample, *b* is the path length of the cell (cm) and *c* is the molar sample concentration.

The most of aromatic compounds (strong bonds) absorb below 260nm, compounds with one or more double bonds at 215 nm, and aliphatic components at 205 nm, a consequence of the transition of electrons in molecular orbitals [37]. Therefore, by a calibration of the system with standards, the amount of a component may be quantitated, if the detector's response is linear to the sample concentration [25].

Degassing

The excess of gas in mobile phases creates serious problems during the analysis, causing significant perturbations in the detection and the repeatability of data. A major condition for using high pressure pumps to distribute the liquid, is to ensure that the solvent and the mobile phases are gas-free in HPLC experimentation [38]. To remove the excess of gas, degassing may be accomplished by a few methods, including:

- Degassing the liquid under vacuum-heavy-walled flask;
- Placing the container of liquid in an ultrasonic bath;
- If the mobile phase has not volatile components, sparging a fine stream of helium through the liquid, because it has the ability to remove other gases from the solutions

- Some equipment has already a built-in degasser. This is the case of all HPLCs that were used to perform the tests.

2.2.2 Gas Chromatography

Gas Chromatography (GC) is a technique to separate and to analyse organic and inorganic compounds with different molecular weight, polarity, and volatility of a complex mixture. The mobile phase is a career gas that passes through the column containing the stationary phase.

During the manufacture of drug substances, a lot of organic solvents are used to synthesize excipients. These organic solvents may not be removed, and its existence represents a high risk of toxicity to consumers and can cause other undesirable effects. Therefore, their analysis and control become an extremely important parameter in the residual solvents test. The residual solvents test provides an evaluation of the number of organic solvents present in a specific formulation checking whether a particular product has the permissible concentration in *International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH)*.

Headspace Analysis

For the quantitative and qualitative analysis of volatile components, headspace sampling is the best method. It has gained extreme importance and acceptance in different areas as the pharmaceutical industry to control residual solvents. This analytical method is based on the equilibrium between the vapor phase and the liquid or solid phase.

Using this method, the sample is prepared and transferred to a vial where non-volatile and volatile components are separated by sealing the vial and heat it forming two different phases. The non-volatile compounds tend to remain in the liquid and the volatile components are isolated in a gas phase (headspace), ensuring a liquid-vapor equilibrium in the system, *Figure 2.6.*

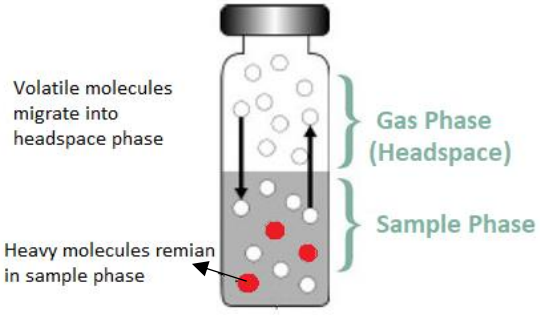


Figure 2.6 - Phases of the headspace vial. Adapted from *[40]*.

Hereupon, the gas phase is conveyed to the system for the separation of volatile components. An important parameter to guarantee an equilibrate distribution of the components between both gas and liquid phases is the partition coefficient (K) of the solvent. The lower the value of K, the biggest the concentration of the analyte in the gas phase and the faster the component passes through the column. The compounds will tend to divide easier into the gas phase and provide high responses and low detection limits. However, it has to be considered the affinity of the partition coefficient with the unwanted compounds [39, 40].

The gas phase, sample to be analysed, is carried through the mobile phase into the column. The mobile phase is a common gas, normally helium, hydrogen, argon or nitrogen, that facilities the separation and detection of the components. The components with higher boiling points move faster than the components with lower boiling points.

Column

Several factors influence the suitability of a GC column. The stationary phase (selectivity variable), column tubing material, inside diameter and length, percent liquid loading and temperature are examples of this. Two types of columns are used: packed or capillary columns. The latter have an extremely small diameter for very high resolution and are usually fabricated from fused silica. Capillary columns can be divided into megabore (wide-bore), normal bore (high-resolution) and microbore (high speed). To analyse residual solvents, the columns used are the capillary.

Detector

There are a large range of GC detectors. The most used are thermal conductivity (TCD), flame ionization (FID) and electron capture (ECD).

The detector used in GC method validated was a FID. A flame ionization detector uses a hydrogen/air flame to oxidise the organic molecules (carbon-containing material) present in the sample, producing ionized particles (*Figure 2.7*). The ions are directed to a collector by a polarizing voltage action and a signal is generated after amplification [41].

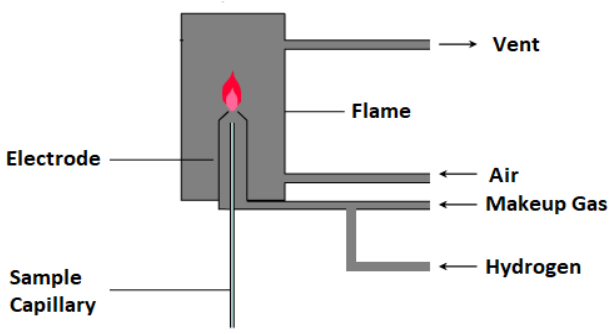


Figure 2.7 - Flame ionization detector. Adapted from *[41]*.

2.2.3 Chromatographic Definitions

The chromatographic definitions described will be supported by *Figure 2.8*.

Chromatogram

Chromatogram is a graphical representation of the detector response according to the elapsed time of the sequence that includes analyte's concentration in the eluent, volume and a range of parameters over time, thus representing the chemical separation of the analytes that occurs in the HPLC system. The ideal chromatograms are represented as a sequence of symmetric Gaussian peaks in a baseline [23, 42].

Peak

Each peak represents the detector response for different compound, and it is generated when the analyte is eluted from column and it has passed through the detector. The first peaks eluting from the column are those who move faster because of the lower affinity with the stationary phase. The peak area and height increase generally linearly following the amount of injected solution and are estimated by an integration, as well as other parameters and calculations. In Gaussian peaks is verified the *Equation 2.2* [23, 43]:

$$w_h = 1.18 w_i \qquad (2.2)$$

Where:

w_h - Peak width at half height;

w_i - Peak width between inflection points.

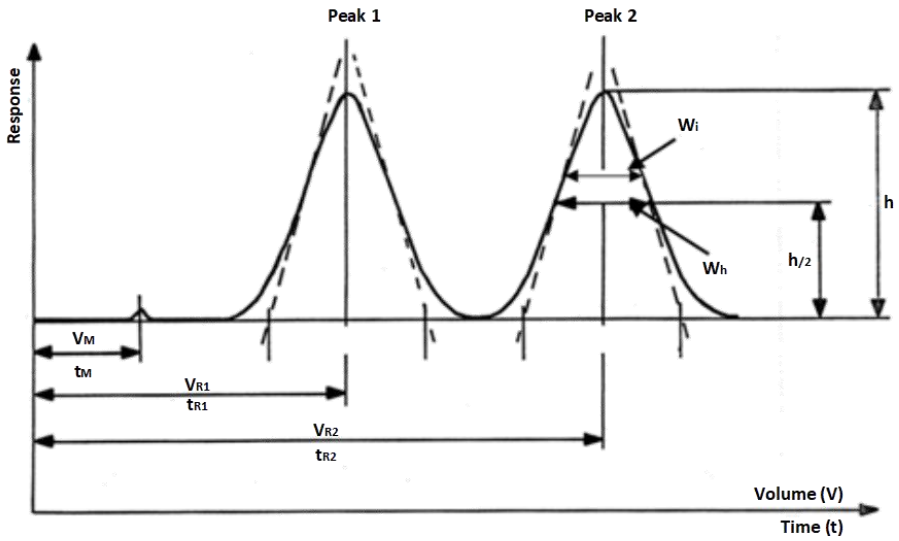


Figure 2.8 - Chromatographic Separations. Adapted from [43].

Retention Time (t_R) and Retention Volume (V_R)

Retention time is the time required for the elution of an analyte defined between the moment of injection and the appearance of the peak maximum. The retention volume is the volume of mobile phase required for the elution of an analyte and it can be obtained from the multiplication of the retention time by the flow rate of the mobile phase [43].

Hold Up Time (t_M) and Hold Up Volume (V_M)

Hold up time is the time required for the elution of an unretained analyte. Otherwise, hold up volume is the volume of the mobile phase required for the elution of an analyte whose concentration in the stationary phase is imperceptible when compared to the concentration in the mobile phase. Hold up volume includes any contributing volumes by the injection system, detector, and connectors and it can be calculated by *Equation 2.3* [43]:

$$V_M = t_M \times F \qquad (2.3)$$

Where F represents the flow rate of mobile phase in mL/min.

Dwell Volume

Dwell volume as known as gradient delay volume is characterized as a pump function and it represents the volume difference between the eluent mixing point and the top of the column. This delay is influenced by the length and internal diameter of the tubes and columns and by the valves and mixers up to the head of the column [44].

Peak-to-Valley ratio (p/v)

In related compounds tests, when two peaks are too close and the separation between them is not achieved due to baseline issues, the peak to valley ratio can be used as a system suitability criterion. *Figure 2.9* demonstrates a partial separation of two components, where H_p represents the height above the extrapolated baseline of the minor peak and H_v is the height above the extrapolated baseline at the lowest point of the curve separating the minor and the major peaks and it can be calculated by *Equation 2.4* [43]:

$$p/v = \frac{H_p}{H_v} \qquad (2.4)$$

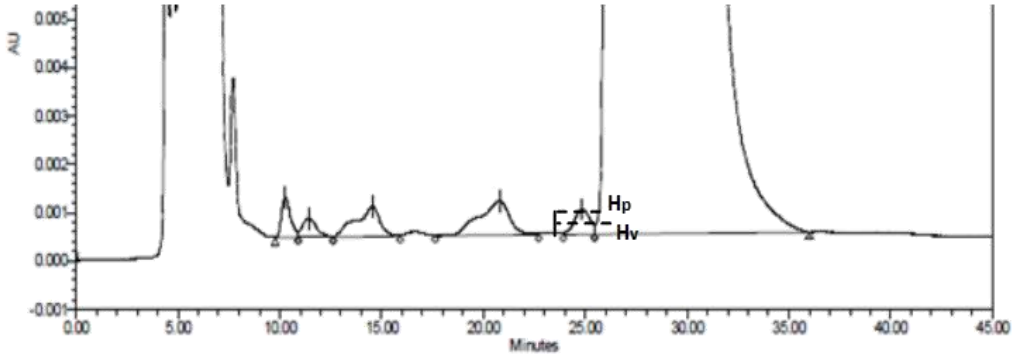


Figure 2.9 - Peak-to-Valley ratio. Adapted from *[43]* .

2.3 Non-Chromatographic Techniques

2.3.1 Karl Fisher Titration – Water Semi-Micro Determination

Normally, to define the quality and shelf life of pharmaceutical products it is necessary to determine the water content [45]. It can be determined by Karl Fisher or by Loss on Drying (LOD). Karl Fisher Titration is a method exclusively to determine the water content in a sample. It involves adding a reagent to the product causing a reaction converting water in a non-conductive chemical. The only limitation is the reactivity of some samples with the reagent and the limited solubility with the alcohol. On the other hand, LOD besides water content, can also measure volatiles impurities and it involves comparing the weight of a product before and after a drying [46, 47].

The semi-micro determination of water is established as the quantitative reaction of water with a solution of sulfur dioxide and iodine in a suitable anhydrous medium in a presence of a base with enough buffering capacity, commercially designated Karl Fisher reagent. The Karl Fisher reagent is composed of methanol, sulfur dioxide and a base (pyridine, imidazole or diethanolamine), represented in *Equation 2.5*, and by iodine.

$$CH_3OH + SO_2 + RN \leftrightarrow [RNH]SO_3CH_3 \quad (2.5)$$

The iodine reacts with the sulfur dioxide in the presence of water originating iodide acid and sulfuric anhydride, represented in *Equation 2.6*:

$$H_2O + I_2 + [RNH]SO_3CH_3 + 2RN \leftrightarrow [RNH]SO_4CH_3 + 2[RNH]I \quad (2.6)$$

The base (*RN*) has the function of stabilizing the reaction as well as helping to solubilize the iodine. The anhydrous methanol is the solvent but will also react with one of the intermediate compounds of the reaction yielding methylpyridonium sulfate (brownish color). It is responsible for the reactivity, end-point indication, and shelf-life [45].

The end-point of the reaction is evidenced by the passage of current in the circuit (two identical indicator electrodes connected to an electrical source that maintains a constant current between them), which occurs when the water is depleted and the iodine introduced by Karl Fisher reagent depolarizes the cathode. As a result of a reaction with water in a sample, the water content is determined by measuring the amount of iodine consumed [48].

The equipment (*Figure 2.10)* consists of a titration vessel composed of two identical platinum electrodes, solvent and titrant introduction inlets, an air passageway through a desiccant and an orifice with a stopper which allows the introduction of the sample. KF instrument should not be set up in areas with high humidity, next to heating or cooling devices, influencing titer stability [49].

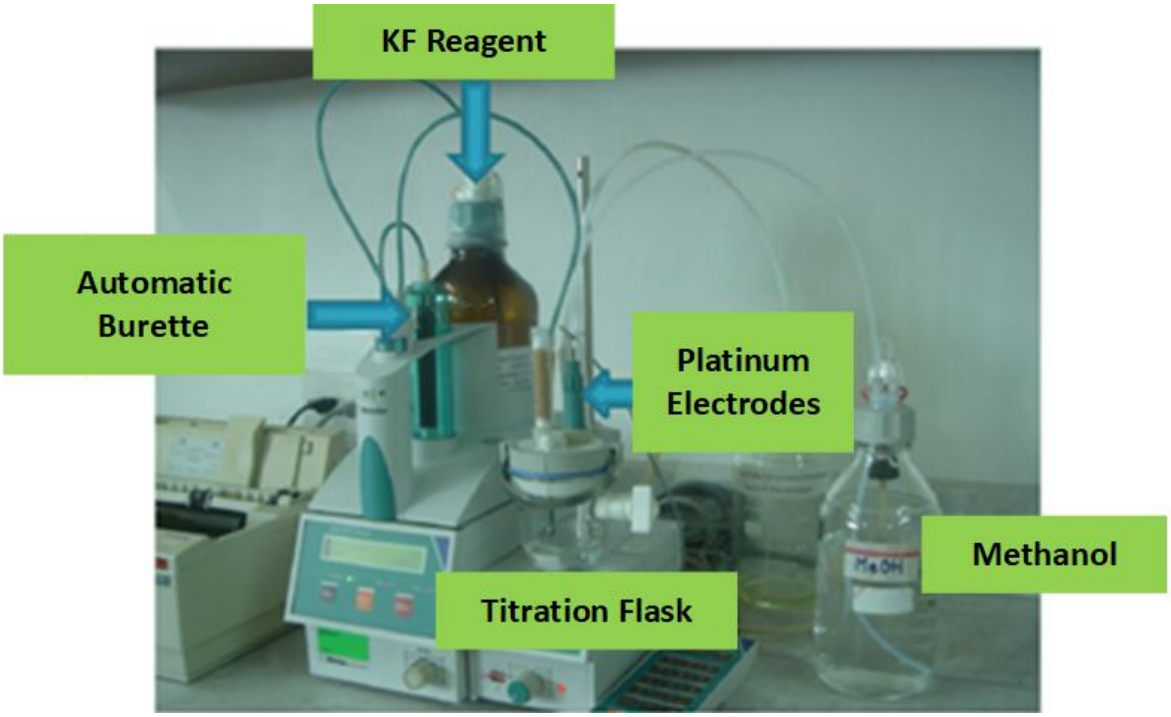


Figure 2.10 - Karl Fisher System. Adapted from *[49].*

The titer of reagent is a fundamental parameter to define the product specification and it is calculated by the *Equation 2.7*:

$$Titer = \frac{Weight\ of\ titrated\ water\ (mg)}{Consumption\ of\ reagent\ (mL)} \qquad (2.7)$$

Depending on the percentage of water present in the sample to be analysed, the titrant reagent to be used and its burette must be adjusted. If the samples contain low water content, it is advisable to use reagent composite 1. In order to reach the equivalence point, successive amounts of purified water or sodium tartrate are added.

Following the validation protocol, after evaluating the water content present in the sample to be analysed, successive known amounts of water are added to the same titration vessel, corresponding to about 100% of the content of water discover in the substance in order to establish the accuracy of the determination. To each addiction, the *Equation 2.8* it is used to calculate the percentage recovery [48]:

$$r = 100 \times \frac{W_2}{W_1} \quad (2.8)$$

Where,

– amount of water added, mg

– amount of water found, mg

If the mean percentage recovery is between 97.5% and 102.5%, then the result is considered acceptable.

In addition, the percentage errors (e_1 and e_2) are also calculating from the results of linear regression by *Equation 2.9* and *Equation 2.10*:

$$e_1 = 100 \times \frac{a-M}{M} \quad (2.9)$$

$$e_2 = 100 \times \frac{|d|-M}{M} \quad (2.10)$$

Where,

a – the y-axis intercept, in milligrams of water;

$|d|$ – the x-axis intercept, in milligrams of water;

M – water content of the substance, in milligrams of water.

Acceptance Criteria:

- The percentage errors and are not greater than 2.5%;
- *b* is between 0,975 and 1,025 (standard deviation ± 2,5%);
- $\bar{r}$ is between 97,5 and 102,5.

2.3.2 Potentiometric Titration

The potentiometric titration is a volumetric method useful for the characterization of many organic and inorganic compounds containing an acidic or a basic component. It is based on the measure of the potential difference between two electrodes (reference electrode and indicator) as a function of the volume of the reagent added. A reference electrode is a half-cell which remains constant regardless of analyte solution composition, with known potential and constant temperature. On the other hand, an indicator electrode has a potential which differs according to the variation of the composition of the analyte solution. Besides that, there is a salt bridge preventing the components of the solution mixing with reference electrode [50].

Potentiometric titration it will be used for the estimation of N-methyl D-glucamine content of API. The content will be determined on an anhydrous basis, so the titration will be non-aqueous. In this specific method, perchloric acid, a very strong acid, is used in glacial acetic acid as titrant [51].

After calibration, each electrode is immersed in an analyte solution. The indicator is selective for H_3O^+ and the reference electrode is stable. Acetic acid has protophillic (proton acceptor) and protogenic (protor donor). As perchloric acid is dissolved in acetic acid, weaker acid, the latter is forced to act as a base (acetate) accepting a proton from perchloric acid, originating acetate acidium ($CH_3OOH_2^+$), as representing in *Equation 2.11* [52, 53].

$$HCLO_4 + CH_3COOH \longrightarrow CH_3COOH_2^+ + CLO_4^- \qquad (2.11)$$

The titration becomes the neutralization of acetate acidium and acetate, *Equation 2.12*: [53]

$$RR'RHN + CH_3OO^- + ClO_4^- + CH_3OOH_2^+ \longrightarrow RR'RNH + ClO_4^- + 2CH_3COOH \qquad (2.12)$$

The potential difference is measured after successive addictions of known increments of acid or titrant base. As the titrant is added, the pH of a solution is measured, until the endpoint which determines a change point.

At least, the *Equations 2.13* and *2.14* are used:

$$\%\ N-methyl\ D-glucamine\ (as\ such) = \frac{A \times 19.521 \times Actual\ Molarity\ of\ 0.1M\ Perchloric\ Acid}{Weight\ of\ sample\ in\ mg \times 0.1} \times 100 \qquad (2.13)$$

$$A = Corrected\ burette\ reading$$

$$\%\ N-methyl\ D-glucamine\ (on\ anhydrous\ basis) = \frac{\%\ N-methyl\ D-glucamine\ (as\ such) \times 100}{(100 - \%\ Water\ content)} \qquad (2.14)$$

2.4 Analytical validation

An analytical procedure is a test in which it is proved that a particular characteristic of a drug product or a drug substance is accepted according to establish criteria. To demonstrate that it is suitable for the purpose for which it is intended, a validation is developed. In the case of some validation data is available to a particular analysis, a transfer of the method is carried on. The transfer of analytical procedures (TAP) is an entire process where a laboratory (the receiving unit) is qualified to use a particular analytical test procedure developed and originated in another laboratory (the transferring unit), ensuring that the receiving unit has the ability to perform the entire procedure properly [19-21].

All of the parameters described below have been taken into consideration aspects of the ICH Topic Q2(R1) Validation of Analytical Procedures: Text and Methodology (CPMP/ICH/381/95) [21].

2.4.1 System Suitability testing

System suitability tests are an integral and fundamental part of liquid chromatography and gas chromatography methods and are used to ensure the performance required for the test.

- **Evaluation of Column Performance**

- ***Relative retention (r)***

Relative retention is used as an estimate and it is translated by the ratio between the retention time of a component to another, used as a reference *Equation 2.15* [43]:

$$r = \frac{(t_{Ri} - t_M)}{(t_{Rst} - t_M)} \qquad (2.15)$$

Where t_{Ri} is the retention time of the concerned peak, t_{Rst} is the retention time of the peak used as a reference, normally the substance to be analysed and t_M is the hold-up time.

- ***Number of Theoretical Plates (N)***

The column is composed of many consecutive segments, designated theoretical plates and for each plate is considered a balance between the solute in the stationary and mobile phase.

The performance of a column can be evaluated on basis of your efficiency and mechanical separation power, connecting the magnitude of a peak's retention to its width. This is an extremely important parameter since a high efficiency is needed to resolve the narrow peaks in the drug analysis. Hence, the higher the value of theoretical plates of a column (*N*) the less the height of the plate (*H*) and therefore the more efficient it will be. It is calculated by *Equation 2.16* [43, 54]:

$$H = \frac{L}{N} \tag{2.16}$$

Where *L* represents the column length.

To the calculations is assumed that if the peak follows a Gaussian distribution, the *Equation 2.17* is applied (*Figure 2.11*) [55]:

$$N = 16\left(\frac{t_r}{W}\right)^2 \tag{2.17}$$

Where, t_r is the retention time of the analyte and, W the peak width at its base.

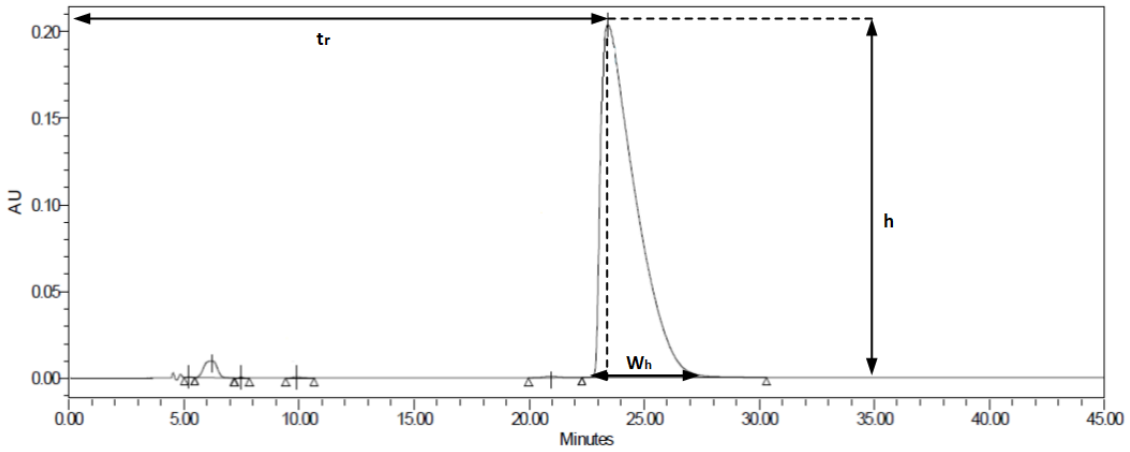


Figure 2.11 - Calculation of the number of Theoretical Plates per meter (USP method). Adapted from *[55]*.

As the number of theoretical plates depends on the conditions that the analytes are submitted, the peaks have great variations according to the flow rate, column temperature, mobile phases, the uniformity of the packing within the column, column characteristics, retention times, as well as the substance itself being chromatographed. For that, the *Equation 2.17* is adapted to *Equation 2.18*, per meter *(Figure 2.12)* [55]:

$$N = 5.54\left(\frac{t_r}{W_{h/2}}\right)^2 \tag{2.18}$$

Where, $W_{h/2}$ is the peak width in the zone corresponding to half-height

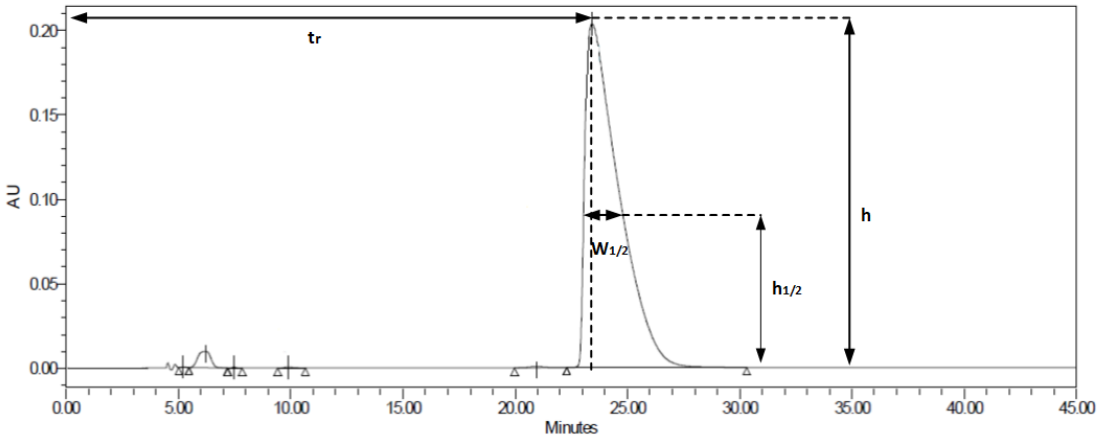


Figure 2.12 - Calculation of the number of Theoretical Plates per meter (half-height method). Adapted from *[55]*.

- ***Capacity Factor / Retention Factor***

A time measurement that relates the residence time of an analyte in the stationary phase with the time that it resides in the mobile phase. It demonstrates how much longer the stationary phase delays an analyte than would be necessary if it only passed at the speed of the mobile phase [23].

It is calculated by *Equation 2.19*:

$$k = \frac{t_r - t_M}{t_M} \qquad (2.19)$$

- ***Resolution***

Resolution is the degree of separation of two components in a mixture, expressed as the distance between the signals relative to the signal width. In other words, it is the difference in their corresponding retention times, divided by their average peak width at the baseline as described in *Equation 2.20* and *Figure 2.13* [23].

$$R_S = \frac{2\,(t_{R2} - t_{R1})}{(W_{b(2)} + W_{b(1)})} \qquad (2.20)$$

Besides that, the resolution can also be calculated using the peak width at half height. It is assumed a Gaussian peak shape and calculated by *Equation 2.21* [23, 56]:

$$R_S = \frac{1.18\,(t_{R2} - t_{R1})}{(W_{h(2)} + W_{h(1)})} \qquad (2.21)$$

Where:

t_{R1} – Retention time of first peak

t_{R2} – Retention time of second peak

$W_{b(1)}$ – Peak width at the base of the first peak

$W_{b(2)}$ – Peak width at the base of the second peak

$W_{h(1)}$ – Peak with at half height of the first peak

$W_{h(2)}$ – Peak with at half height of the first peak

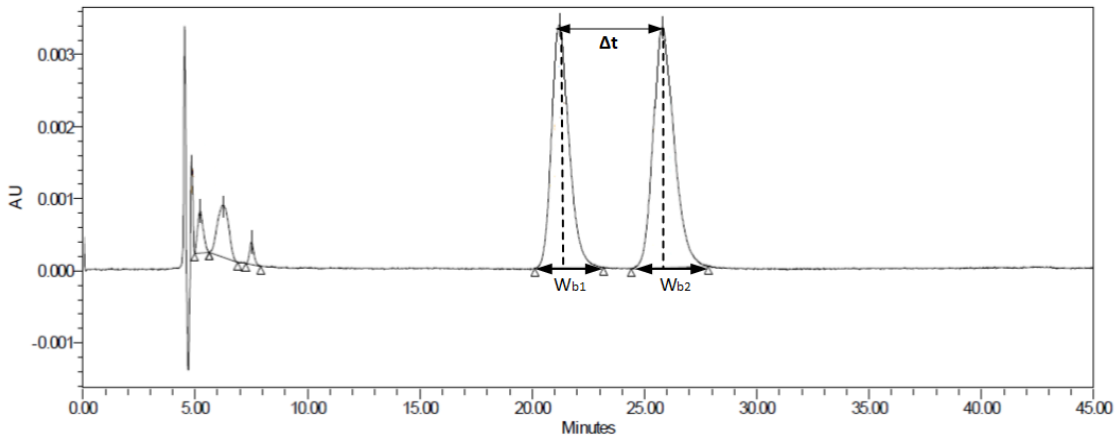


Figure 2.13 - Chromatographic Resolution. Adapted from *[56]*.

The higher the resolution, the greater the separation of the peaks in the baseline. Resolution is influenced by two parameters, efficiency, and selectivity. Higher efficiency columns produce narrower peaks and improve resolution for difficult separations. One way to raise the resolution is by increasing the selectivity, by conjugating the mobile phase with the stationary phase, assertively.

- ***Symmetry Factor (As)***

The symmetry factor is known as the “tailing factor” is a coefficient that allows determining the degree of peak symmetry. It is defined as the distance from the front slope of the peak to the back slope divided by twice the distance from the centreline of the peak to the front slope, with all measurements made at 5% of the maximum peak height (*Figure 2.14*) [23]. It is calculated by *Equation 2.22*:

$$A_s = \frac{W_{0.05}}{2f} \quad (2.22)$$

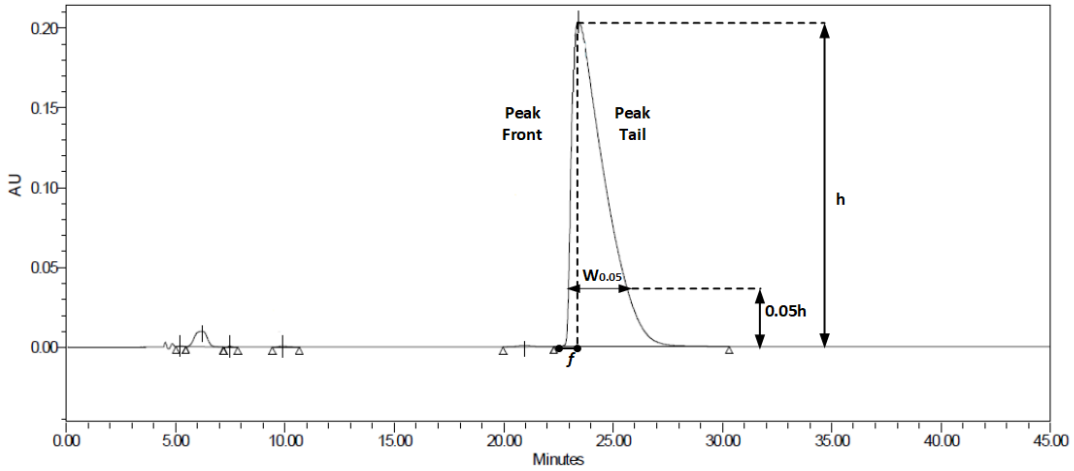


Figure 2.14 - Asymmetrical chromatographic peak. Adapted from *[23]*.

For values equal to 1.0 means symmetry (Gaussian distribution). When A_S > 1.0, the peak is tailing. When A_S < 1.0, the peak is fronting.

- **Signal-To-Noise (S/N)**

The signal-to-noise ratio is important system suitability that influences the precision of quantitation. It is calculated by the *Equation 2.23*:

$$S/N = \frac{2H}{h} \quad (2.23)$$

Where *H* is the height between the maximum of the concerned peak and the minimum height of the baseline noise. The variation between the maximum and minimum height of baseline noise peaks is given by *h (Figure 2.15)* [23, 43].

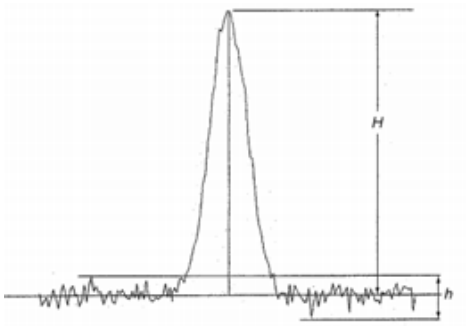


Figure 2.15 - Noise and chromatographic peak, components of the S/N ratio *[43]*.

- **System Repeatability**

Relative Standard Deviation (RSD)

To evaluate the repeatability of a system, successive injections of a reference solution are required to evaluate the relative standard deviation (S_r (%) or RSD (%)) between their respective responses before the beginning of an analytical procedure, and is calculated using the *Equation 2.24* [43]:

$$S_r(\%) = \frac{100}{\bar{y}} \sqrt{\frac{\sum(y_1 - \bar{y})^2}{n-1}} \qquad (2.24)$$

Where:

y_1 = individual values relative to peak area, peak height or ratio of areas by the internal standardization method.

$\bar{y}$ = mean of individual values

n = number of individual values

Response Factor

A response factor is a ratio between the concentration of a component being analysed and the response of the detector to that component. Normally two solutions are prepared equally, a working standard and a control standard. It is calculated by *Equation 2.25*:

$$R_F = \frac{R_c}{R_W} \times \frac{C_W}{C_c} \qquad (2.25)$$

Where:

R_c – Response (area) of control standard solution;

R_W – Response (area) of working standard solution;

C_W – Concentration of working standard solution (µg/mL);

C_c – Concentration of control standard solution (µg/mL);

2.4.2 Specificity

Specificity is the ability of a test to be discriminative in the identification of a particular compound, relative to another with similar structure, present in the same matrix. The analyte should have no interference in the presence of components which may be expected to be present, as impurities present in the active drug, from the synthesis process, degradation products, excipients, solvents, and other extraneous components.

In the *Table 2.13* are the acceptance criteria for all the tests evaluated with this parameter.

Table 2.1 - Summary of acceptance criteria for the specificity parameter

Tests	Acceptance Criteria
Identification and Assay	No peaks interfering with the peaks of the substances to quantify should be detected.
Related Substances – Test 1 and Test 2	No peaks interfering with the peaks of the substances to quantify should be detected.
Stereochemical Purity	No peaks interfering with the peaks of the substances to quantify should be detected.
Content of Acetic Acid	No peaks interfering with the peaks of the substances to quantify should be detected.
Residual Solvents	No peaks interfering with the peaks of the substances to quantify should be detected.
Content of N-methyl D-glucamine	Quantifying the analyte with no interference from excipients or other substances present in the sample matrix.

2.4.3 Linearity

Linearity is the ability to obtain test results directly proportional to analyte concentrations in a sample, within a given range. Primarily, it should be assessed visually from a signal plot as a function of analyte concentration. If a linearity relation is confirmed, the results are analysed by statistical methods, such as a regression line by the method of least squares.

To determine the degree of linearity is necessary to consider some factors as the correlation coefficient, y-intercept, slope of the regression line and residual sum of squares. To be ideal results, the slope of the linear regression should be as close to one. A slope less than one indicates a smaller test response than the assumed with higher analyte concentrations. If the slope of the regression line is equal to one and the y-interception is greater or less than zero, then systematic errors probably could have existed. A correlation coefficient less than one expresses inaccuracy, imprecision or some linearity failure. The reasonable criteria is: r ≥ 0,98 and the 95% confidence interval of the regression line slope should contain one.

In the *Tables 2.14 and 2.15* are the acceptance criteria for all the tests evaluated with this parameter.

Table 2.2 - Summary of acceptance criteria for the linearity parameter.

Tests	Acceptance Criteria
Identification and Assay	- r ≥ 0,999; - Slope – ideal value should approach factor F (F = y(x) / x); - Interception – interception limits (95% confidence interval) must contain zero; - |Intercept| < 5% response obtained for the lowest concentration level; - Random residuals distribution.
Related Substances – Test 1 and Test 2	- r ≥ 0,99; - Slope – ideal value should approach factor F (F = y(x) / x); - Interception limits (95% confidence interval) must contain zero; - |Intercept| < 10% response obtained for the lowest concentration level; - Random residuals distribution.
Stereochemical Purity	- r ≥ 0,99; - Slope – ideal value should approach factor F (F = y(x) / x); - Interception limits (95% confidence interval) must contain zero; - |Intercept| < 10% response obtained for the lowest concentration level; - Random residuals distribution.
Content of Acetic Acid	- r ≥ 0,995; - Slope – ideal value should approach factor F (F = y(x) / x); - Interception limits (95% confidence interval) must contain zero; - |Intercept| < 10% response obtained for the lowest concentration level; - Random residuals distribution.

Table 2.3 - Summary of acceptance criteria for the linearity parameter (continuation).

Tests	Acceptance Criteria
Residual Solvents	- r ≥ 0,995; - Slope – ideal value should approach factor F (F = y(x) / x); - Interception limits (95% confidence interval) must contain zero; - |Intercept| < 10% response obtained for the lowest concentration level; - Random residuals distribution.
Content of N-Methyl D-Glucamine	- r ≥ 0,99; - Slope – ideal value should approach factor F (F = y(x) / x); - Interception limits (95% confidence interval) must contain zero; - |Intercept| < 5% response obtained for the lowest concentration level; - Random residuals distribution.

2.4.4 Precision

Precision reflects the degree of agreement between results obtained from multiple samples prepared from one homogeneous sample and reflects the ability of the system or test to report similar results. Thus, the precision of analytical procedures is often used as the relative standard deviation (coefficient of variation) of a data set and it includes system repeatability, method repeatability, intermediate precision and reproducibility.

System repeatability is a measure of variability inherent to the chromatographic system. It is determined by analysing the same sample five or more times into the system and verifying that the RSD meets the acceptance criteria.

Method repeatability is a measure of variability inherent to the test, incorporating the variability of the experimental procedure used in sample preparation. It is determined by consecutively analysing at least six samples, prepared at 100% concentration level, from the same solution/batch against the standard or analysing at least nine samples, prepared at three different concentration levels, covering the entire range to validate.

Intermediate precision translates the measure of intra-laboratory variability of the procedure with some variations as a different operator, equipment and day of analysis. It is determined on a set of results from the consecutive analysis of two replicate samples. Both samples follow the method

repeatability procedure, with the difference that they are analysed on different days and different equipment and with two different operators.

Reproducibility is a measure of test variability when performed in different laboratories. After analysing one or more batches, it is verified whether the differences in the results are statistically significant [21, 43].

In the *Table 2.16* are the acceptance criteria for all the tests evaluated with this parameter.

Table 2.4 -Summary of acceptance criteria for the precision parameter.

Tests	**Acceptance Criteria**	
	System Repeatability	**Method Repeatability**
Identification and Assay	RSD ≤ 2,0 %	RSD ≤ 2,0 %
Related Substances – Test 1 and Test 2	RSD ≤ 5,0 %	RSD ≤ 10,0 %
Stereochemical Purity	RSD ≤ 5,0 %	RSD ≤ 10,0 %
Content of Acetic Acid	RSD ≤ 5,0 %	RSD ≤ 10,0 %
Residual Solvents	RSD ≤ 15,0 %	RSD ≤ 15,0 %
Content of N-Methyl D-Glucamine	RSD ≤ 5,0 %	RSD ≤ 5,0 %

2.4.5 Accuracy

Accuracy is a method of the exactness of the analytical method. It is a measure of the closeness of test results obtained by a method to the true value and it means that there is no systematic error or bias in the method.

In the *Table 2.17* are the acceptance criteria for all the tests evaluated with this parameter.

Table 2.5 - Summary of acceptance criteria for the accuracy parameter.

Tests	Acceptance Criteria
Identification and Assay	- r ≥ 0,99; - residuals < 5% (5% to the lower limit of the line); - 95% confidence interval of slope contains unit; - 95% confidence interval of intercept contains zero; - random residuals distribution.
Related Substances – Test 1 and Test 2	- r ≥ 0,99; - residuals < 10% (15% to the lower limit of the line); - 95% confidence interval of slope contains unit; - 95% confidence interval of intercept contains zero; - random residuals distribution.
Stereochemical Purity	- r ≥ 0,99; - residuals < 10% (15% to the lower limit of the line); - 95% confidence interval of slope contains unit; - 95% confidence interval of intercept contains zero; - random residuals distribution.
Content of Acetic Acid	- r ≥ 0,99; - random residuals distribution.
Residual Solvents	- r ≥ 0,995; - random residuals distribution.
Content of N-Methyl D-Glucamine	- r ≥ 0,99; - residuals < 2% (5% to the lower limit of the line); - 95% confidence interval of slope contains unit; - 95% confidence interval of intercept contains zero; - random residuals distribution.

2.4.6 Quantitation Limit

The quantitation limit is a quantitative parameter and expresses the lower concentration of an analyte in a sample with suitable accuracy and precision. Thus, it is ensured that the method is accurate, precise and linear at a certain point. It should normally be less than or equal to the specified test limit, preferably ≤ 50% of this level and it should be proven by calculating the RSD and appropriate accuracy measurements using at least five distinct dilutions of a standard solution.

The quantitation limit can be determined for several processes. It can be based on a visual evaluation (by analysis of samples with known concentrations of the analyte, establishing the minimum level to which it is quantified with accuracy and precision), on the signal-to-noise approach (by comparing the measured signals for samples with known minimum concentrations to those of blank samples. It is generally considered a 10:1 signal to noise ratio) and on the standard deviation of the response and the slope (from regression values in the low concentration range, by application of the equation $LQ = (10 \times \sigma)/S$, in which S represents the standard deviation of the response and represents the slope of the calibration curve).

In the *Table 2.18* are the acceptance criteria for all the tests evaluated with this parameter.

Table 2.6 - Summary of acceptance criteria for the quantitation limit parameter.

Tests	Acceptance Criteria
Related Substances – Test 1 and Test 2	RSD ≤ 10,0 %
Stereochemical Purity	RSD ≤ 10,0 %
Content of Acetic Acid	RSD ≤ 10,0 %
Residual Solvents	RSD ≤ 15,0 %

3. Materials and Methods

The active substance analytical method tests transferred take into consideration aspects of the ICH Topic Q2 (R1) Validation of Analytical Procedures: Text and Methodology (CPMP/ICH/381/95) [21]. Information contained in the supplier's DMF, specifications and results present in the CoA of the active substance was also considered.

For chromatography techniques by HPLC, reversed-phase partition chromatography was used. Furthermore, except related substances – test 1, in which a photodiode detector was used (PDA), all methods were analyzed with a diode detector (DAD).

3.2 Materials and Instruments

All equipment used in the procedures are described in *Table 3.1* and substances and their specifications are described in *Table 3.2.*

3.2.1 Equipment

Table 3.1 - Equipment involved in the transfer of analytical methods.

Name	Brand	Model
HPLC	Waters	Alliance 2695 2996 PDA detector
GC-HS	Agilent	7890 (G3440A) FID detector G1888A Headspace Sampler
Automated titrator	Metrohm	751 GPD Titrino (806 Exchange unit)

3.2.2 Reference Substances

Table 3.2 – References Substances.

Identification	Source	Purity (%)	Expiry/retest date
API	Supplier	99,3	2020/Ago/08
Impurity A	Supplier	84,2	2019/Ago/12
Impurity B	Supplier	91,0	2019/Ago/12
Impurity C	Supplier	99,9	2019/Ago/12
Impurity D	Supplier	86,5	2019/Ago/12
Impurity E	Supplier	89,2	2019/Jul/25
Impurity F	Supplier	69,4	2019/Mai/06
Other isomer API	Supplier	92,5	2018/Nov/28
Acetone	Sigma-Aldrich	99,9	2020/Mai/31
Cyclohexane	Sigma-Aldrich	99.9	2020/Abr/30
Ethanol	Sigma-Aldrich	99,9	2023/Mar/13
Ethyl acetate	Sigma-Aldrich	100	2020/Abr/30
Isopropyl alcohol	Sigma-Aldrich	99,9	2019/Nov/30
Methylene chloride	Sigma-Aldrich	99,9	2020/Set/30
Methanol	Sigma-Aldrich	99,9	2020/Fev/28
Tetrahydrofuran	Sigma-Aldrich	99.9	2020/Fev/28
Toluene	Sigma-Aldrich	99,9	2021/Jan/31
Acetic acid	Sigma-Aldrich	99,9	2021/jan/31

Note: All the reference substances were used before the end of expiry date.

3.2.3 Column details

The HPLC columns used and their specifications are:

- Zorbax Eclipse XDB C-18, Agilent;
- Xterra RP-18, Waters;
- Chiral AGP, Chiralpak;
- Inertsil ODS 3V, Shinawa.

The GC column used, and its specification is:

- DB624, Macherey-Nagel.

3.3 Tests Procedures

3.3.1 Identification by HPLC

In the test for assay, the retention time of principle peak from the sample should match with that from API working standard.

3.3.2 Assay by HPLC

Assay is a quantity procedure with the objective of measure the content of the active substance in a sample.

The method was applied at temperatures designated by the supplier, with mobile phases of mixed composition (aqueous and organic) and the elution by gradient, according to the chromatographic conditions given in the *Table 3.3*.

Table 3.3 - Chromatographic conditions (same as described in related substances: Test 1)

Apparatus	A HPLC equipped with quaternary gradient pumps, variable wavelength UV detector attached with data recorder and integrator software, or equivalent
Column	Eclipse XDB C-18
Flow Rate	1,0 mL/min
Diluent	Water:Acetonitrile

Preparation of Test solution: Transfer the sample accurately weighed into a volumetric flask, adding diluent. $C_{Test\ solution}$ = 0.3 mg/mL.

Preparation of Standard Solution: Transfer API working standard accurately weighed into a volumetric flask, adding diluent. $C_{Standard\ solution}$ = 0.3 mg/mL.

System suitability test: The relative standard deviation determined from the standard solution in five replicate injections is not more than 2.0%.

Calculation:

$$\%\ Assay\ (as\ such) = \frac{Area\ of\ test\ solution}{Average\ area\ of\ standard\ solution} \times \frac{Standard\ Weight}{10} \times \frac{5}{50} \times \frac{10}{Sample\ Weight}\frac{50}{5} \times Potency\ of\ Working\ Standard \quad (3.1)$$

$$\%\ Assay\ (on\ anhydrous\ basis) = \frac{\%\ Assay\ (as\ such) \times 100}{(100 - \%\ Water\ content)} \quad (3.2)$$

3.3.3 Related Substances by HPLC – Test 1

Related substances are a quantification procedure with the objective of measuring the other substances in a sample, including impurities. The method was applied at temperatures designated by the supplier, with mobile phases of mixed composition (aqueous and organic) and the elution by gradient, according to the chromatographic conditions given in the *Table 3.4*.

Table 3.4 - Chromatographic Conditions (Test 1)

Apparatus	A HPLC equipped with quaternary gradient pumps, variable wavelength UV detector attached with data recorder and integrator software or equivalent
Column	Eclipse XDB C-18
Flow Rate	1,0 mL/min
Diluent	Water:Acetonitrile

Preparation of Test solution (Prepare the test solution in duplicate): Transfer the sample accurately weighed into a volumetric flask, adding diluent. $C_{Test\ solution}$ = 3.0 mg/mL.

Note: Test solution should be prepared freshly for every analysis.

Preparation of Reference solution (a): Transfer API working standard accurately weighed into a volumetric flask, adding diluent. C_{API} = 0.3 mg/mL.

Preparation of Reference solution (b): Transfer some Impurity C standard, accurately weighed into a volumetric flask. C_{IMP} c = 0.3 mg/mL.

Preparation of Reference solution (c): Transfer some of reference solution (a) and reference solution (b) into a volumetric flask, adding diluent. C_{API} =15.0 µg/mL; $C_{IMP\ C}$ = 15.0 µg/mL.

Preparation of Reference solution (d): Transfer some of Impurity A standard, accurately weighed into a volumetric flask. $C_{IMP\ A}$ = 0.2 mg/mL.

Preparation of Reference solution (e): Transfer some of Impurity B standard, accurately weighed into a volumetric flask. $C_{IMP\ B}$ = 0.2 mg/mL.

Preparation of Reference solution (f): Transfer some of Impurity C standard, accurately weighed into a volumetric flask. $C_{IMP\ C}$ = 0.2 mg/mL.

Preparation of Reference solution (g): Transfer some of Impurity D standard, accurately weighed into a volumetric flask. $C_{IMP\ D}$ = 0.2 mg/mL.

Preparation of Reference solution (h): Transfer some API working standard, accurately weighed into a volumetric flask, adding diluent. Add some of each reference solution (d), reference solution (e), reference solution (f) and reference solution (g) into it. C_{API} = 3.0 mg/mL; $C_{IMP\ A,\ B,\ C,\ D,\ F}$ = 9.0 µg/mL.

System Suitability:

The relative standard deviation for Impurity C peak and API peak determined from six replicate injections of reference solution (c) should not be more than 5.0%. In reference solution (h), resolution between main peak and Impurity D peak should not be less than 2.5.

Calculations:

$$\%\ Impurity\ C = \frac{Area\ of\ Impurity\ C\ peak\ in\ test\ solution}{Average\ area\ of\ Impurity\ C\ peak\ in\ reference\ solution\ (c)} \times \frac{Weight\ of\ Impurity\ C\ standard\ in\ Reference\ solution\ (b)}{100} \times \frac{5}{100} \times \frac{10}{Weight\ of\ Sample\ in\ test\ solution} \times \frac{P_1}{100} \times 100 \quad (3.3)$$

$$\%\ Impurity\ A = \frac{Area\ of\ Impurity\ A\ in\ test\ solution}{Average\ area\ of\ API\ peak\ in\ reference\ solution\ (c)} \times \frac{Weight\ of\ API\ working\ standard\ in\ Reference\ solution\ (a)}{10} \times \frac{5}{50} \times \frac{5}{100} \times \frac{10}{Weight\ of\ Sample\ in\ test\ solution} \times Response\ factor \times \frac{P_2}{100} \times 100 \quad (3.4)$$

$$\%\ Impurity\ B = \frac{Area\ of\ Impurity\ B\ in\ test\ solution}{Average\ area\ of\ API\ peak\ in\ reference\ solution\ (c)} \times \frac{Weight\ of\ API\ working\ standard\ in\ Reference\ solution\ (a)}{10} \times \frac{5}{50} \times \frac{5}{100} \times \frac{10}{Weight\ of\ Sample\ in\ test\ solution} \times Response\ factor \times \frac{P_2}{100} \times 100 \quad (3.5)$$

$$\%\ Impurity\ D = \frac{\frac{Area\ of\ Impurity\ D\ in\ test\ solution}{Average\ area\ of\ API\ peak\ in\ reference\ solution\ (c)}}{} \times \frac{\frac{Weight\ of\ API\ working\ standard\ in\ Reference\ solution\ (a)}{10}}{} \times \frac{5}{50} \times \frac{5}{100} \times \frac{10}{Weight\ of\ Sample\ in\ test\ solution} \times Response\ factor \times \frac{P_2}{100} \times 100$$

(3.6)

$$\%\ Impurity\ F = \frac{\frac{Area\ of\ Impurity\ F\ in\ test\ solution}{Average\ area\ of\ API\ peak\ in\ reference\ solution\ (c)}}{} \times \frac{\frac{Weight\ of\ API\ working\ standard\ in\ Reference\ solution\ (a)}{10}}{} \times \frac{5}{50} \times \frac{5}{100} \times \frac{10}{Weight\ of\ Sample\ in\ test\ solution} \times Response\ factor \times \frac{P_2}{100} \times 100$$

(3.7)

$$\%\ Any\ other\ unspecified\ impurity = \frac{\frac{Area\ of\ Any\ other\ Unspecified\ impurity\ in\ test\ solution}{Average\ area\ of\ API\ peak\ in\ reference\ solution\ (c)}}{} \times \frac{\frac{Weight\ of\ API\ working\ standard\ in\ Reference\ solution\ (a)}{10}}{} \times \frac{5}{50} \times \frac{5}{100} \times \frac{10}{Weight\ of\ Sample\ in\ test\ solution} \times \frac{P_2}{100} \times 100$$

(3.8)

$$\%\ Total\ unkown\ impurities = \frac{\frac{Area\ of\ Total\ unkown\ impurities\ in\ test\ solution}{Average\ area\ of\ API\ peak\ in\ reference\ solution\ (c)}}{} \times \frac{\frac{Weight\ of\ API\ working\ standard\ in\ Reference\ solution\ (a)}{10}}{} \times \frac{5}{50} \times \frac{5}{100} \times \frac{10}{Weight\ of\ Sample\ in\ test\ solution} \times \frac{P_2}{100} \times 100$$

(3.9)

P_1 = Potency of Impurity C standard

P_2 = Potency of API working standard.

3.3.4 Related Substances by HPLC – Test 2 (Content of Impurity E)

Related substances are a quantity procedure with the objective of measure the other substances in a sample, including impurities. The method was applied at temperatures designated by the supplier, with mobile phases of mixed composition (aqueous and organic) and the elution by gradient, according to the chromatographic conditions given in the *Table 3.5*.

Table 3.5 - Chromatographic conditions (Test 2)

Apparatus	A HPLC equipped with quaternary gradient pumps, variable wavelength UV detector attached with data recorder and integrator software or equivalent
Column	Xterra RP-18
Flow Rate	1,0 mL/min
Diluent	Buffer:Acetonitrile

Preparation of Test solution (Prepare the test solution in duplicate): Transfer of sample accurately weighed, into a volumetric flask, adding diluent. $C_{Test\ Solution}$ = 2.0 mg/mL.

Note: Test solution should be prepared freshly for every analysis.

Preparation of Reference solution (a): Transfer some of Impurity E standard, accurately weighed into a volumetric flask, adding diluent. $C_{IMP\ E}$ = 2.0 mg/mL.

Preparation of Reference solution (b): Transfer some of reference solution (a) into a volumetric flask, adding diluent. $C_{IMP\ E}$ = 10.0 µg/mL.

Preparation of Reference solution (c): Transfer some of Impurity E standard, accurately weighed into a volumetric flask, adding diluent. $C_{IMP\ E}$ = 0.1 mg/mL

Preparation of Reference solution (d): Transfer some of API working standard, accurately weighed into a volumetric flask, adding diluent. Add some of reference solution (c). C_{API} = 2.0 mg/mL; $C_{IMP\ E}$ = 6.0 µg/mL.

System Suitability: The relative standard deviation determined from six replicate injections of reference solution (b) should not be more than 5.0%. In reference solution (d), resolution between main peak and Impurity E peak should not be less than 2.0.

Calculation:

$$\%\ Impurity\ E = \frac{\frac{Area\ of\ Impurity\ E\ Peak\ in\ test\ solution}{Average\ area\ of\ Impurity\ E\ peak\ in\ reference\ solution\ (b)}}{} \times \frac{\frac{Weight\ of\ Impurity\ E\ standard\ in\ Reference\ solution\ (a)}{10}}{} \times \frac{5}{50} \times \frac{5}{100} \times \frac{20}{\frac{Weight\ of\ Sample}{in\ test\ solution}} \times \frac{P}{100} \times 100 \qquad (3.10)$$

P = Potency of Impurity E standard

3.3.5 Stereochemical Purity by HPLC

The method was applied at temperatures designated by the supplier, with mobile phases of mixed composition (aqueous and organic) and isocratic elution, according to the chromatographic conditions given in the *Table 3.6*.

Table 3.6 - Chromatographic conditions (Stereochemical purity)

Apparatus	A HPLC equipped with quaternary gradient pumps, variable wavelength UV detector attached with data recorder and integrator software or equivalent
Column	Chiral AGP
Flow Rate	0,3 mL/min
Diluent	Buffer:Acetonitrile

Preparation of Reference Solution (a): Transfer some of API working standard accurately weighed, into volumetric flask, adding diluent. C_{API} = 30.0 µg/mL.

Preparation of Reference Solution (b): Transfer some of API stereochemical purity standard, accurately weighed, into volumetric flask, adding diluent. $C_{API\ Stereochemical\ Purity}$ = 1.2 mg/mL.

Preparation of Reference Solution (c): Transfer some of reference solution (b), into a volumetric flask, adding diluent. $C_{API\ Stereochemical\ Purity}$ = 12.0 µg/mL

Preparation of Reference Solution (d): Transfer some of API working standard accurately weighed, into volumetric flask, adding diluent. Add some of reference solution (c). Make up to the mark with diluent and mix. C_{API} = 0.6 mg/mL; $C_{API\ Stereochemical\ Purity}$ = 2.4 µg/mL.

Preparation of Test solution (in duplicate) Transfer spme of sample accurately weighed, into a volumetric flask, adding diluent. C_{API} = 0.6 mg/mL

System suitability test: The relative standard deviation determined from six replicate injections of reference solution (a) should not be more than 5.0%. Resolution between the main peak and other isomer peak from reference solution (d) chromatogram should not be less than 1.0.

Calculation:

$$\%\ Other\ isomer = \frac{\frac{Area\ of\ other\ isomer\ in\ test\ solution}{Average\ area\ of\ Reference\ solution\ (a)}}{} \times \frac{\frac{Weight\ of\ working\ standard\ in\ Reference\ solution\ (a)}{25}}{} \times \frac{1}{100} \times \frac{4}{20} \times \frac{25}{Weight\ of\ Sample\ in\ test\ solution} \times \frac{P}{100} \times 100 \quad (3.11)$$

P = Potency of working standard

3.3.6 Content of Acetic Acid by HPLC

The method was applied at temperatures designated by the supplier, with mobile phases of mixed composition (aqueous and organic) and the elution by gradient, according to the chromatographic conditions given in the *Table 3.7*.

Table 3.7 - Chromatographic conditions (Content of Acetic Acid)

Apparatus	A HPLC equipped with quaternary gradient pumps, variable wavelength UV detector attached with data recorder and integrator software or equivalent
Column	Inertsil ODS 3V
Flow Rate	1,0 mL/min
Diluent	Buffer:Methanol

Preparation of Test solution (in duplicate): Transfer some of sample accurately weighed, into a volumetric flask, adding diluent. $C_{Test\ Solution}$ = 25.0 mg/mL

Preparation of Reference solution (a): Transfer some of Glacial acetic acid standard, accurately weighed into a volumetric flask, adding diluent. $C_{Acetic\ Acid}$ = 1.0 mg/mL.

Preparation of Reference solution (b): Transfer some of reference solution (a) into a volumetric flask, adding diluent. $C_{Acetic\ Acid}$ = 125,0 µg/mL.

System Suitability: The relative standard deviation from six replicate injections of reference solution (b) should not be more than 5.0%.

Calculation:

$$Content\ of\ Acetic\ Acid = \frac{Area\ of\ acetic\ acid\ peak\ in\ test\ solution}{Average\ area\ of\ acetic\ acid\ peak\ in\ Reference\ solution\ (a)} \times \frac{Weight\ of\ acetic\ acid\ standard\ in\ Reference\ solution\ (a)}{100} \times \frac{2.5}{20} \times \frac{10}{Weight\ of\ Sample\ in\ test\ solution} \times Potency\ of\ Acetic\ Acid\ Standard \times 1000 \quad (3.12)$$

3.3.7 Residual Solvents by GC

The method was applied at temperatures designated by the supplier, according to the chromatographic conditions given in the *Table 3.8*.

Table 3.8 - Chromatographic conditions (Residual Solvents)

Instrument	Gas chromatograph equipped with FID detector and Headspace	
Injector	*Carrier gas:*	Nitrogen
	Linear velocity:	30 cm/sec
	Temperature:	200ºC
Detector	*Type:*	FID
	Temperature:	250ºC
Oven	*Temperature:*	*Restricted Part*
Column	*Type:*	Rtx-624 or equivalent
Head-space Sampler	*Incubation time:*	10 minutes
	Incubation temperature:	70ºC
	Agitation speed:	600 rpm
	Syringe temperature:	115ºC
	Injection volume	1 mL

Diluent: Dimethylformamide

Standard Solution should be prepared freshly for every analysis.

Preparation of Standard Stock Solution: Take 510µL of Acetone, 400µL Cyclohexane, 510µL Ethanol, 440µL Ethyl acetate, 510µL of Isopropyl alcohol, 36µL of Methylene chloride, 300µL of Methanol, 65µL of Tetrahydrofuran and 82µL of Toluene into a 100 mL of volumetric flask containing 80 mL of Dimethylformamide and mix. Dilute up to the mark with Dimethylformamide and mix.

Preparation of Standard solution: Take 5mL of Standard stock solution in a 100mL volumetric flask and dilute up to the mark with Dimethylformamide and mix.

Preparation of Sample Solution: Transfer about 200mg of sample accurately weighed in 20 mL Head space vial. Add 5 mL of Dimethylformamide and mix.

System suitability: The resolution between each peak must be more than 1.0 and the relative standard deviation (RSD) for each solvent peak response (area) must be less than 15% from replicate injections of standard solution.

Calculation:

$$Content\ of\ Solvent\ in\ ppm = \frac{\frac{Area\ of\ Solvent\ in\ sample\ solution}{Average\ area\ of\ Solvent\ in\ standard\ solution}}{} \times \frac{\frac{\mu l\ of\ Solvent\ standard}{100} \times density\ of\ solvent}{} \times \frac{5}{100} \times \frac{5}{\frac{Sample\ Weight\ (mg)}{}} \times 10^6$$

(3.14)

Solvent = Acetone, Cyclohexane, Ethanol, Ethyl acetate, Isopropyl alcohol, Methylene chloride, Methanol, Tetrahydrofuran, Toluene.

3.3.8 Content of N-Methyl D-Glucamine (On anhydrous basis)

Transfer about 200mg of sample accurately weighed, into a 250mL beaker. Add about 60mL of glacial acetic acid and sonicate to dissolve. Titrate it against 0.1M Perchloric acid, using suitable electrode with autotitrator to the potentiometric end point. Perform blank by omitting the sample. The corrected burette reading is obtained by subtracting blank from sample reading.

Determine the content of N-methyl D-glucamine by using the *Equations 3.15* and *3.16*:

$$\%\ N-methyl\ D-glucamine\ (as\ such) = \frac{A \times 19.521 \times Actual\ Molarity\ of\ 0.1M\ Perchloric\ Acid}{Weight\ of\ sample\ in\ mg \times 0.1} \times 100$$

(3.15)

$$A = Corrected\ burette\ reading$$

$$\%\ N-methyl\ D-glucamine\ (on\ anhydrous\ basis) = \frac{\%\ N-methyl\ D-glucamine\ (as\ such) \times 100}{(100 - \%\ Water\ content)}$$

(3.16)

3.3.9 Water Content by KF

Clean the titration vessel, stirrer and electrode using methanol or other specified solvent.

Switch on the instrument. Fill the titration vessel with 15-20 mL of fresh methanol or other specified solvent. Add 35 to 40mL of anhydrous methanol or the solvent specified in the procedure to the titration vessel. Neutralize Methanol or other specified solvent with KF potentiometrically.

Quickly transfer prescribed volume/weight of the substance to be examined to the titration vessel and start the titration with KF Potentiometrically.

When titration is complete, record the volume of K.F. used.

Calculate the water content of the solid sample with the help of given *Equation 3.17*:

$$Water\ content = \frac{Volume\ of\ KF\ used \times KF\ factor \times 100}{Weight\ of\ sample\ in\ g \times 1000} \quad (3.17)$$

Note:

1. When water content is determined using Autotitrator, end point is determined electrometrically.
2. Sample quantity: about 100 mg
3. Solvent media: Methanol

4. Results and Evaluation of the Validation Parameters

In this chapter, experimental work and obtained results will be discussed. Each section corresponds to each method transferred and the analyzed parameters. In section 4.1, drug substance identification and assay are tests to identify the analyte and to establish an exact result of the content of the analyte in the sample, respectively. In section 4.2, the first test of related substances guarantees the analysis of the content of all known and unknown impurities, except the impurity E, which in turn is evaluated in section 4.3 by the second method of related substances, for having the same retention time of the active substance. In section 4.4, a method to evaluate the stereochemical isomer of the substance is described, followed by sections 4.5 and 4.6, in which the content of acetic acid and residual solvents present in a sample are evaluated. To confirm the content of dimeglumine in a sample, a method by potentiometry is also transferred (section 4.7). Finally, in section 4.8, the results of the semi-micro determination of water content are presented.

4.2 Identification and Assay by HPLC

The parameters that were considered for the method transfer includes system suitability, specificity, linearity, and precision (system repeatability and method repeatability).

4.2.1 System Suitability

To evaluate the system suitability, API standard solution was injected six times. The results are shown in the *Table 4.1*.

Besides, a second API standard solution, at 0,3 mg/mL, was prepared and injected at three times, to determine the match factor which confirms if each solution was well prepared. For Assay test, the acceptance criteria of the match factor shall be within the range of 0.98-1.02.

Table 4.1 - System suitability results of API standard solution

Standard solution	Injection number	API Peak retention time (min)	API Peak Area (AU)	API peak tailing factor
1	1	29.37	3215346	1.83
	2	29.38	3218589	1.84
	3	29.41	3238065	1.83
	4	29.40	3221278	1.85
	5	29.39	3225383	1.83
	6	29.09	3248309	1.83
Average		**29.34**	**3227828**	**1.84**
SD		**0.12**	**12761.72**	**--**
% RSD		**0.42**	**0.40**	**--**
2	1	29.38	3027737	--
	2	29.37	3052705	--
	3	29.38	3047554	--
Match Factor			**0.997 (< 2.0)**	

The system proved to be suitable for API quantitation since %RSD for area of API peak is not more than 2,0.

The match factor determined complies with the acceptance criterion.

4.2.2 Specificity

The method is considered to be specific if it is capable of accurately quantifying the analyte with no interference from excipients or other substances present in the sample matrix.

A diluent solution was injected where the method baseline is visible, *Figure 4.1*. Also, an API standard solution was shown in *Figure 4.2* and a test solution in *Figure 4.3*. Each known impurity (Impurity A, Impurity B, Impurity C, Impurity D and Impurity F) solution was prepared individually at 15% of the API working concentration, *Figures 4.9-4.13* and a solution of all known impurities spiked with the API (Spiked Test solution) was also prepared, *Figure 4.17*. API and impurities retention times are summarized in *Table 4.2*.

As the chromatographic conditions are the same as in the chromatographic method Related Substances – Test 1 and as it is a test to verify whether the method is specific or not and due to the small amount of impurities available it has been decided that the specificity of the known impurities would be performed at the same time in two methods and therefore all the relative chromatograms will be present in the specificity section of Related Substances – Test 1.

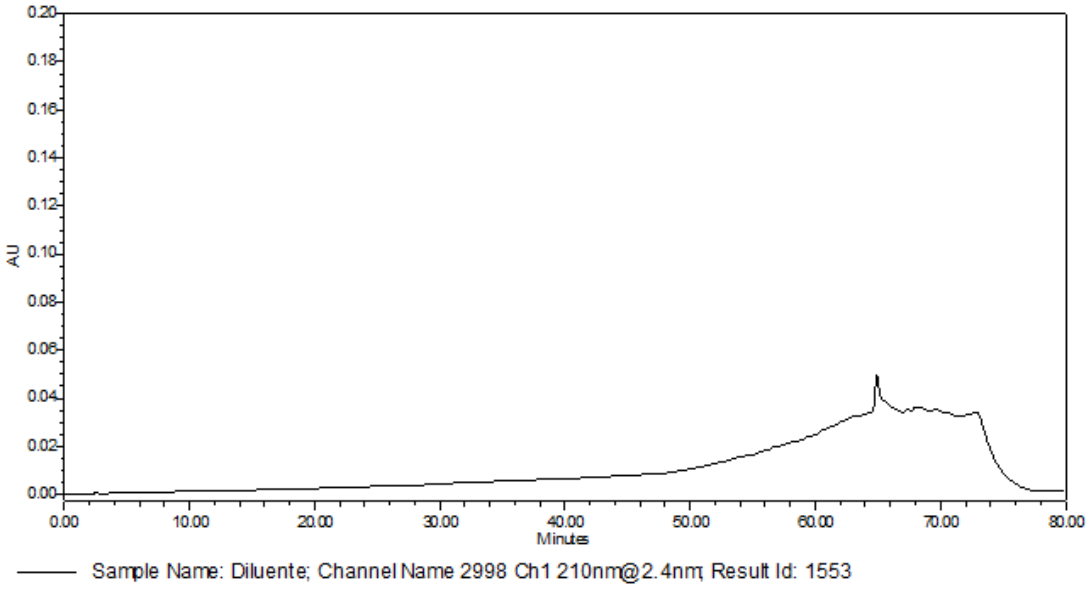


Figure 4.1 - Chromatogram of Blank: Water:Acetonitrile (70:30, v/v).

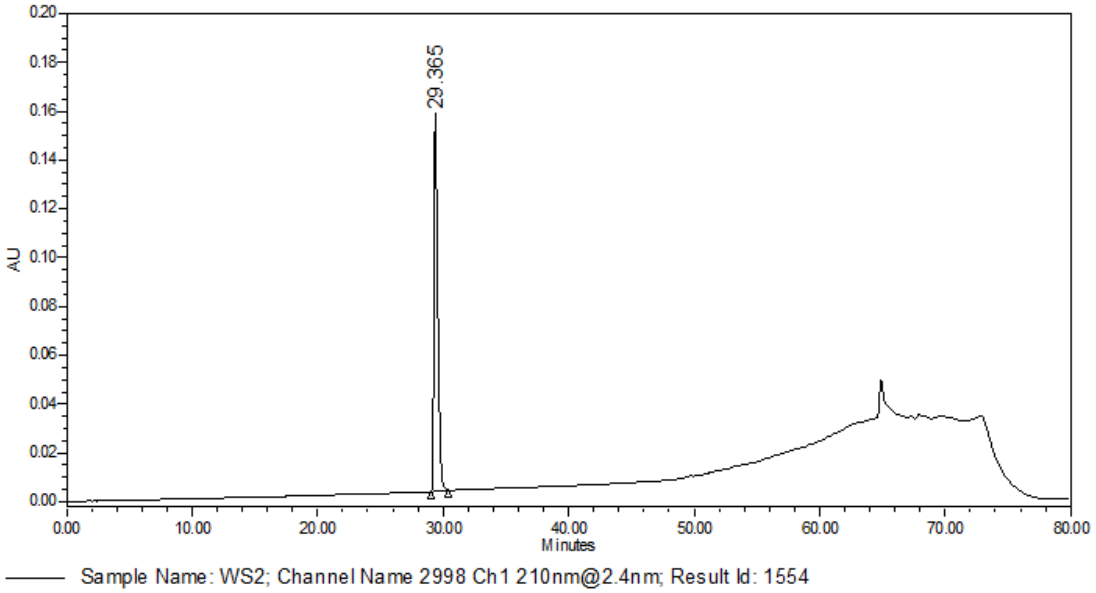


Figure 4.2 - Chromatogram of API standard solution, C_{API} = 0,3 mg/mL.

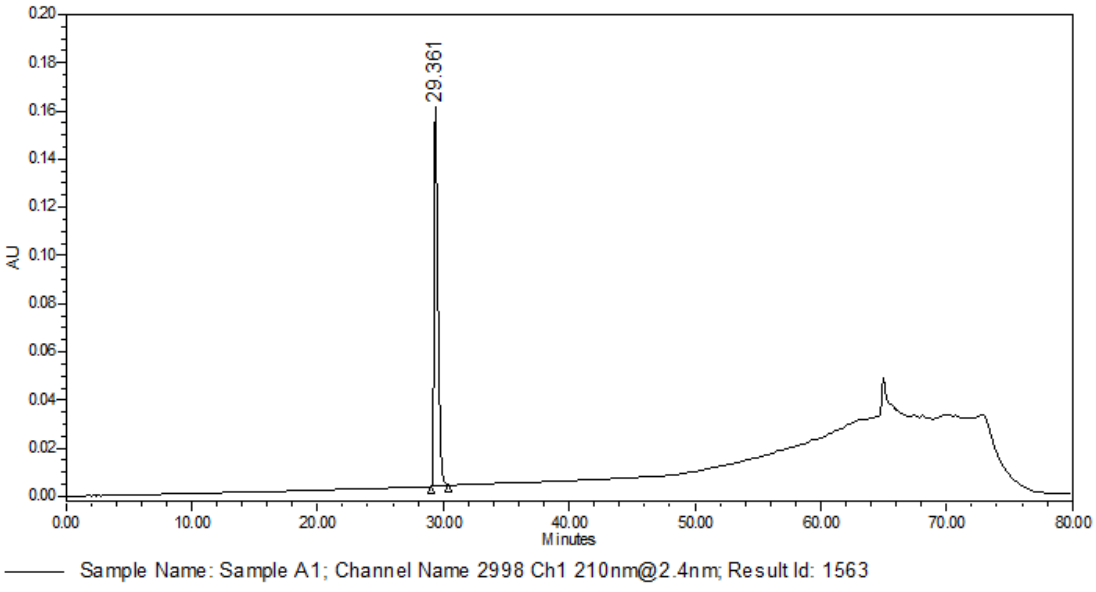


Figure 4.3 - Chromatogram of API test solution, C_{API} = 0,3 mg/mL.

Table 4.2 - Retention times of API and impurity peaks, in individual and spiked test solutions

Substance	Retention time (aprox) in minutes	
	Individual solution	**Spiked test solution**
API	31.45	31.14
Impurity A	54.16	54.15
Impurity B	40.06	40.16
Impurity C	51.71	51.79
Impurity D	34.51	34.53
Impurity F	49.91	49.97

The system met the specificity requirements.

In the chromatograms corresponding to Blank, Standard solution, Sample solution and Impurities solution it is observed that there is no peak interfering with API identification and quantitation.

4.2.3 Linearity

Linearity test studies the proportionality between analyte concentration and instrument response.

To evaluate the linearity of this method, several reference solutions were prepared at different concentrations, ranging from 50 % to 150 % of API concentration used in the sample preparation (0,2 mg/mL). The linearity test was done in triplicate.

The linearity results are present in *Table 4.3* and the regression plot in *Figure 4.4*.

Table 4.3 – API linearity results.

% Nominal Concentration	Concentration (µg/mL)	API Average Peak Area (AU)	Peak Area Response RSD (%)
50.8	152.3	1649282	0.15
76.1	228.4	2446007	0.27
101.5	304.6	3332497	0.27
126.9	380.7	4232193	0.11
152.3	456.8	4958832	0.09

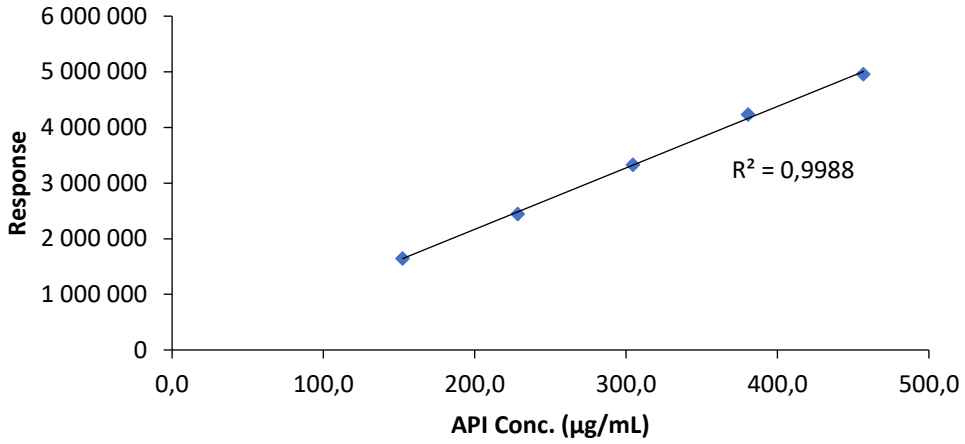


Figure 4.4 – Linear regression plot of API.

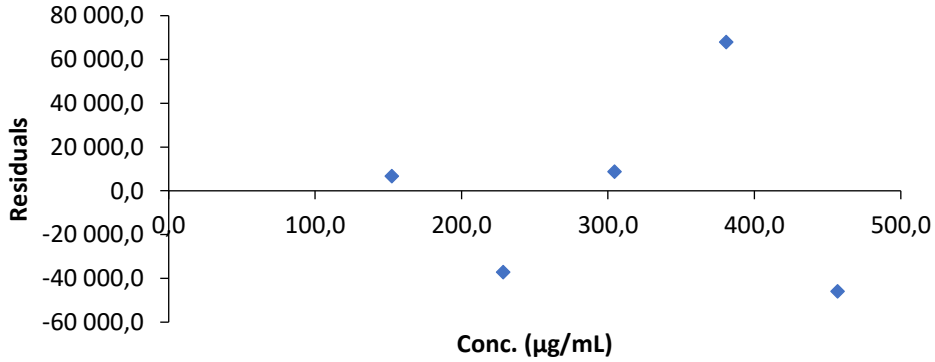


Figure 4.5 – Residual plot of regression analysis for API.

Table 4.4 - Parameters of the API linearity study

Parameter	Value	Result
Correlation coefficient	0.999	Complies
R Squared	0.999	Complies
Interception	-38351.87	---
Slope	11039.50	---
% y-intercept respect to response at 100% concentration	-1.15	--

Analysing the *Figure 4.5*, there is no systematic trend in residual plot. Besides that, all of parameters present in *Table 4.4* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is linear in the concentration range tested.

4.2.4 Accuracy

Accuracy can be inferred from linearity. In this case, a new straight line shall be calculated in which the calculated concentration is reported, by interpolation of the straight line obtained in the linearity study, as a function of the experimentally prepared concentration. Besides that, also recovery was calculated to demonstrate how close the results are from theoretical values. The results are present in *Table 4.5* and the regression plot in *Figure 4.6*.

Table 4.5 - Results of API accuracy test.

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
152.3	145,9	104,4
228.4	218,1	104,7
304.6	298,4	102,1
380.7	379,9	100,2
456.8	445,7	102,5

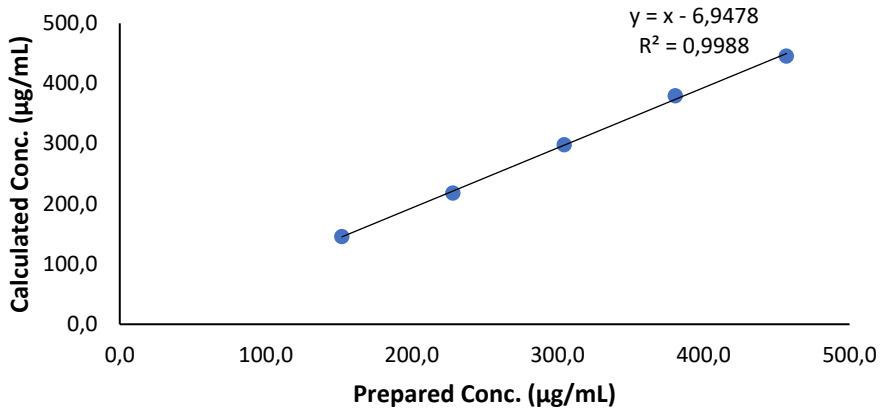


Figure 4.6 - Linear regression plot for accuracy test of API

Table 4.6 - Parameters of the API accuracy study

Parameter	Value	Result
Correlation coefficient	0,999	Complies
R Squared	0.999	Complies
Interception	-6,948	---
Slope	0,999	---

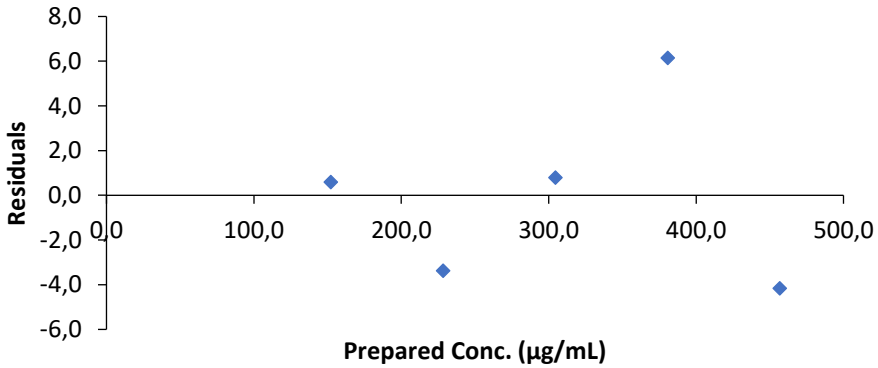


Figure 4.7 - Residual plot of regression analysis for accuracy test of API

Analysing the *Figure 4.7*, there is no systematic trend in residual plot. Besides that, all of parameters present in *Table 4.6* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is accurate in the concentration range tested.

4.2.5 Precision (System Repeatability)

This parameter tests the variability due only to the instrument under the chromatographic conditions indicated in the method. For this, the API standard solution, at 0,3 mg/mL was injected six times and the results are shown in *Table 4.7*.

Table 4.7 - System repeatability results

Injection number	API Peak Area (AU)
1	3215346
2	3218589
3	3238065
4	3221278
5	3225383
6	3248309
Average	**3227828**
SD	**12761.72**
RSD (%)	**0.40 (< 2.0%)**

As the relative standard deviation for the API peak area is less than 2,0 %, it is possible to confirm, that there are no significant differences between the 6 injections of the same assay standard solution.

The results obtained met the requirements and the test complied with the system suitability criteria.

4.2.6 Precision (Method Repeatability)

The method repeatability tests the variability of the method by means of a series of tests on the same homogeneous sample.

To evaluate the repeatability of the method, 6 independent sample solutions at 0,3 mg/mL were prepared and quantified against a standard solution. The results are shown in *Table 4.8*.

Table 4.8 – Method repeatability results

Injection number	API Assay (%)
1	101.0
2	100.9
3	101.4
4	101.5
5	98.0
6	101.0
Average	**100.7**
SD	**1.32**
RSD (%)	**1.31 (< 2.0 %)**

There are no significant differences among the values obtained for the API assay results as the relative standard deviation obtained is not more than 2,0 %.

The test complied with the acceptance criteria and the results obtained met the requirements for method repeatability.

4.3 Related Substances by HPLC – Test 1

The method transfer included the following parameters: system suitability, specificity, confirmation of quantitation limits, linearity and precision (system repeatability and method repeatability).

4.3.1 System Suitability

To evaluate the system suitability, Reference solution (c) with API at 15.0 µg/mL and Impurity C at 15.0 µg/mL, was injected six times. For Related Substances, the values of match factor shall be within the range of 0.95-1.05. The results are shown in *Table 4.9*.

Table 4.9 - System suitability results of Reference solution (c)

Standard solution	Injection number	API Peak retention time (min)	API Peak Area (AU)	Imp. C Peak retention time (min)	Imp. C Peak Area (AU)
1	1	31.97	161694	51.78	310771
	2	31.99	162220	51.79	310476
	3	31.99	162111	51.79	311211
	4	31.92	162290	51.75	310870
	5	31.92	161382	51.74	311761
	6	31.89	162162	51.72	311433
Average		**31.95**	**161977**	**51.76**	**311087**
SD		**0.04**	**358.70**	**0.03**	**471.10**
RSD (%)		**0.14**	**0.22**	**0.06**	**0.15**
2	1	31.88	163468	51.70	313420
	2	31.87	162402	51.69	313524
	3	31.87	164760	51.70	313014
Match factor		**1.011**		**1.020**	

The system suitability complies since the relative standard deviation for Impurity C and API peak determined from six replicate injections of reference solution (c) is less than 5.0%. In reference solution (h), resolution between API and Impurity D peaks is 3.87 (≥ 2.5).

4.3.2 Specificity

The method is considered to be specific if it is capable of accurately quantify the analyte with no interference from impurities or other substances present in the sample matrix.

A diluent solution was injected where the method baseline is visible, *Figure 4.8*. The reference solutions (h) and (c) are shown in *Figures 4.14* and *4.15*, respectively, and the test solution in *Figure 4.16*. Each known impurity (Impurity A, Impurity B, Impurity C, Impurity D and Impurity F) and API solution were prepared individually at specification limit concentration*, Figure 4.9-4.13* and a solution of all known impurities spiked with the API (Spiked test solution) was also prepared, *Figure 4.17*. The API and impurities retention times are summarized in *Table 4.10*.

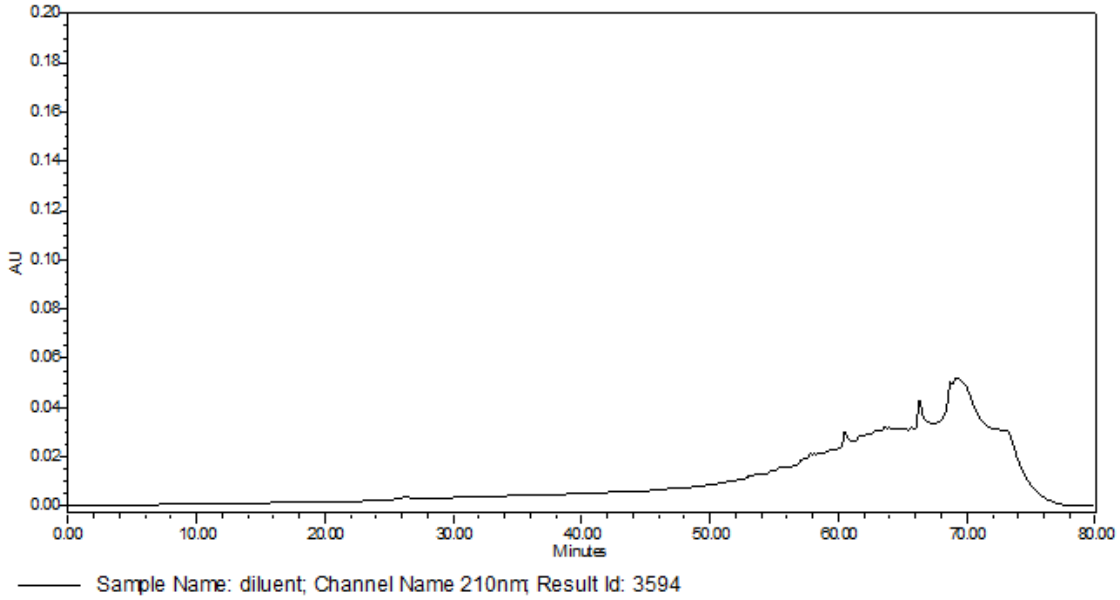
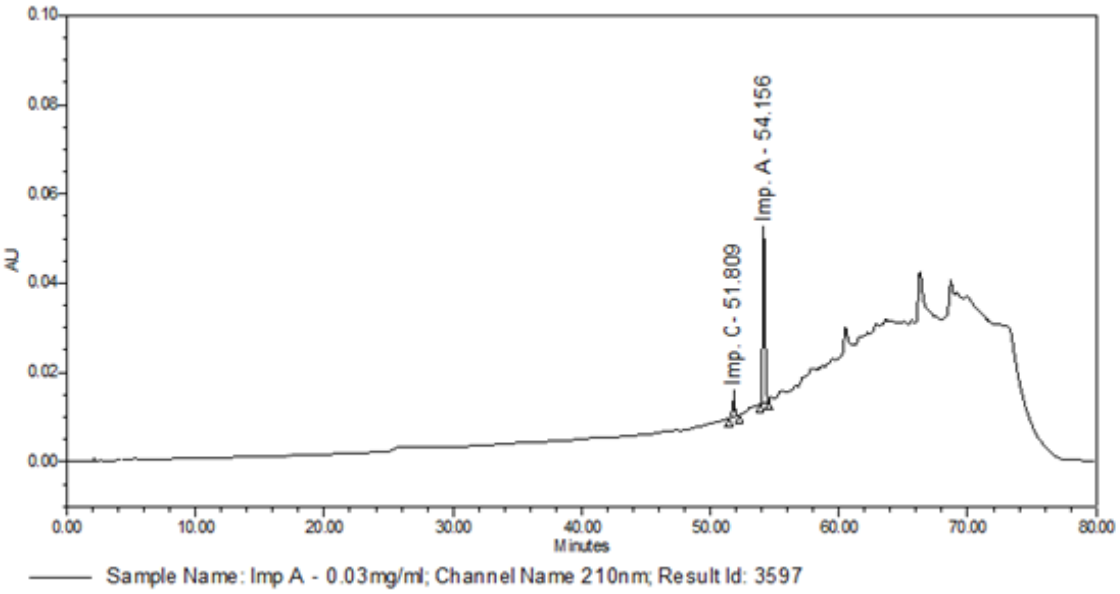


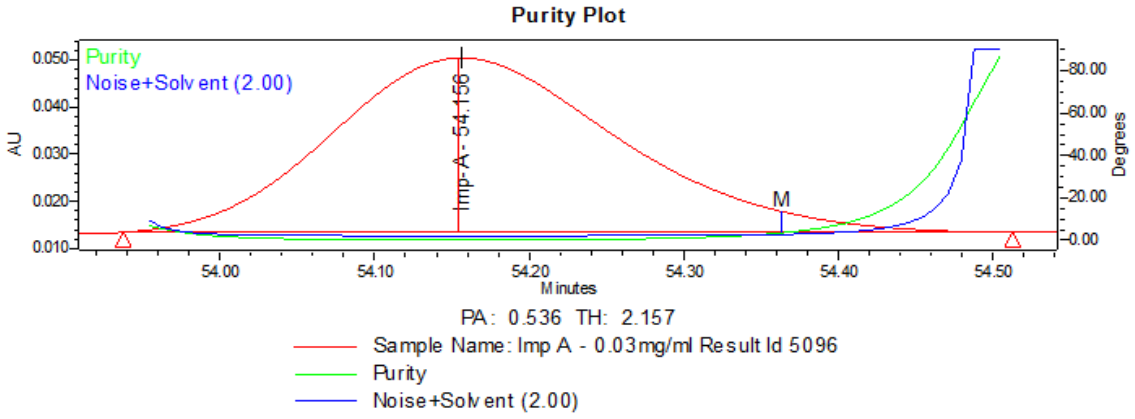
Figure 4.8 - Chromatogram of diluent (Water:Acetonitrile (70:30, v/v)).

All these solutions were analysed using the PDA detector as per the HPLC method described in the protocol. Besides that, it was also analysed the peak purity for impurities A, B, C, D and F. This test determines whether a peak is spectrally (not chemically) pure, that is, whether it represents a single compounds or multiple compounds. When the purity angle (PA) exceeds the purity threshold (PT), a detectable impurity is present within the peak. All impurities demonstrate to be purities and the results are shown in *Figures 4.9-4.13, Subfigure B*. Also, a match plot shows the peak spectrum with all possible wavelengths and calculate the maximum wavelength that the impurities absorb, *Figures 4.9-4.13, Subfigure C*.

A



B



C

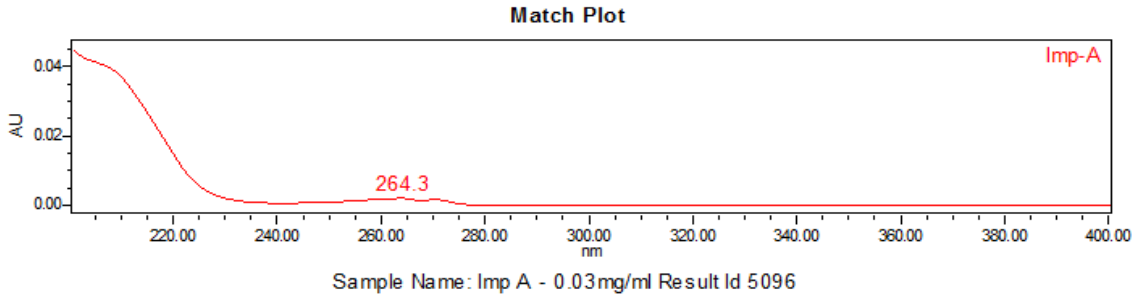


Figure 4.9 – A: Chromatogram of Impurity A solution, 30.0 µg/mL; B: Purity Plot of Impurity A (Purity Angle < Purity Threshold) ; C: Match Plot of Impurity A.

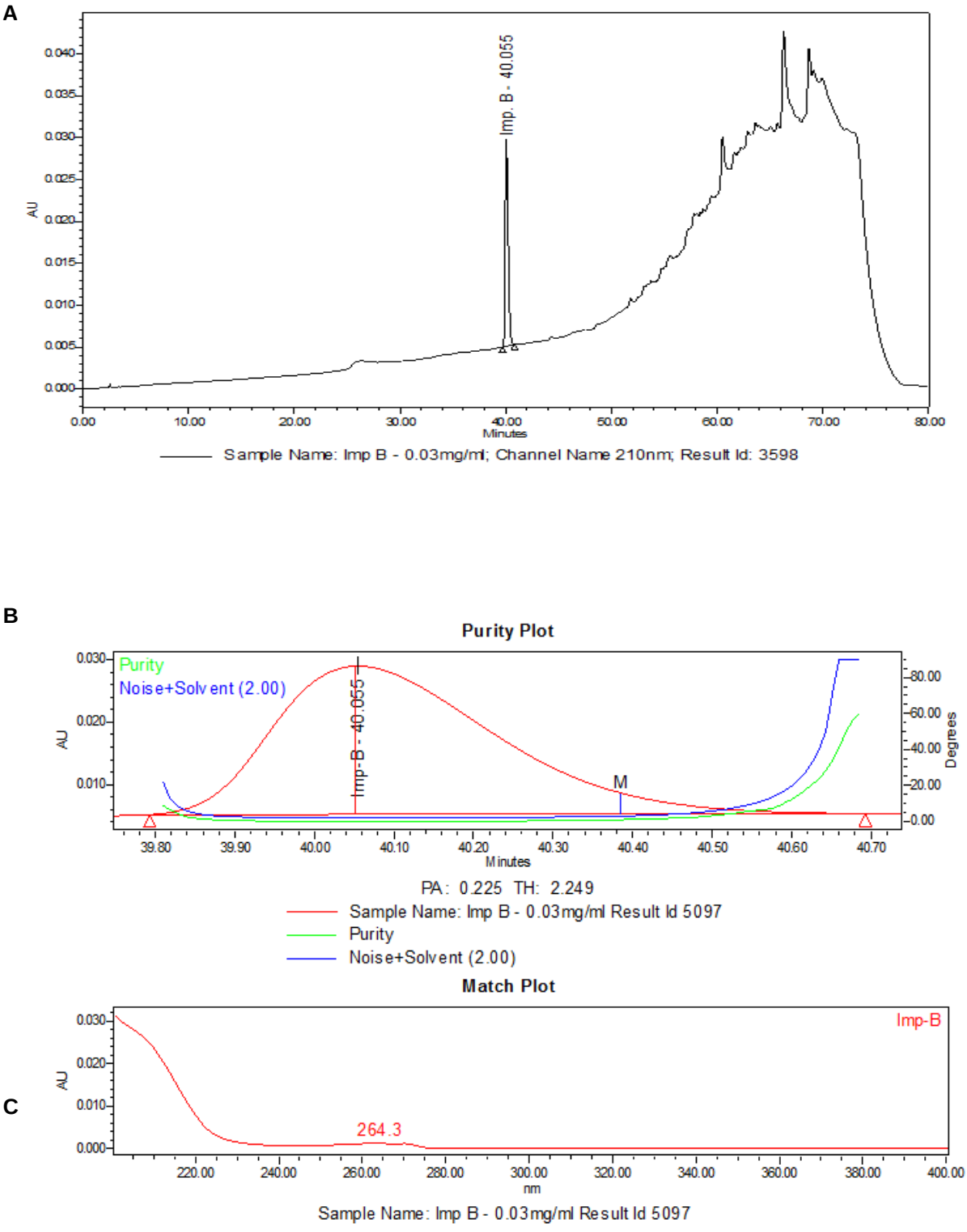


Figure 4.10 – A: Chromatogram of Impurity B solution, 30.0 µg/mL; B: Purity Plot of Impurity B (Purity Angle < Purity Threshold) ; C: Match Plot of Impurity B

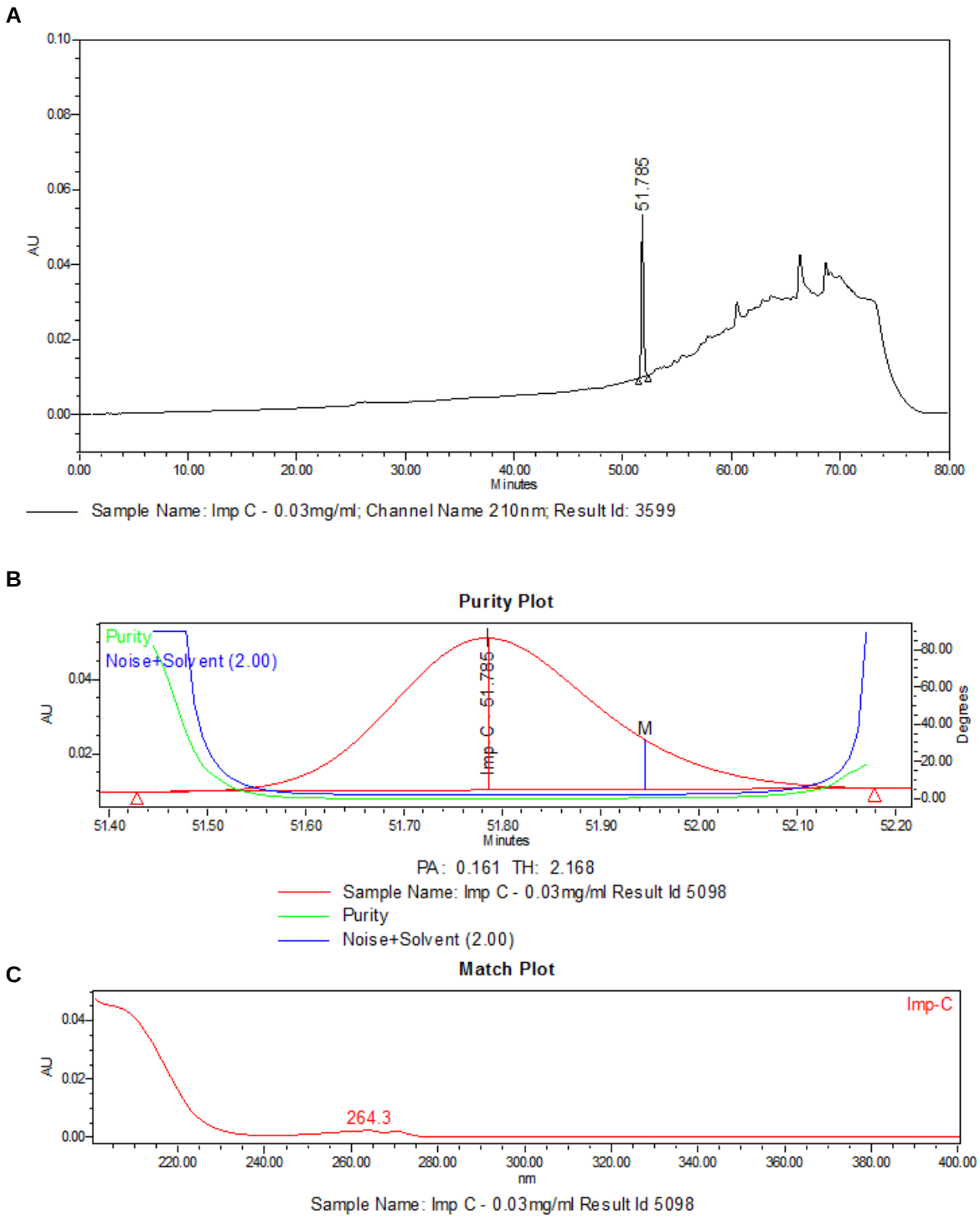


Figure 4.11 - A: Chromatogram of Impurity C solution, 30.0 µg/mL; B: Purity Plot of Impurity C (Purity Angle < Purity Threshold) ; C: Match Plot of Impurity C.

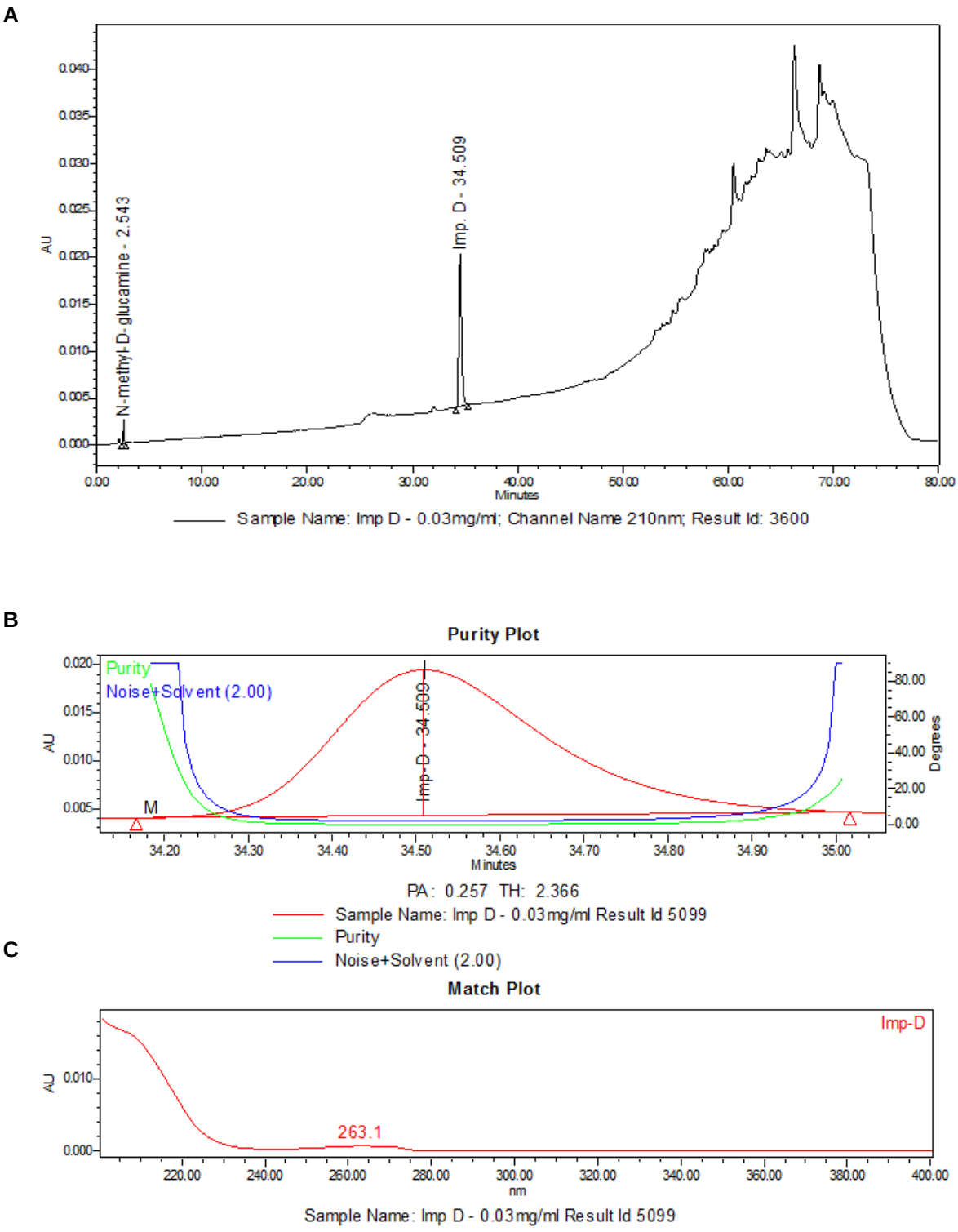
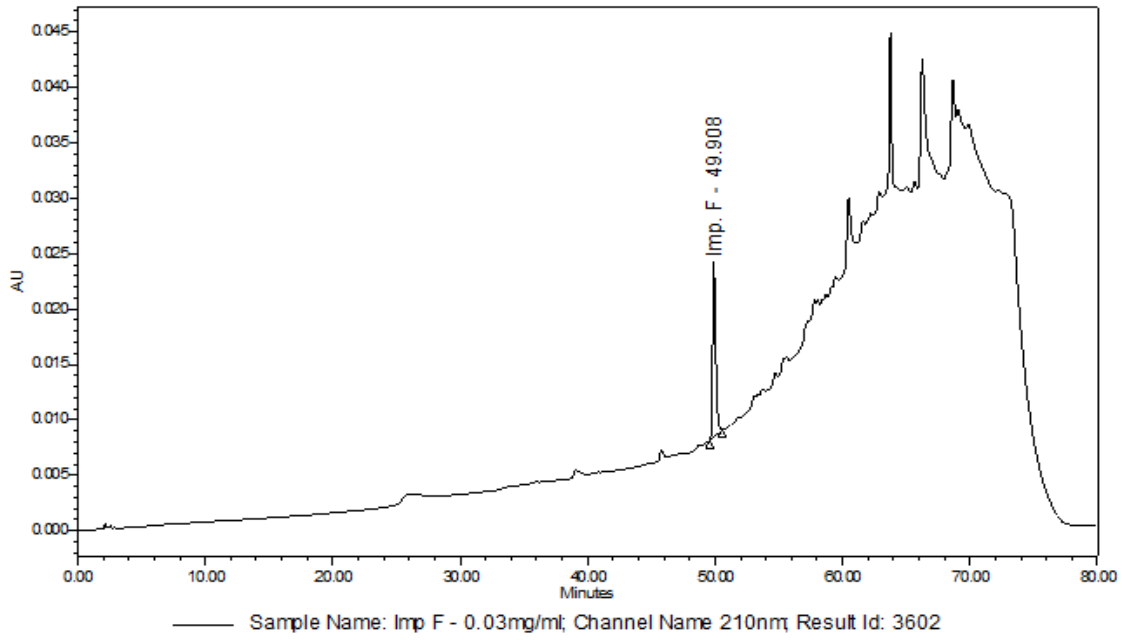
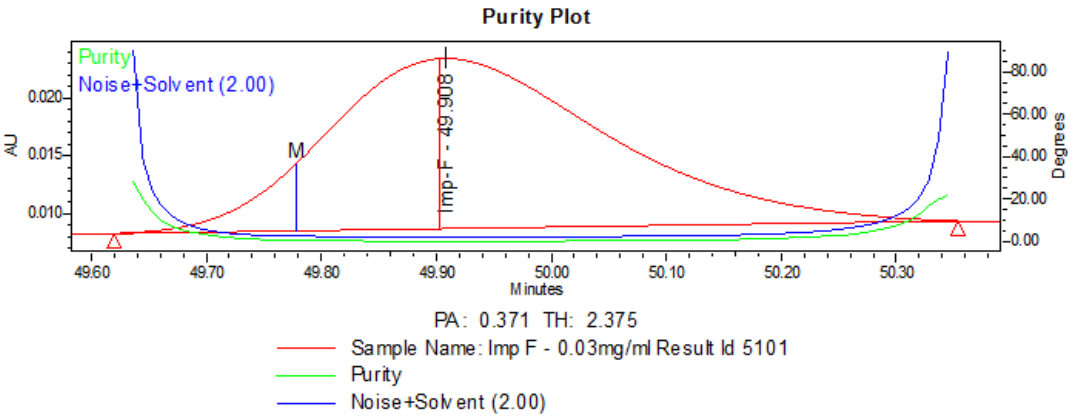


Figure 4.12 - A: Chromatogram of Impurity D solution, 30.0 µg/mL; B: Purity Plot of Impurity D (Purity Angle < Purity Threshold) ; C: Match Plot of Impurity D.

A



B



C

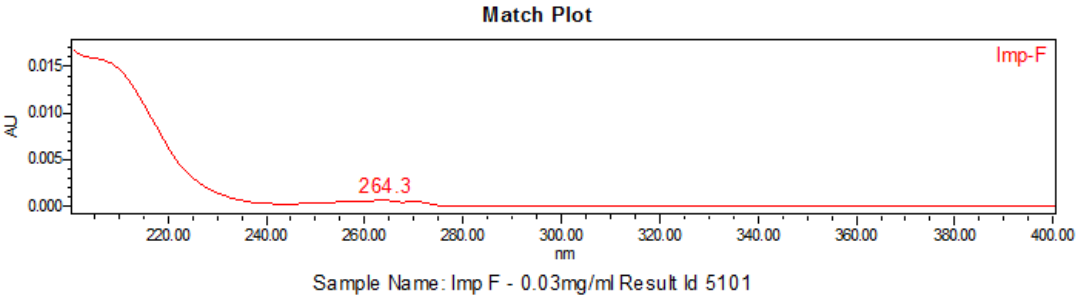


Figure 4.13 - A: Chromatogram of Impurity F solution, 30.0 µg/mL; B: Purity Plot of Impurity F (Purity Angle < Purity Threshold) ; C: Match Plot of Impurity F.

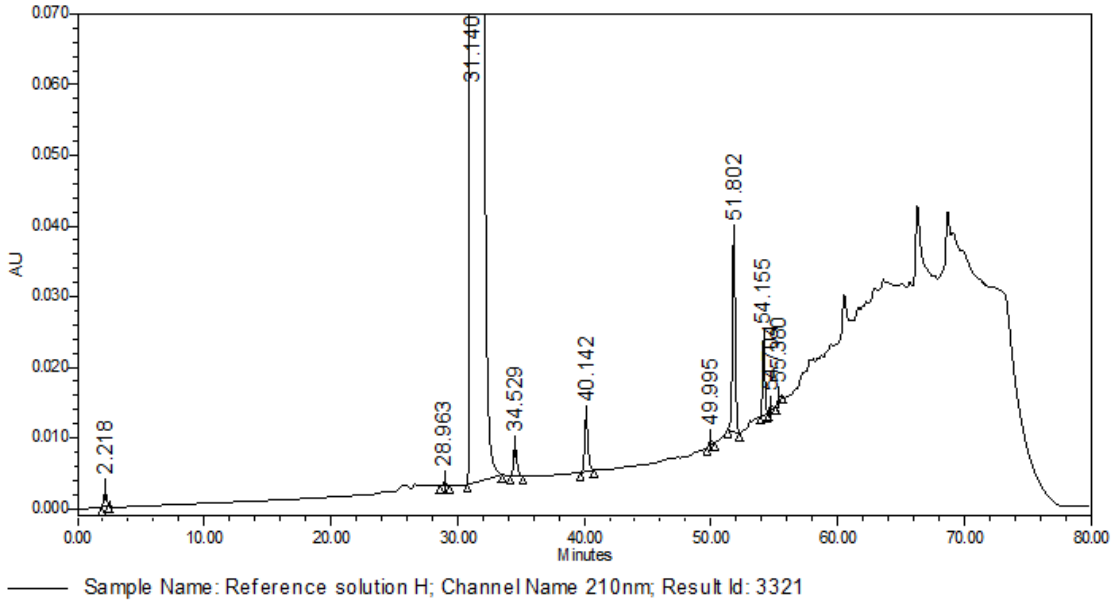


Figure 4.14 - Chromatogram of Reference solution (h), API (31.140 min) at 3 mg/mL and Impurities D (34.529 min), B (40.142 min), C (51.802 min) and A (54.155 min) at 0.009 mg/mL

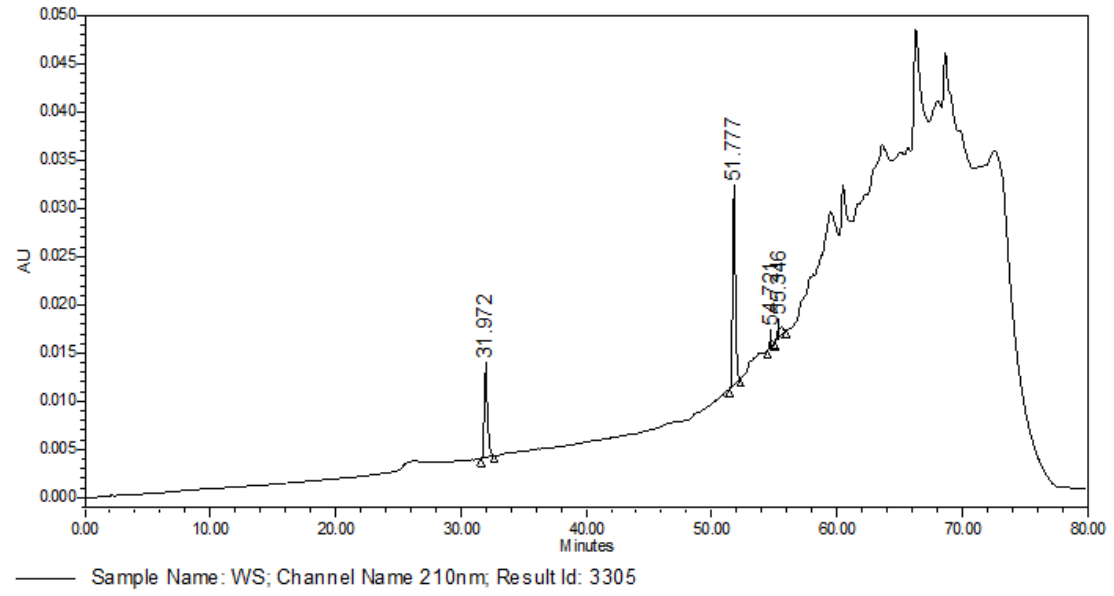


Figure 4.15 - Chromatogram of Reference solution (c), API (31.97 min) at 3.0 mg/mL and Impurity C (51.78 min) at 4.5 µg/mL

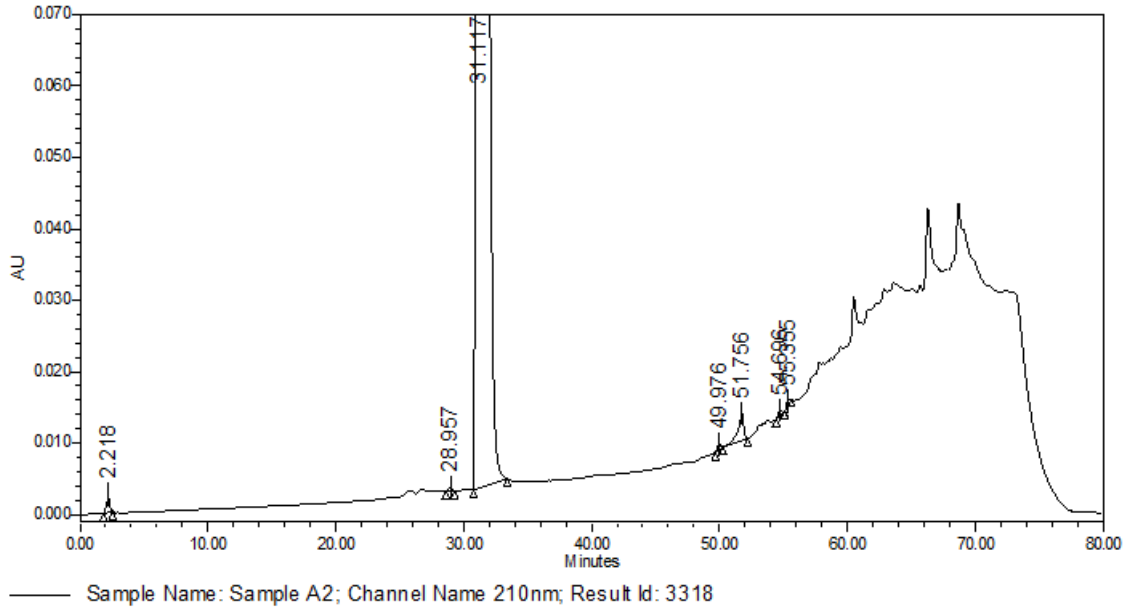


Figure 4.17 **-** Chromatogram of Test solution, 3.0 mg/mL

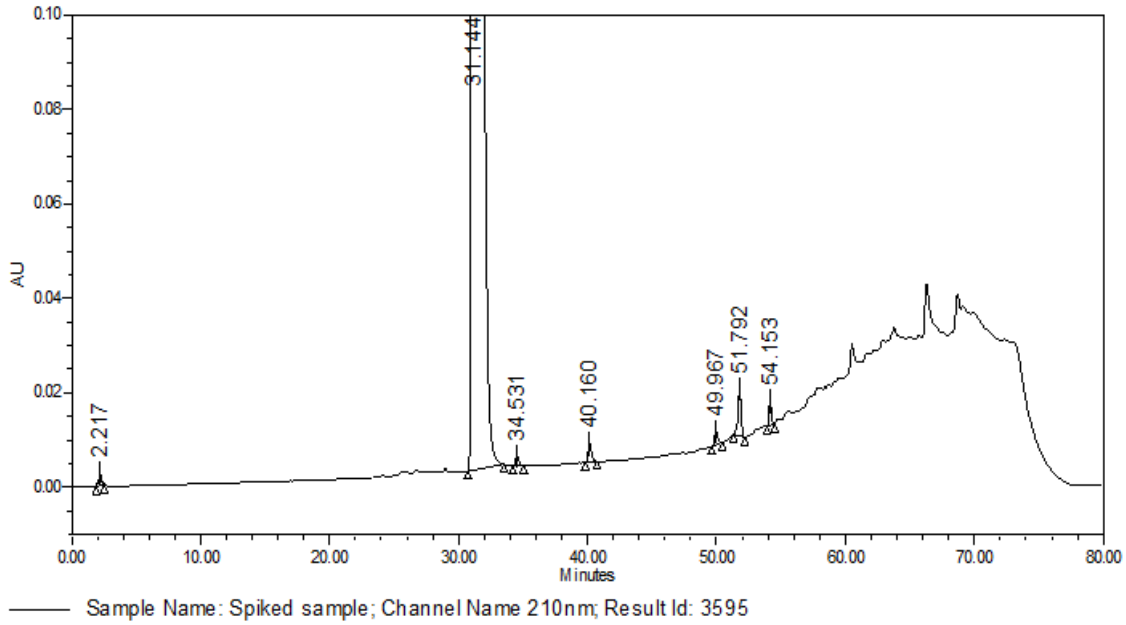


Figure 4.16 - Chromatogram of Spiked Test solution, API at 3.0 mg/mL and Impurities A (54.15 min), B (40.16 min), C (51.79 min), D (34.53 min) and F (49.97 min) at 0.0045 µg/mL.

Table 4.10 - Retention times of API and impurity peaks, in individual and spiked sample solutions

Substance	Retention time (aprox) in minutes	
	Individual solution	**Spiked test solution**
API	31.45	31.14
Impurity A	54.16 51.81 (Imp. C)	54.15
Impurity B	40.06	40.16
Impurity C	51.71	51.79
Impurity D	34.51 2.54 (N-methyl D-glucamine)	34.53
Impurity F	49.91	49.97

The system suitability complies, and the method proved to be specific since there is any interfering peak at the retention time of Impurities A, B, C, D and F and API due to the blank solution.

In *Figure 4.9* it is visible the presence of impurity C in impurity A chromatogram which could be also a sign of degradation of impurity A. However, as impurity A is only a process impurity, some contamination may have occurred. In addiction in chromatogram of impurity D (*Figure 4.12*), N-methyl D-glucamine peak appear due to its chemical structure (*Table 2.4*).

The elution order and the relative retention time obtained from individual solution and spiked sample solution present in *Table 4.10* are comparable and the peak purity comply for Impurities A, B, C, D, F and API obtained from individual solution.

4.3.3 Quantitation Limit

The solution should contain the minimum analyte (active substance and impurities) concentration quantified by the chromatographic system, which should correspond to the lower concentration in the low range linearity.

The QL concentrations shown in the *Table 4.11* were confirmed by injecting six replicates of individual solutions of each known impurity and API at the determined concentration.

Table 4.11 – Limit of quantitation of API and impurities.

Substance	QL (µg/mL)	QL (%)	Average Area	RSD (%)
API (unknown imp.)	0.6	0.02	5693	3.81
Impurity A	0.5	0.02	9886	5.79
Impurity B	0.8	0.03	7390	5.28
Impurity C	0.6	0.02	12804	1.72
Impurity D	0.6	0.02	6699	4.46
Impurity F	0.2	0.01	5623	4.98

The system met the requirements. The RSD of the peak areas of API and its impurities are less than 10 %.

4.3.4 Low Range Linearity

Linearity test study the proportionality between analyte concentration and instrument response.

To evaluate the linearity of this method, API standard solutions were prepared at a minimum of five different concentrations, ranging from QL to 150 % of specification limit (3.0 µg/mL). Linearity test was performed in triplicate.

The following figure represents the linear plot of API. Linear regression analysis confirmed the acceptability of the HPLC method for quantitative determination of API impurities over the concentration range of ≈ 0.6 – 4.7 µg/mL, corresponding to ≈ 20 – 155% of 3.0 µg/mL (0,10 % of the nominal concentration in API, 3.0 mg /mL). Results of the linearity study for API are shown in the *Table 4.12* and the regression plot in *Figure 4.18*.

Table 4.12: Results of API linearity study.

Level	Concentration in % (relative to test)	API Concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	0.02	0.6	5899	6.00
2	0.05	1.6	15093	2.60
3	0.08	2.3	22937	2.95
4	0.10	3.1	30816	1.69
5	0.13	3.9	38833	1.56
6	0.16	4.7	46272	0.91

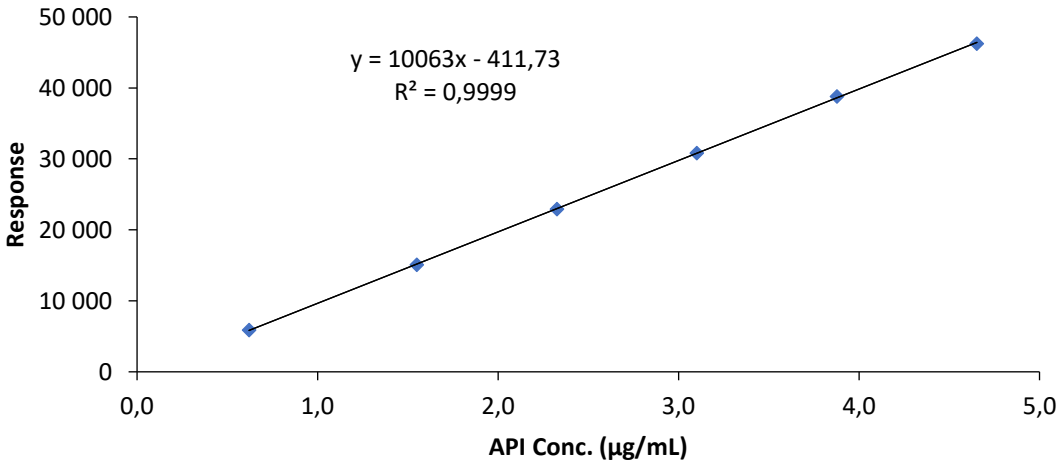


Figure 4.18 – Linear regression plot of API.

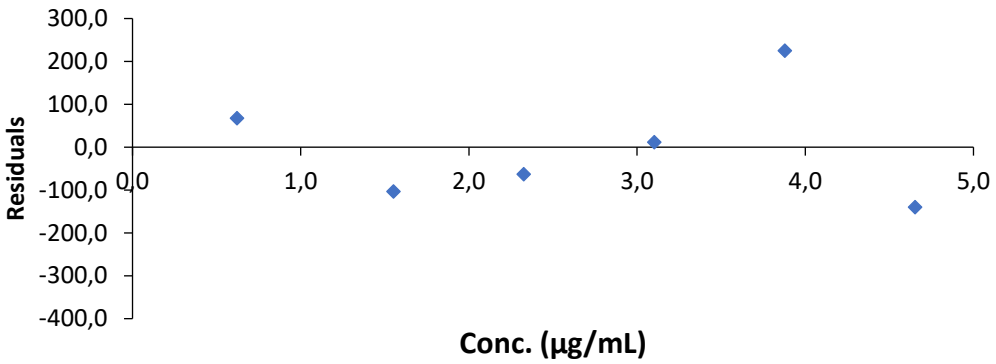


Figure 4.19 – Residual plot of low range linearity of API.

Table 4.13 - Parameters of the API linearity study

Parameter	Value	Result
Correlation coefficient	1.000	Complies
R Squared	1.000	---
Interception	-411.73	---
Slope	10062.62	---
% y-intercept respect to response at 100% concentration	1.34	--
10% of 100% concentration response	3081.6	Residuals are within 10% value

There is no systematic trend in residuals as shown in *Figure 4.19* and Residuals are within 10% of 100% concentration response. Besides that, all of parameters present in *Table 4.13* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is linear in the concentration range tested.

4.3.5 Accuracy

Accuracy can be inferred from linearity. In this case, a new straight line shall be calculated in which the calculated concentration is reported, by interpolation of the straight line obtained in the linearity study, as a function of the experimentally prepared concentration.

Besides that, also recovery was calculated to demonstrate how close the results are from theoretical values. The results are present in *Table 4.14* and the regression plot in *Figure 4.20*.

Table 4.14 - Results of API accuracy test.

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
0.62	0,63	98,9
1.55	1,54	100,7
2.33	2,32	100,3
3.10	3,10	100,0
3.88	3,90	99,4
4.65	4,64	100,3

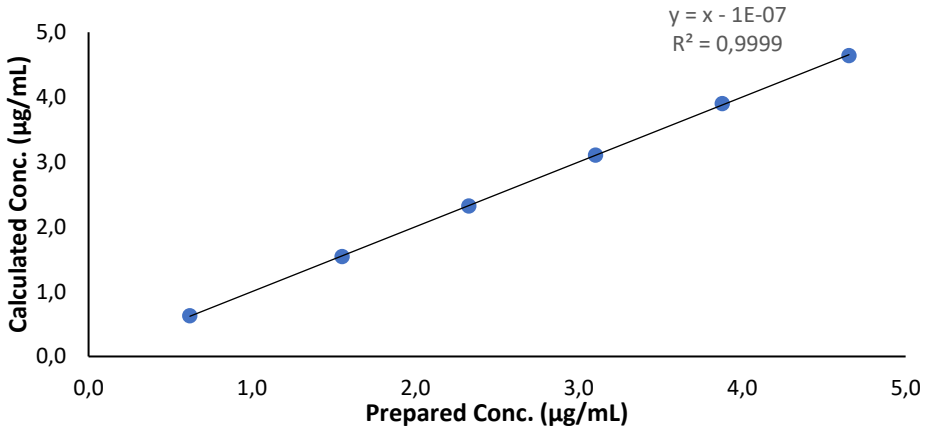


Figure 4.20 **-** Linear regression plot for accuracy test of API

Table 4.15 - Parameters of the API accuracy study

Parameter	Value	Result
Correlation coefficient	0,999	Complies
R Squared	0.999	Complies
Interception	-1,406E-07	---
Slope	0,999	---

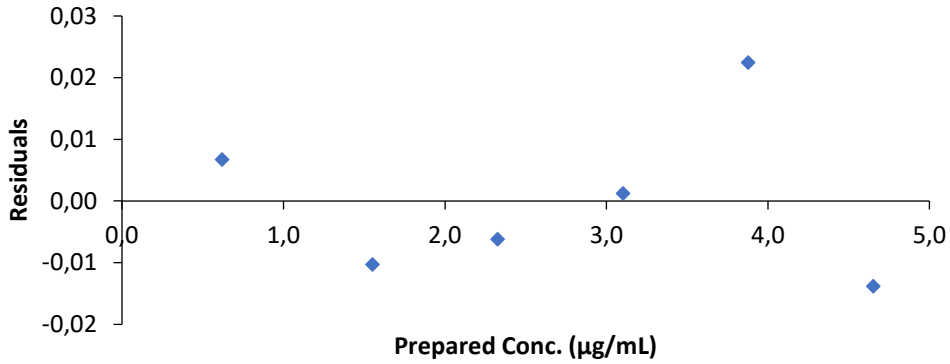


Figure 4.21 - Residual plot of regression analysis for accuracy test of API.

Analysing the *Figure 4.21*, there is no systematic trend in residual plot. Besides that, all of parameters present in *Table 4.15* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is accurate in the concentration range tested.

4.3.6 Precision (System Repeatability)

This parameter tests the variability due only to the instrument under the chromatographic conditions indicated in the test procedure.

To evaluate the system repeatability, a reference solution at specification limits, API at 3.0 µg/mL and Impurities A, B, C, D and F at 4.5 µg/mL, was injected six times. The results are shown in the *Table 4.16*.

Table 4.16 – System repeatability of API at 0.003 mg/mL and impurities A, B, C, D and F at 4.5 µg/mL.

Identification		1	2	3	4	5	6	Average	RSD (%)
API	**R_t**	32.01	32.03	32.04	32.07	32.11	32.12	32.06	0.14
	Area	27971	26850	27442	27200	26807	26968	**27206**	**1.63**
Imp. A	**R_t**	54.13	54.13	54.15	54.17	54.20	54.21	54.166	0.07
	Area	70493	70776	70378	70132	70073	70255	**70351**	**0.37**
Imp. B	**R_t**	40.14	40.15	40.17	40.20	40.24	40.26	40.19	0.12
	Area	68032	67753	67368	67713	64082	64187	**66523**	**2.80**
Imp. C	**R_t**	51.78	51.79	51.80	51.83	51.86	51.87	51.82	0.08
	Area	102741	102508	101895	101980	102057	101527	**102118**	**0.43**
Imp. D	**R_t**	34.55	34.55	34.57	34.60	34.64	34.66	34.59	0.13
	Area	38630	39257	38342	38537	37370	37216	**38225**	**2.06**
Imp. F	**R_t**	49.96	49.97	49.98	50.00	50.04	50.05	49.99	0.08
	Area	36438	36014	36280	35921	36496	36497	**36274**	**0.70**

The system met the system repeatability requirements. % RSD of peak area responses for each component is not more than 5,0.

4.3.7 Precision (Method Repeatability)

For evaluating the method repeatability, six individual API test solutions diluted at the specification limit concentration (3.0 µg/mL) were prepared and analysed against reference solution. The results are shown in *Table 4.17*.

Table 4.17 – Method repeatability results of API, 0.003 mg/mL.

Substance	API (%)
% Recovery	96.9
	100.2
	96.8
	96.1
	97.8
	95.5
Average ± RSD	**97.2 % ± 1.71%**
C. I. *95%*	**95.3% – 99.2%**

The RSD value obtained is lower than 10,0% and the percentage of recovery obtained is within 90% and 110%. The system met the method repeatability requirements.

4.4 Related Substances by HPLC – Test 2 (Content of Impurity E)

The method transfer included the following parameters: system suitability, specificity, confirmation of quantitation limit, linearity and precision (system repeatability and method repeatability).

4.4.1 System Suitability

To evaluate the system suitability, reference solution (b) with Impurity E at 10.0 µg/mL was injected six times. For Related Substances, the results of match factor shall be within the range of 0.95-1.05. The system suitability results are presented in the following *Table 4.18*.

Table 4.18 - System suitability results of Reference solution (b)

Standard solution	Injection number	Impurity E Peak retention time (min)	Impurity E Peak Area (AU)
1	1	25.29	84288
	2	25.32	84089
	3	25.36	81383
	4	25.39	75906
	5	25.43	80085
	6	25.46	76545
Average		**25.37**	**80383**
SD		**0.07**	**3601.28**
RSD (%)		**0.26**	**4.48**
2	1	25.50	74613
	2	25.53	79486
	3	25.57	78888
Match factor		**0.985**	

The system suitability complies since the relative standard deviation for Impurity E determined from six replicate injections of reference solution (b) is less than 5.0%. In reference solution (d), resolution between API and Impurity D peaks is 2.47 (≥ 2.0).

4.4.2 Specificity

The method is considered to be specific if it is capable of accurately quantify the analyte with no interference from impurities or other substances present in the sample matrix. A solution of diluent is shown in *Figure 4.22* and the reference solution (d), which API concentration is 2.0 mg/mL and Impurity E at 6.0 µg/mL is presented in *Figure 4.23*. Also, Impurity E solution was prepared at specification limit concentration at 10.0 µg/mL, *Figure 4.24*, and solutions of API (Test solution at 2

mg/mL and spiked test solution at 2.0 mg/mL spiked with impurity E at 3.0 µg/mL) was also prepared, *Figures 4.25 and 4.26*. The API and impurity E retention times are summarized in *Table 4.19.*

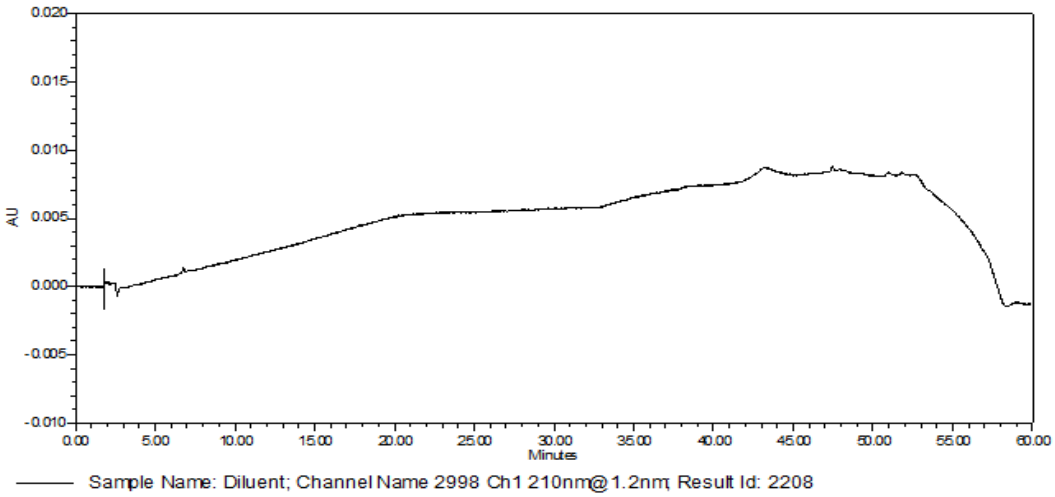


Figure 4.22 - Chromatogram of diluent (Buffer:Acetonitrile (50:50, v/v)).

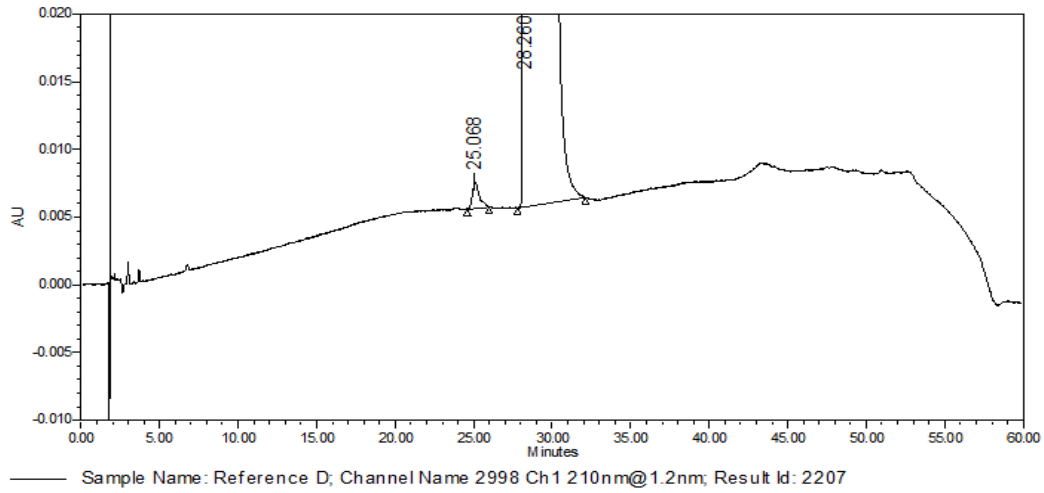


Figure 4.23 - Chromatogram of Reference solution (d), API at 2.0 mg/mL and Impurity E at 6.0 µg/mL

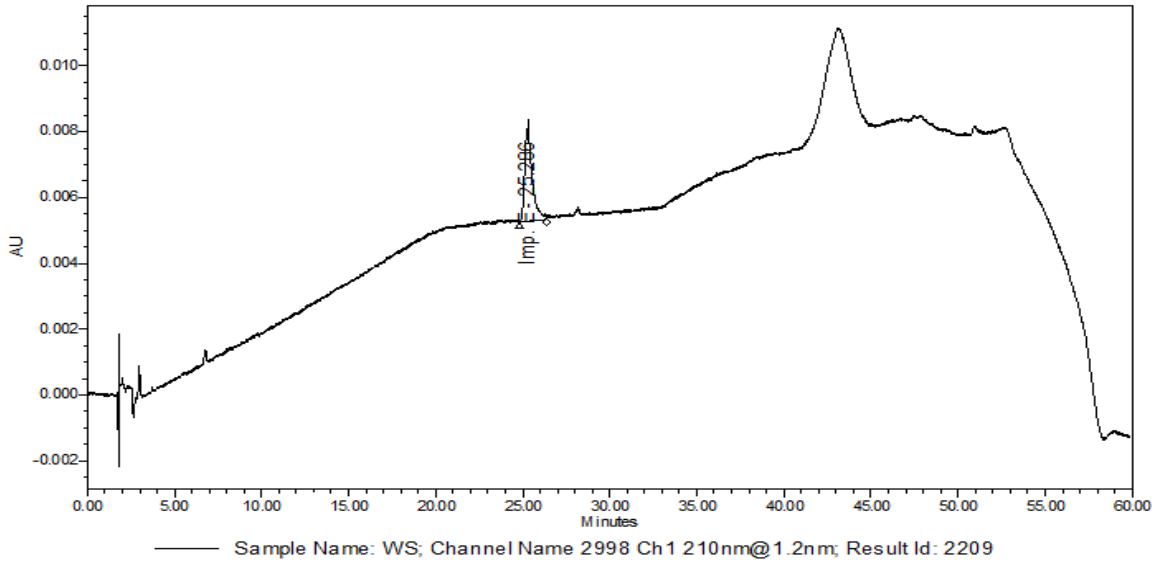


Figure 4.24 - Chromatogram of Reference solution (b), Impurity E at 10.0 µg/mL

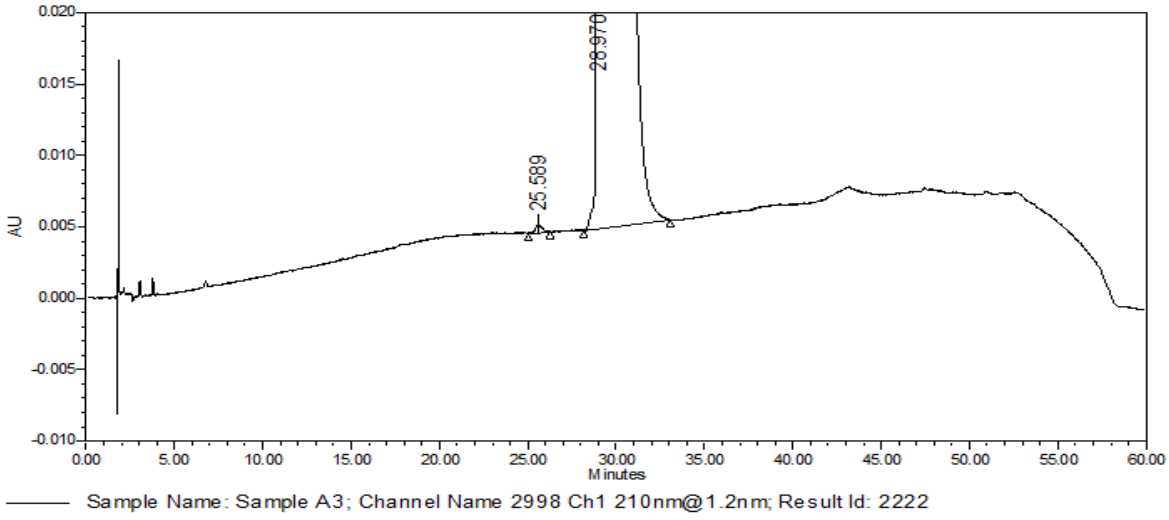


Figure 4.25 - Chromatogram of Test solution, 2.0 mg/mL

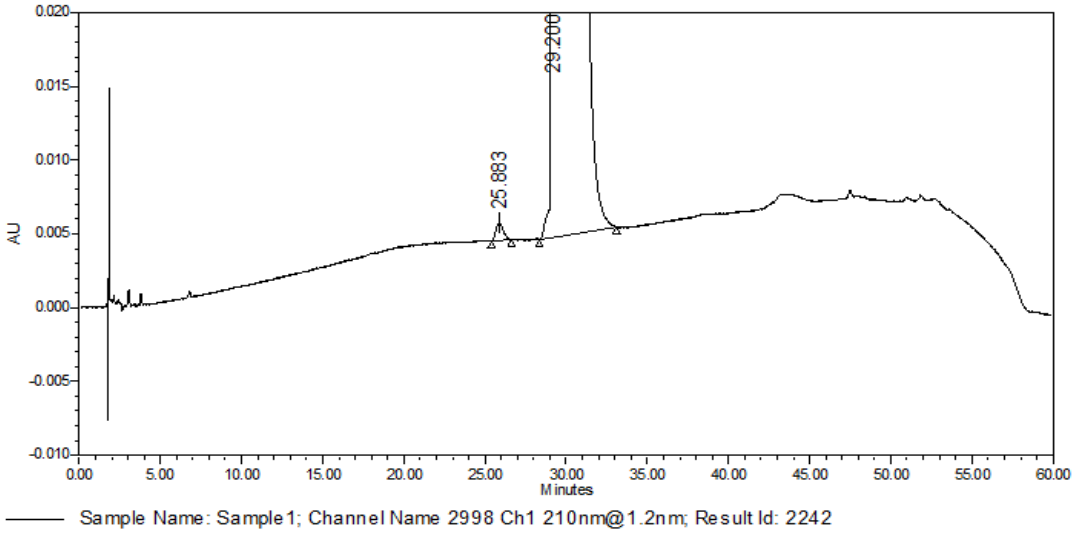


Figure 4.26 - Chromatogram of Spiked Test solution, API at 2.0 mg/mL and Impurity E at 3.0 µg/mL

Table 4.19 - Retention times of API and impurity peaks, in individual and spiked sample solutions

Substance	Retention time (aprox) in minutes	
	Individual solution	Spiked test solution
API	28.97	29.20
Impurity E	25.88	25.88

The system suitability complies, and the method proved to be specific since there is any interfering peak at the retention time of Impurity E due to the blank solution.

4.4.3 Quantitation Limit

The solution should contain the minimum analyte (active substance and impurities) concentration quantified by the chromatographic system, which should correspond to the lower concentration in the low range linearity.

The QL concentration shown in the *Table 4.20* were confirmed by injecting six replicates of individual solution of Impurity E, at the determined concentration (0.2 µg/mL). However, no peak was observed in the chromatogram. For that reason, it was decided to confirm the Reporting threshold (RT) concentration instead.

A solution of Impurity E at 0.07% relative to the nominal concentration (2.0 µg/mL) was prepared and injected six consecutive times.

Table 4.20 – Reporting Threshold of Impurity E.

Substance	RT (µg/mL)	RT (%)	Average Area	RSD (%)
Impurity E	1.4	0.07	8745	6.33

The system met the requirements. The RSD of the peak areas of Impurity E, at the Reporting threshold are less than 10%.

4.4.4 Low Range Linearity

Linearity test study the proportionality between analyte concentration and instrument response. To evaluate the linearity of this method, Impurity E standard solutions were prepared at a minimum of five different concentrations, ranging from RT to 150 % of specification limit (3,0 µg/mL). Linearity test was done in triplicate.

However, it should be noted that impurity E degrades easily with time. A second peak is observed in each chromatogram and its area increases with time. Because of this degradation, the validation of some parameters was quite difficult.

The following figure represents the linear plot of Impurity E. Linear regression analysis confirmed the acceptability of the HPLC method for quantitative determination of Impurity E over the concentration range of ≈ 1.4 – 4.2 µg/mL, corresponding to ≈ 46 – 138.9% of 3 µg/mL (0,15 % of the

nominal concentration in API 2 mg /mL). Results of the linearity study for Impurity E are shown in the *Table 4.21* and the regression plot in *Figure 4.27*.

Table 4.21 - Results of Impurity E linearity study.

Level	Concentration in % (relative to test)	Impurity E concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	0.07	1.4	8539	7.60
2	0.10	2.1	13690	3.24
3	0.14	2.8	20649	8.48
4	0.17	3.5	27022	0.65
5	0.21	4.2	30089	8.82

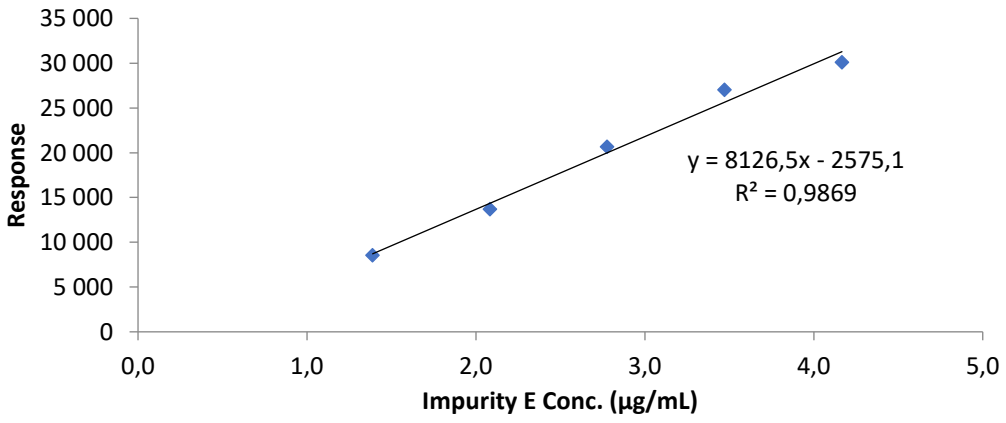


Figure 4.27 *– Linear regression plot of Impurity E.*

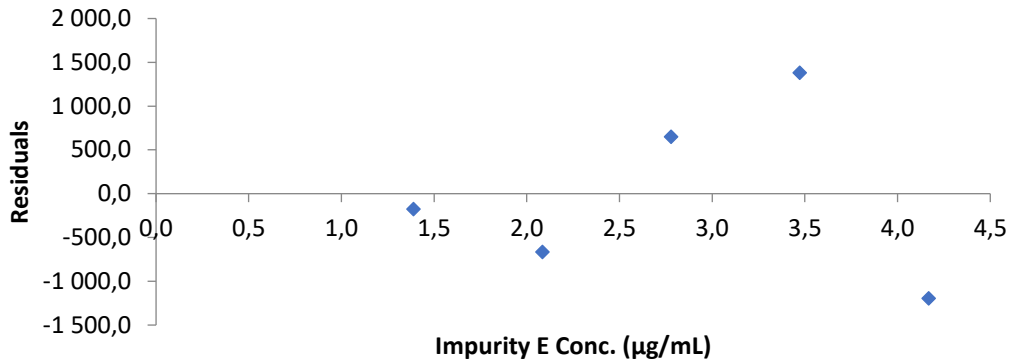


Figure 4.28 – Residual plot of low range linearity of Impurity E.

Table 4.22 - Parameters of the Impurity E linearity study

Parameter	Value	Result
Correlation coefficient	0.990	Complies
R Squared	0.990	---
Interception	-2575.13	---
Slope	8126.52	---

Analysing the *Figure 4.28*, there is no systematic trend in residual plot. Besides that, all of parameters present in *Table 4.22* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is linear in the concentration range tested.

4.4.5 Accuracy

Accuracy can be inferred from linearity. In this case, a new straight line shall be calculated in which the calculated concentration is reported, by interpolation of the straight line obtained in the linearity study, as a function of the experimentally prepared concentration.

Besides that, also recovery was calculated to demonstrate how close the results are from theoretical values. The results are present in *Table 4.23* and the regression plot in *Figure 4.29*.

Table 4.23 - Results of Impurity E accuracy test.

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
1,4	1,4	102,7
2,1	2,0	105,2
2,8	2,8	98,3
3,5	3,6	96,4
4,2	4,0	104,8

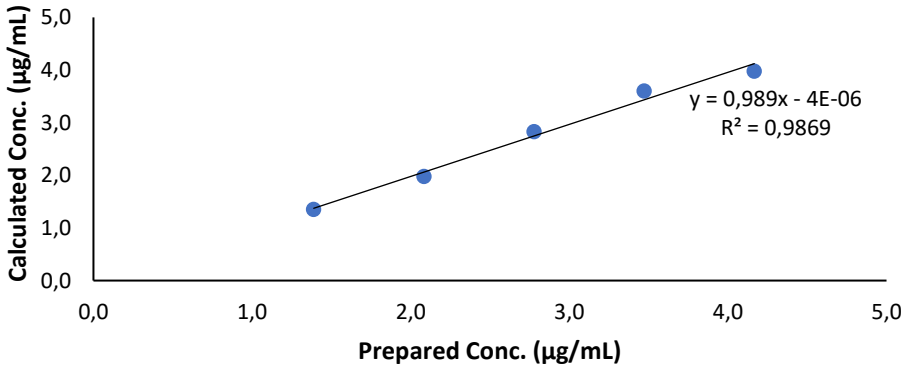


Figure 4.29 - Linear regression plot of Impurity E for accuracy test

Table 4.24 - Parameters of the Impurity E accuracy study

Parameter	Value	Result
Correlation coefficient	0,993	Complies
R Squared	0,987	Complies
Interception	-4,1E-06	---
Slope	0,989	---

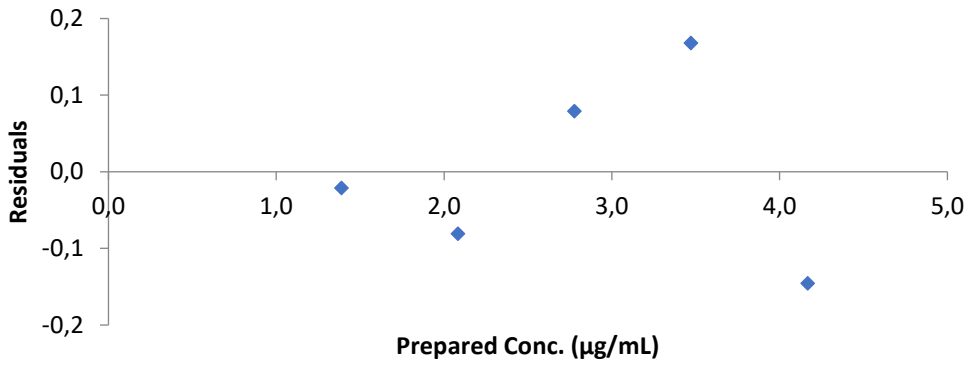


Figure 4.30 -Residual plot of regression analysis for accuracy test of Impurity E

Analysing the *Figure 4.30*, there is no systematic trend in residual plot. Besides that, all of parameters present in *Table 4.24* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is accurate in the concentration range tested.

4.4.6 Precision (System Repeatability)

This parameter tests the variability due only to the instrument under the chromatographic conditions indicated in the test procedure.

To evaluate the system repeatability, a reference solution at specification limit, Impurity E at 3.0 µg/mL, was injected six times. The results are shown in the *Table 4.25*.

Table 4.25 – System repeatability of Impurities E at 3.0 µg/mL.

Identification		**1**	**2**	**3**	**4**	**5**	**6**	**Average**	**RSD (%)**
Imp. E	**R_t**	27.15	27.13	27.12	27.07	27.06	27.06	27.10	0.15
	Area	18946	17095	18853	18799	19464	18444	**18600**	**4.34**

The system met the system repeatability requirements. % RSD of peak area responses for Impurity E is not more than 5,0.

4.4.7 Precision (Method Repeatability)

For evaluating the method repeatability, six individual API test solutions (2.0 mg/mL) spiked with Impurity E, at the specification limit concentration (3.0 µg/mL) were prepared and analysed against reference solution. The results are shown in *Table 4.26*.

Table 4.26 – Method repeatability results of Impurity E, 3.0 µg/mL.

Substance	Impurity E (%)
% Recovery	97.4
	92.4
	100.2
	99.9
	101.2
	89.3
Average ± RSD	**96.7 % ± 4.99%**
C. I. $_{95\%}$	**91.2% – 102.3%**

The RSD value obtained is lower than 10,0% and the % of recovery obtained is within 90% and 110%. The system met the method repeatability requirements.

4.5 Stereochemical Purity by HPLC

The method transfer included the following parameters: system suitability, specificity, confirmation of quantitation limit, linearity and precision (system repeatability and method repeatability).

4.5.1 System Suitability

To evaluate the system suitability, reference solution (a) with API at 1.2 µg/mL was injected six times. The values of match factor shall be within the range of 0.95-1.05. The system suitability results are presented in the following *Table 4.27*.

Table 4.27 - System suitability results of Reference solution (a)

Standard solution	Injection number	API Peak retention time (min)	API Peak Area (AU)
1	1	25.70	43221
	2	25.71	40225
	3	25.64	39947
	4	25.66	40768
	5	25.66	40705
	6	25.71	40083
Average		**25.68**	**40825**
SD		**0.03**	**1219.8**
RSD (%)		**0.13**	**2.99**
2	1	25.72	41010
	2	25.67	40667
	3	25.69	39445
Match factor		**0.990**	

The system suitability complies since the relative standard deviation for API determined from six replicate injections of reference solution (a) is less than 5.0%. In reference solution (d), resolution between API and isomer peaks is 1.24 (≥ 1.0).

4.5.2 Specificity

The method is considered to be specific if it is capable of accurately quantify the analyte with no interference from impurities or other substances present in the sample matrix.

A solution of diluent is shown in *Figure 4.31.* Beside that it was also prepared and injected Stereochemical purity standard solution at 12.0 µg/mL (*Figure 4.32*), reference solution (d) at API at 0.6 mg/mL and Stereochemical purity standard at 2.4 µg/mL(*Figure 4.33*), reference solution (a), at API at 1.2 µg/mL (*Figure 4.34*), a test solution of API (*Figure 4.35*) and spiked test solution with Stereochemical purity standard (*Figure 4.36*). The API and stereochemical isomer retention times are summarized in *Table 4.28.*

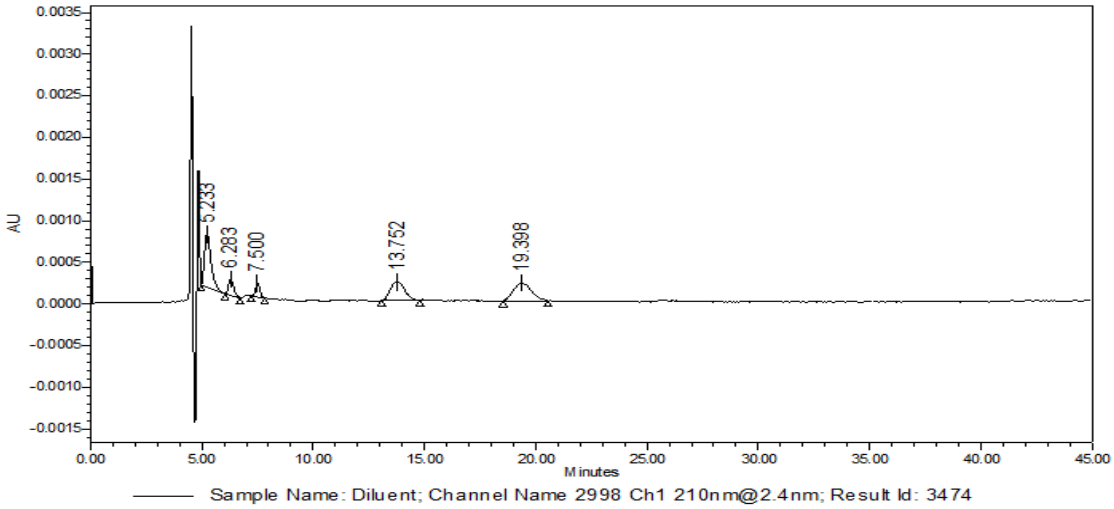


Figure 4.31 - Chromatogram of diluent (Buffer:Acetonitrile (50:50, v/v)).

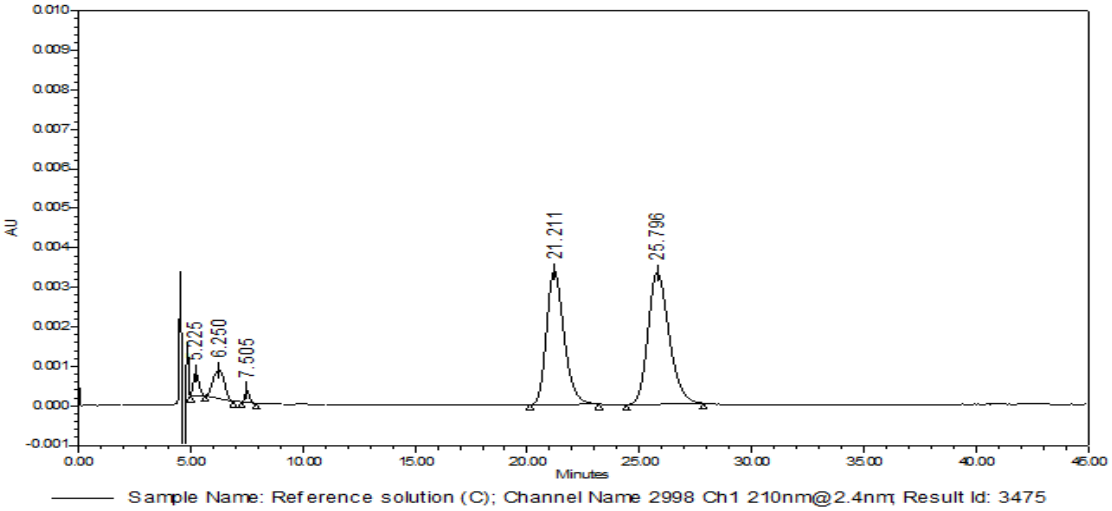


Figure 4.32 - Chromatogram of Reference solution (c), Stereochemical purity standard at 12.0 µg/mL.

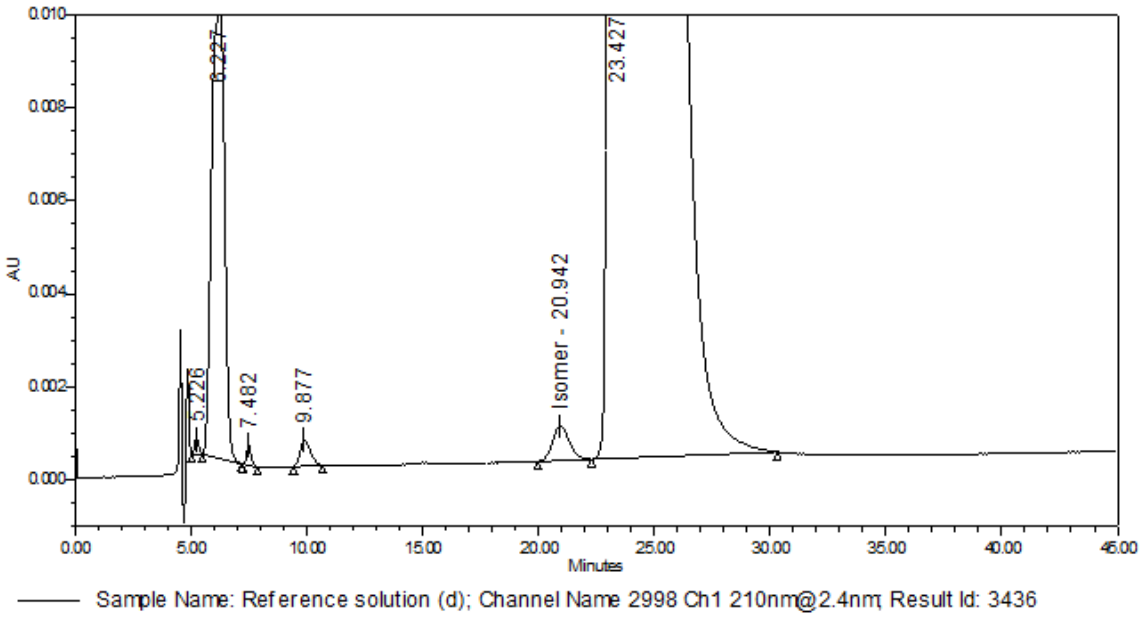


Figure 4.33 - Chromatogram of Reference solution (d), API 0.6 mg/mL and Stereochemical purity standard at 2.4 µg/mL.

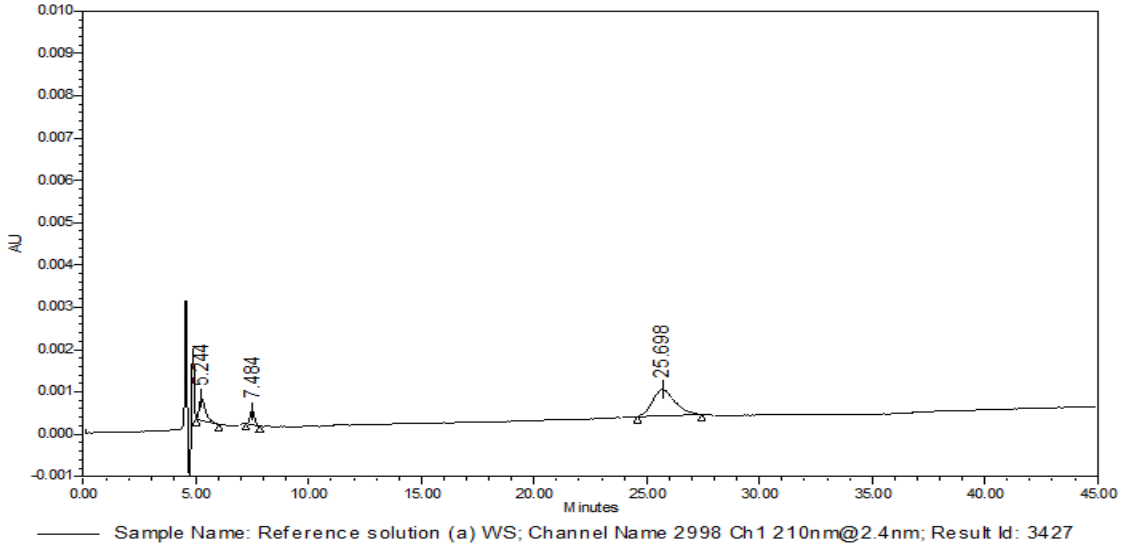


Figure 4.34 - Chromatogram of Reference solution (a), API at 1.2 µg/mL

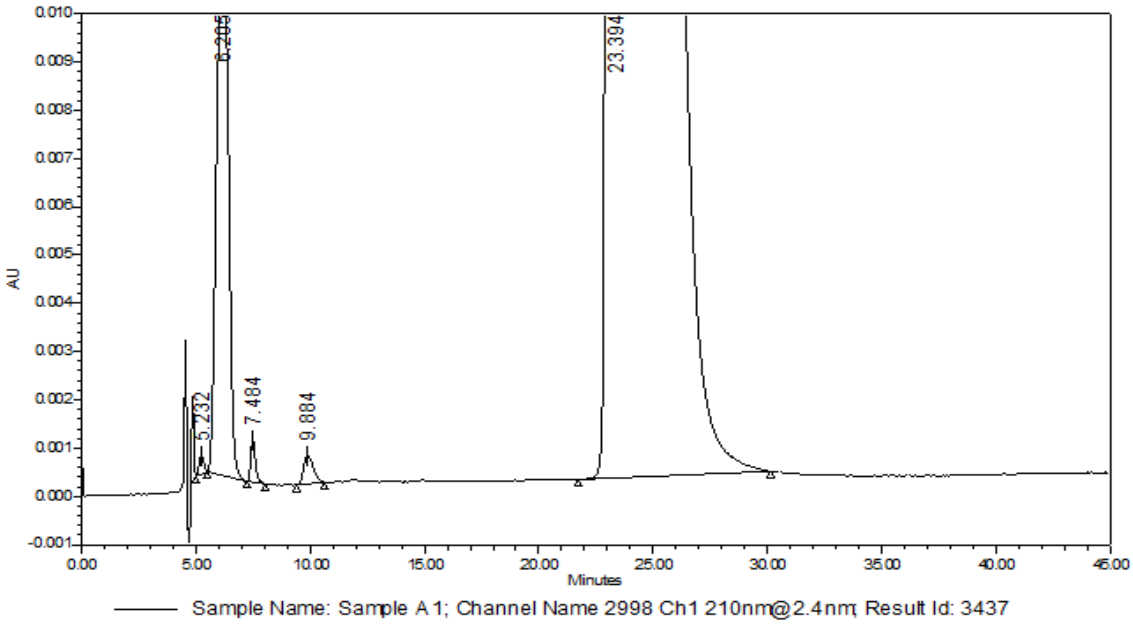


Figure 4.35 - Chromatogram of Test solution, 0.6 mg/mL

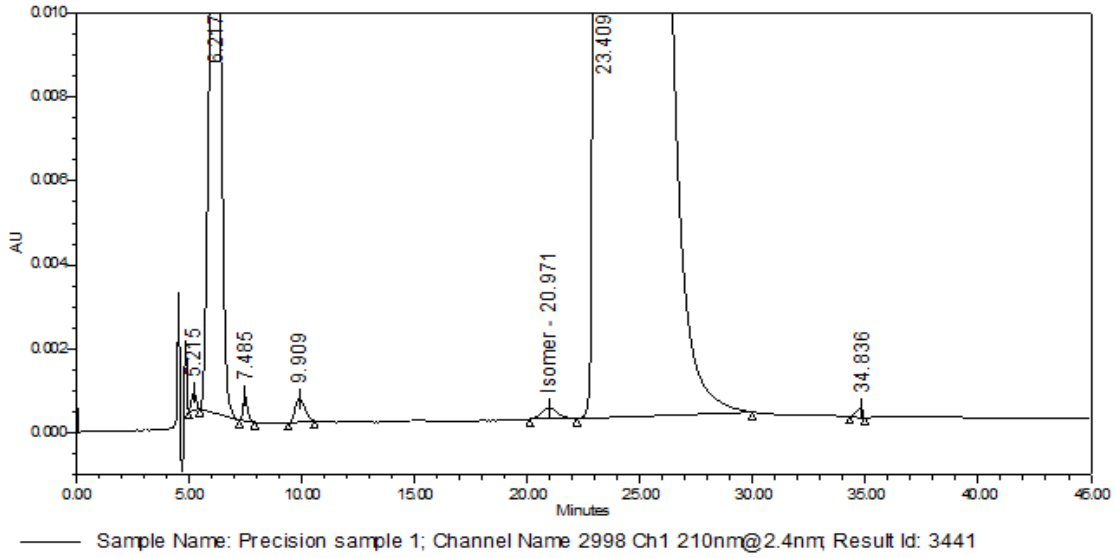


Figure 4.36 - Chromatogram of Spiked Test solution, API 0.6 mg/mL and Stereochemical purity standard at 9.0 µg/mL

Table 4.28 - Retention times of API and isomer peaks, in individual and spiked sample solutions

Substance	Retention time (aprox) in minutes	
	Individual solution	**Spiked test solution**
API	23.39	23.41
Other isomer	21.21	20.97

The system suitability complies, and the method proved to be specific since there is any interfering peak at the retention time of other isomer of API.

4.5.3 Quantitation Limit

The solution should contain the minimum analyte (active substance and impurities) concentration quantified by the chromatographic system, which should correspond to the lower concentration in the low range linearity.

The QL concentration shown in the *Table 4.29* were confirmed by injecting six replicates of individual solution of API, at the determined concentration (0.1 µg/mL).

Table 4.29 – Limit of quantitation of other isomer.

Substance	QL (µg/mL)	QL (%)	Average Area	RSD (%)
API	0.1	0.02	3206	7.76

The system met the requirements. The RSD of the peak areas of API, at quantitation limit concentration is less than 10 %.

4.5.4 Low Range Linearity

Linearity test study the proportionality between analyte concentration and instrument response.

To evaluate the linearity of this method, API standard solutions were prepared at a minimum of five different concentrations, ranging from QL to 150 % of specification limit (0.9 µg/mL). Linearity test was performed in triplicate.

The following figure represents the linear plot of API. Linear regression analysis confirmed the acceptability of the HPLC method for quantitative determination of the other isomer of API over the concentration range of ≈ 0.1 – 1.4 µg/mL, corresponding to ≈ 14 – 153% of 0.9 µg/mL (0,15 % of the nominal concentration in API, 0.6 mg /mL). Results of the linearity study for API are shown in the *Table 4.30* and the regression plot in *Figure 4.37*.

Table 4.30 - Results of the other isomer of API linearity study.

Level	Concentration in % (relative to test)	API concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	0.02	0.1	2501	15.13
2	0.08	0.5	13551	3.06
3	0.11	0.7	20850	2.00
4	0.15	0.9	30919	0.47
5	0.19	1.2	37047	1.20
6	0.23	1.4	44304	1.33

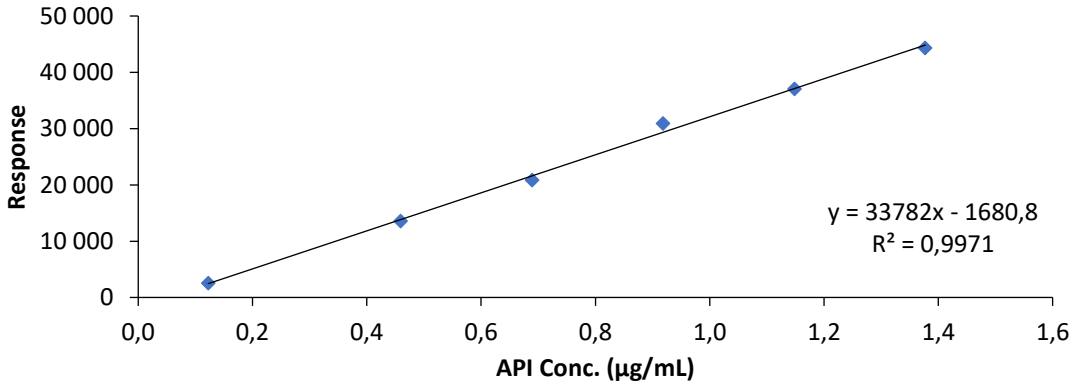


Figure 4.37 – Linear regression plot of API (other isomer).

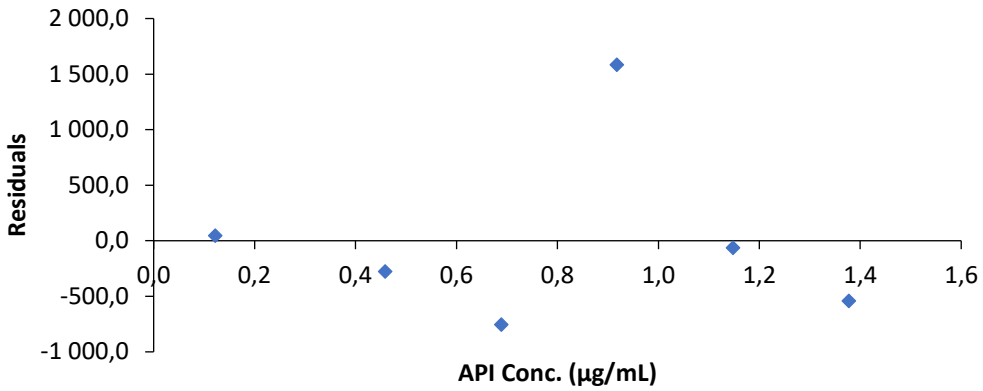


Figure 4.38 – Residual plot of low range linearity of API (other isomer).

Table 4.31 - Parameters of the API (other isomer) linearity study

Parameter	Value	Result
Correlation coefficient	1.000	Complies
R Squared	1.000	---
Interception	-1680.79	---
Slope	33781.9	---

Analysing the *Figure 4.38*, there is no systematic trend in residual plot. Besides that, all of parameters present in *Table 4.31* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is linear in the concentration range tested.

4.5.5 Accuracy

Accuracy can be inferred from linearity. In this case, a new straight line shall be calculated in which the calculated concentration is reported, by interpolation of the straight line obtained in the linearity study, as a function of the experimentally prepared concentration.

Besides that, also recovery was calculated to demonstrate how close the results are from theoretical values. The results are present in *Table 4.32* and the regression plot in *Figure 4.39*.

Table 4.32 - Results of the other isomer of API accuracy study.

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
0,12	0,12	98,9
0,46	0,45	101,8
0,69	0,67	103,3
0,92	0,97	95,1
1,15	1,15	100,2
1,38	1,36	101,2

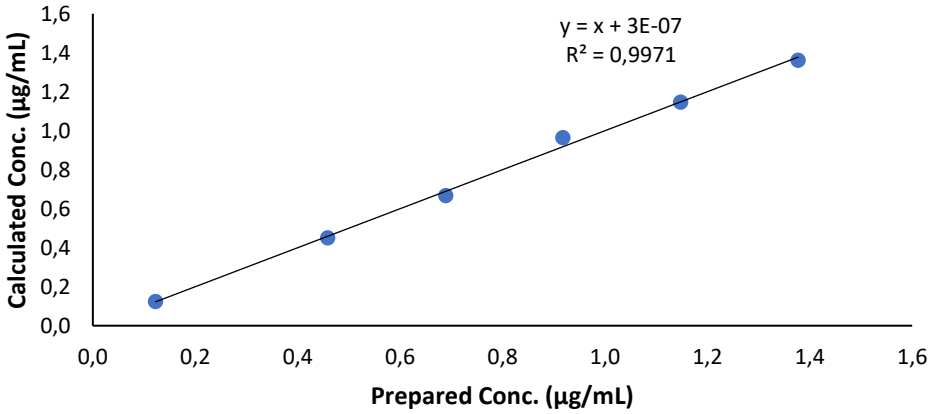


Figure 4.39 - Linear regression plot of API (other isomer) for accuracy test

Table 4.33 - Parameters of the API (other isomer) accuracy study

Parameter	Value	Result
Correlation coefficient	0,999	Complies
R Squared	0,997	Complies
Interception	2,6E-07	---
Slope	0,999	---

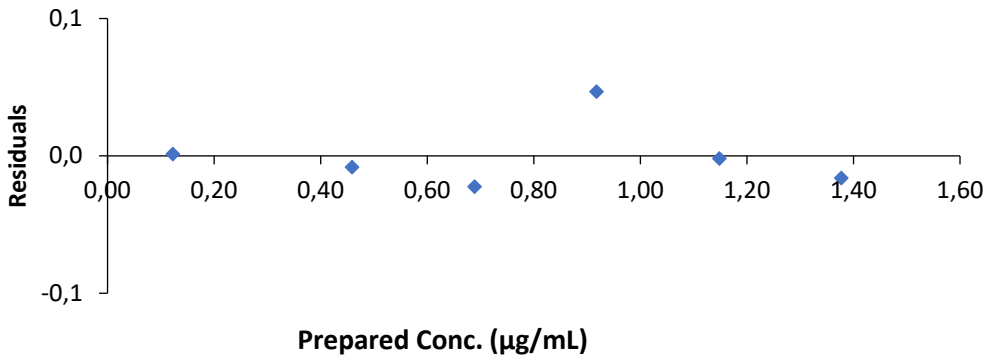


Figure 4.40 - Residual plot of regression analysis for accuracy test

Analysing the *Figure 4.40*, there is no systematic trend in residual plot. Besides that, all of parameters present in *Table 4.33* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is accurate in the concentration range tested.

4.5.6 Precision (System Repeatability)

This parameter tests the variability due only to the instrument under the chromatographic conditions indicated in the test procedure.

To evaluate the system repeatability, a reference solution at specification limit, API at 0.9 µg/mL, was injected six times. The results are shown in the *Table 4.34*.

Table 4.34 – System repeatability of API at 0.9 µg/mL.

Identification		**1**	**2**	**3**	**4**	**5**	**6**	**Average**	**RSD (%)**
API	**R_t**	25.70	25.71	25.64	25.66	25.66	25.71	25.68	0.13
	Area	27511	27240	27135	27657	27588	28187	**28553**	**1.35**

The system met the system repeatability requirements. % RSD of peak area responses for API is not more than 5,0.

4.5.7 Precision (Method Repeatability)

For evaluating the method repeatability, six individual API test solutions (0.6 mg/mL) spiked with Stereochemical purity standard, at the specification limit concentration (0.9 µg/mL) were prepared and analysed against reference solution. The results are shown in *Table 4.35.*

Table 4.35 – Method repeatability results of Stereochemical purity standard, 0.9 µg/mL.

Substance	Other isomer (%)
% Recovery	89.6
	96.0
	92.2
	93.1
	91.3
	98.2
Average ± RSD	**93.4 % ± 3.41%**
C. I. $_{95\%}$	**89.7% – 97.1%**

The RSD value obtained is lower than 10,0% and the % of recovery obtained is within 90% and 110%. The system met the method repeatability requirements.

4.6 Content of Acetic Acid by HPLC

The method transfer included the following parameters: system suitability, specificity, confirmation of quantitation limit, linearity and precision (system repeatability and method repeatability).

4.6.1 System Suitability

To evaluate the system suitability, reference solution (b) with Acetic acid at 125,0 µg/mL was injected six times. The results of match factor shall be within the range of 0.95-1.05. The system suitability results are presented in the following *Table 4.36*.

Table 4.36 - System suitability results of Reference solution (b)

Standard solution	Injection number	Acetic acid Peak retention time (min)	Acetic acid Peak Area (AU)
1	1	6.89	81680
	2	6.88	81346
	3	6.89	81356
	4	6.88	81624
	5	6.87	81494
	6	6.86	81284
Average		**6.88**	**81464**
SD		**0.01**	**161.95**
RSD (%)		**0.13**	**0.20**
2	1	25.50	82471
	2	25.53	82529
	3	25.57	82720
Match factor		**1.014**	

The system suitability complies since the relative standard deviation for Acetic acid determined from six replicate injections of reference solution (b) is less than 5.0%.

4.6.2 Specificity

The method is considered to be specific if it is capable of accurately quantify the analyte with no interference from impurities or other substances present in the sample matrix. Acetic acid solution was prepared at specification limit concentration.

A solution of diluent is shown in *Figure 4.41.* Beside that it was also prepared and injected Reference solution (b) (*Figure 4.42*), test solution of API (*Figure 4.43*) and a spiked test solution with acetic acid (*Figure 4.44*). The API and acetic acid retention times are summarized in *Table 4.37.*

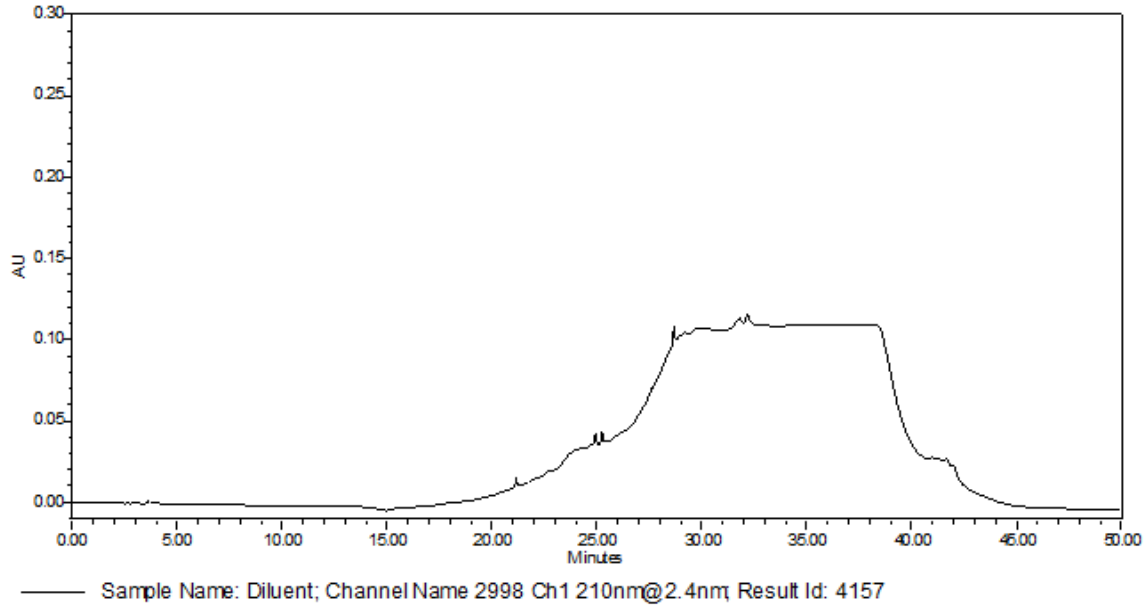


Figure 4.41 - Chromatogram of diluent (Buffer:Methanol (70:30, v/v)).

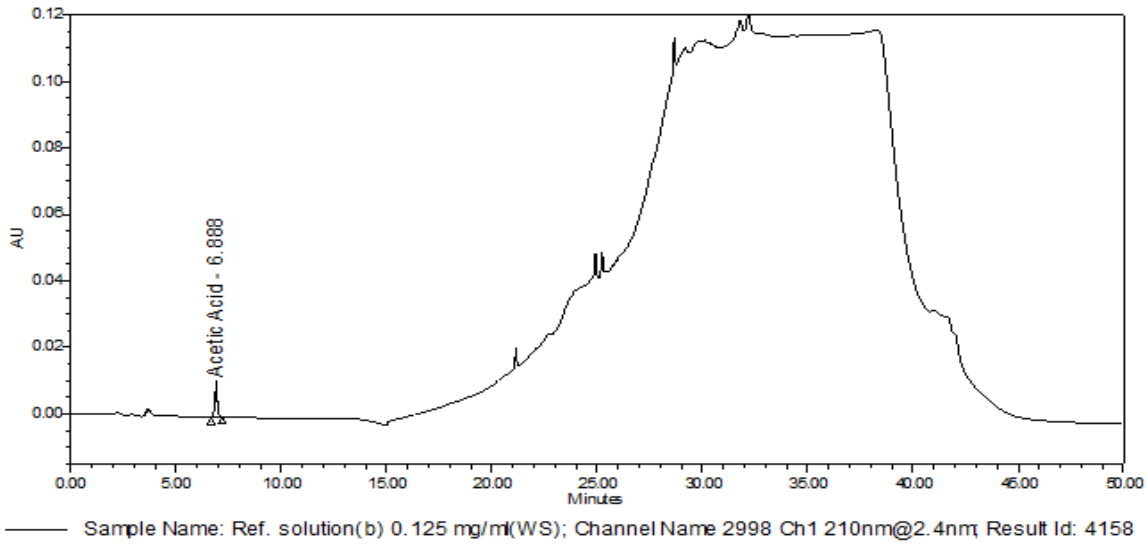


Figure 4.42 - Chromatogram of Reference solution (b), Acetic acid at 125,0 µg/mL.

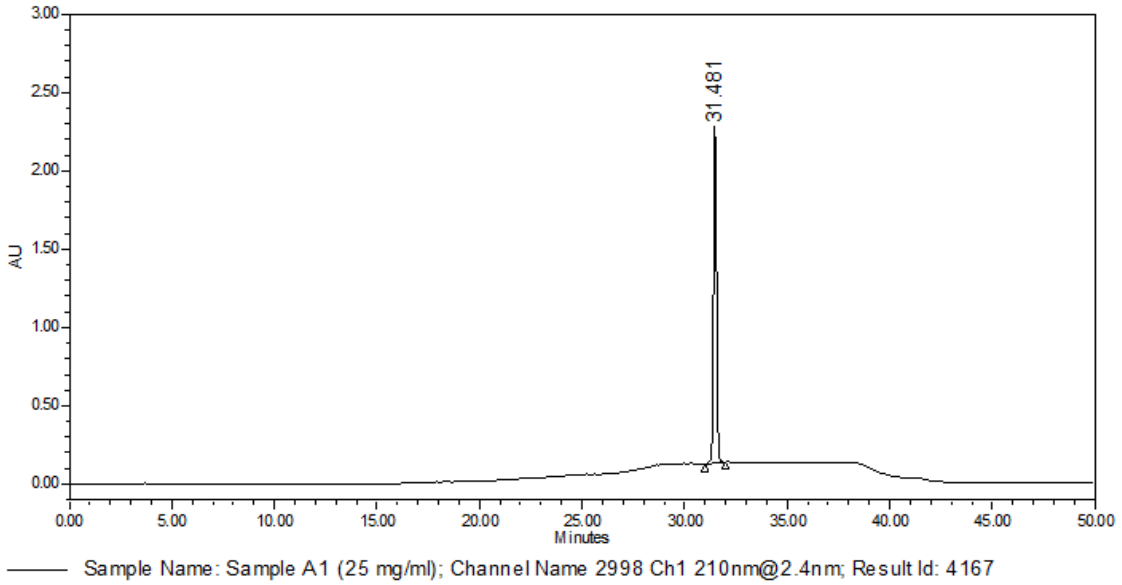


Figure 4.43 - Chromatogram of Test solution, 25.0 mg/mL

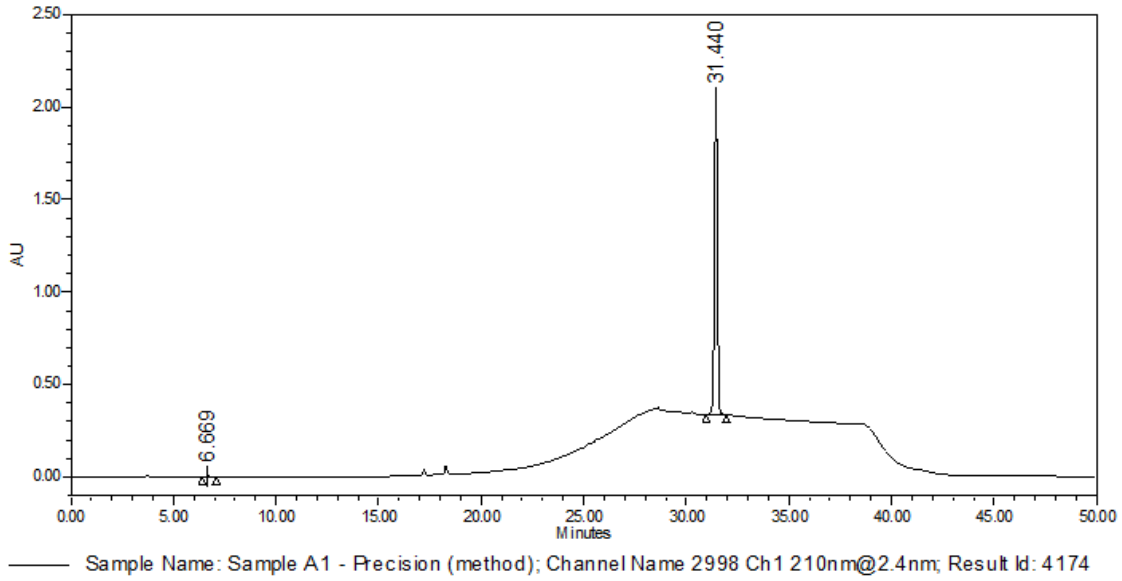


Figure 4.44 - Chromatogram of Spiked Test solution, API at 25.0 mg/mL and Acetic acid at 125,0 µg/mL

Table 4.37 - Retention times of API and impurity peaks, in individual and spiked sample solutions

Substance	Retention time (aprox) in minutes	
	Individual solution	**Spiked test solution**
API	31.48	31.44
Acetic acid	6.89	6.67

The system suitability complies, and the method proved to be specific since there is any interfering peak at the retention time of Acetic acid.

4.6.3 Quantitation Limit

The solution should contain the minimum analyte (active substance and impurities) concentration quantified by the chromatographic system, which should correspond to the lower concentration in the low range linearity.

The QL concentration shown in the *Table 4.38* were confirmed by injecting six replicates of individual solution of Acetic acid, at the determined concentration (170 ppm).

Table 4.38 – Limit of quantitation of Acetic acid.

Substance	QL (µg/mL)	QL (%)	Average Area	RSD (%)
Acetic Acid	4.3	0.02	2282	6.17

The system met the requirements. The RSD of the peak areas of Acetic acid, at the Quantitation limit concentration is less than 10 %.

4.6.4 Low Range Linearity

Linearity test study the proportionality between analyte concentration and instrument response.

To evaluate the linearity of this method, Acetic acid standard solutions were prepared at a minimum of five different concentrations, ranging from QL to 150 % of specification limit (125,0 µg/mL or 5000ppm). Linearity test was done in triplicate.

The following figure represents the linear plot of Acetic acid. Linear regression analysis confirmed the acceptability of the HPLC method for quantitative determination of acetic acid over the concentration range of ≈ 4.2– 187.2 µg/mL, corresponding to ≈ 3 – 150% of 125.0 µg/mL (0,5 % of the nominal concentration in API, 25.0 mg /mL). Results of the linearity study for Acetic acid are shown in the *Table 4.39* and the regression plot in *Figure 4.45*.

Table 4.39 - Results of Acetic acid linearity study.

Level	Concentration in % (relative to test)	Acetic acid concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	0.02	4.2	2335	6.67
2	0.25	62.4	52760	0.18
3	0.37	93.6	81534	0.11
4	0.50	124.8	104677	0.19
5	0.62	156.0	132771	0.04
6	0.75	187.2	161116	0.10

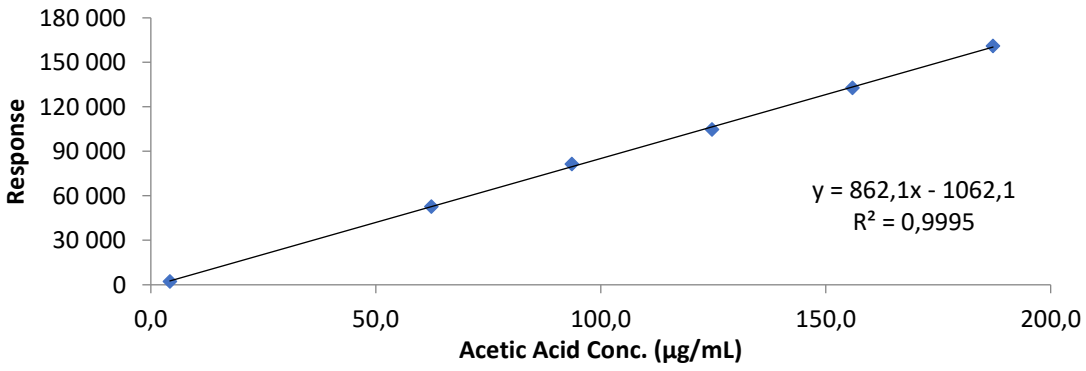


Figure 4.45 – Linear regression plot of Acetic acid.

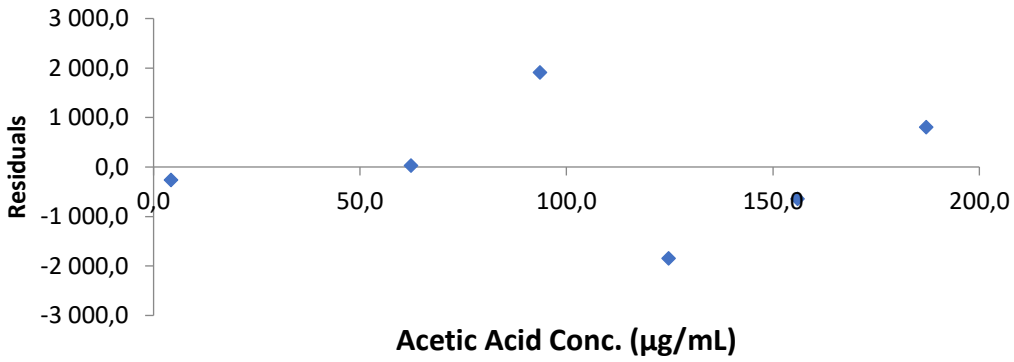


Figure 4.46 - Residual plot of low range linearity of Acetic acid.

Table 4.40 - Parameters of the Acetic acid linearity study

Parameter	Value	Result
Correlation coefficient	1.000	Complies
R Squared	1.000	---
Interception	-1062.13	---
Slope	862.10	---

Analysing the *Figure 4.46*, there is no systematic trend in residual plot. Besides that, all of parameters present in *Table 4.40* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is linear in the concentration range tested.

4.6.5 Accuracy

Accuracy can be inferred from linearity. In this case, a new straight line shall be calculated in which the calculated concentration is reported, by interpolation of the straight line obtained in the linearity study, as a function of the experimentally prepared concentration.

Besides that, also recovery was calculated to demonstrate how close the results are from theoretical values. The results are present in *Table 4.41* and the regression plot in *Figure 4.47*.

Table 4.41 - Results of Acetic Acid accuracy test.

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
4,2	3,9	107,7
62,4	62,4	99,9
93,6	95,8	97,7
124,8	122,7	101,7
156.0	155,2	100,5
187,2	188,1	99,5

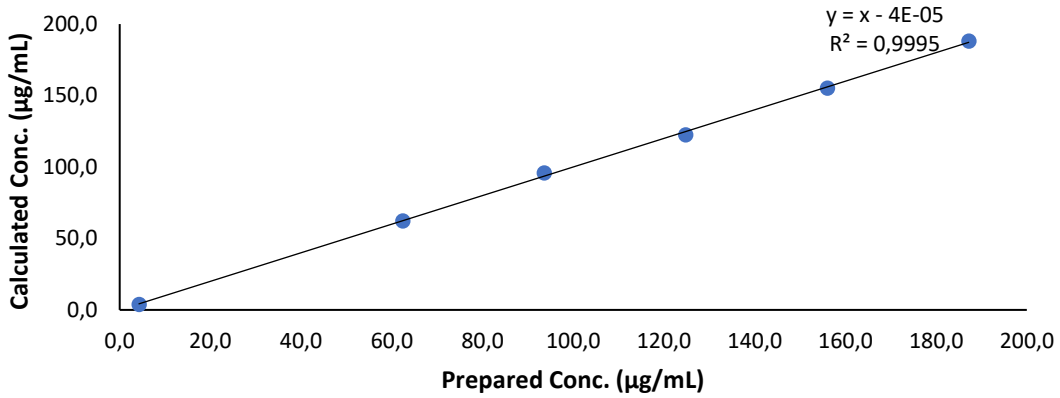


Figure 4.47 **-** Linear regression plot for Acetic Acid accuracy test

Table 4.42 - Parameters of the API accuracy study

Parameter	Value	Result
Correlation coefficient	0,999	Complies
R Squared	0,999	Complies
Interception	-3,754E-05	---
Slope	1	---

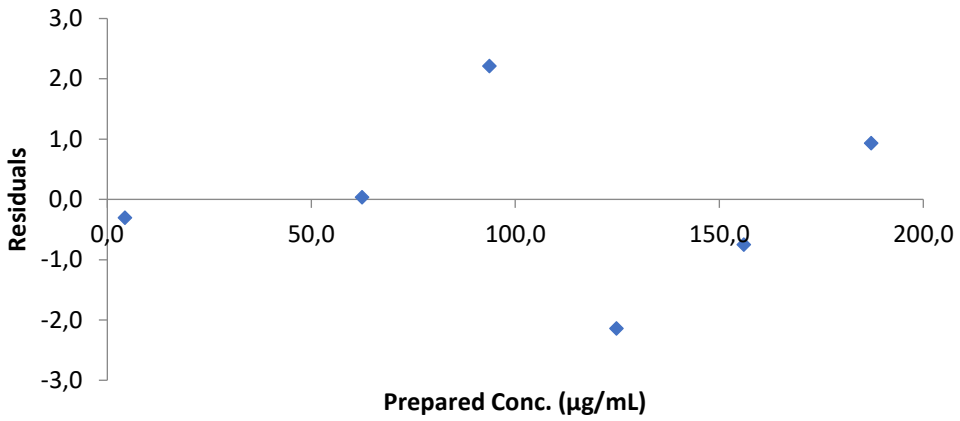


Figure 4.48 - Residual plot of accuracy test of Acetic acid.

Analysing the *Figure 4.48*, there is no systematic trend in residual plot. Besides that, all of parameters present in *Table 4.42* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is accurate in the concentration range tested.

4.6.6 Precision (System Repeatability)

This parameter tests the variability due only to the instrument under the chromatographic conditions indicated in the test procedure.

To evaluate the system repeatability, a reference solution at specification limit, Acetic acid at 125,0 µg/mL, was injected six times. The results are shown in the *Table 4.43*.

Table 4.43 – System repeatability of Acetic Acid at 125.0 µg/mL.

Identification		**1**	**2**	**3**	**4**	**5**	**6**	**Average**	**RSD (%)**
Acetic Acid	**R_t**	6.89	6.88	6.89	6.88	6.87	6.86	6.88	0.13
	Area	81680	81346	81356	81624	81494	81284	**81464**	**0.20**

The system met the system repeatability requirements. % RSD of peak area responses for Acetic acid is not more than 5,0.

4.6.7 Precision (Method Repeatability)

For evaluating the method repeatability, six individual API test solutions (25.0 mg/mL) spiked with Acetic acid, at the specification limit concentration (125,0 µg/mL) were prepared and analysed against reference solution. The results are shown in *Table 4.44.*

Table 4.44 – Method repeatability results of Acetic acid, 125,0 µg/mL.

Substance	Acetic acid (%)
% Recovery	104.1
	101.6
	101.7
	101.0
	101.9
	101.8
Average ± RSD	**102.0 % ± 1.04%**
C. I. $_{95\%}$	**100.8% – 103.2%**

The RSD value obtained is lower than 10,0% and the % of recovery obtained is within 90% and 110%. The system met the method repeatability requirements.

4.7 Residual Solvents by GC

The method transfer included the following parameters: system suitability, specificity, confirmation of quantitation limits, linearity, and precision (system repeatability and method repeatability).

4.7.1 System Suitability

To evaluate the system suitability, residual solvents standard solution was injected six times. To determine the Match factor, a second standard solution was prepared and injected 3 consecutive times. For residual solvents the results of match factor shall be within the range of 0.85-1.15. The system suitability results are presented in the following *Table 4.45*.

Table 4.45 – System suitability results of Residual Solvents standard solution.

Solvent	Concentration (µg/mL)	% RSD (Peak area, n=6)	% RSD (retention time, n=6)	Match Factor	Resolution (related to previous peak)
Methanol	118.6	2.26	0.01	1.061	15.4
Ethanol	201.2	2.43	0.01	1.058	12.7
Acetone	201.6	0.88	0.01	1.062	7.0
Isopropyl alcohol	200.1	2.42	0,02	1.009	2.9
Methylene Chloride	23.9	1.17	0.01	0.987	2.4
Ethyl acetate	198.4	1.08	0.00	1.011	23.6
Tetrahydrofuran	28.9	0.79	0.01	1.008	3.7
Cyclohexane	155.6	0.63	0.01	1.108	3.9
Toluene	35.4	2.23	0.00	0.996	32.1

The system met the suitability requirements. The % RSD of peak area responses of each solvent from six replicate injections of standard solution is not more than 15,0 and the resolution between adjacent peaks is not less than 1,0.

4.7.2 Specificity

The method is considered to be specific if it is capable of accurately quantifying the residual solvents with no interference from other substances present in the sample matrix.

Specificity of the method was verified by injecting several solutions (see the *Figures 4.49-4.61*), checking if there is no peaks coelution. Elution order of Residual Solvents in individual and standard solutions are shown in *Table 4.46*.

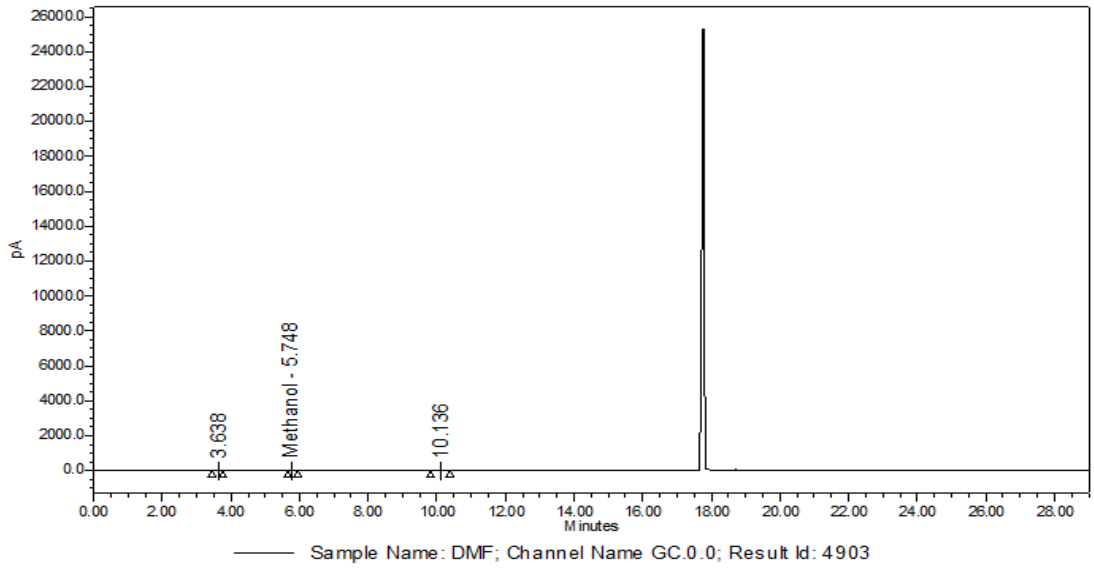


Figure 4.49 - Chromatogram of solvent (Dimethylformamide).

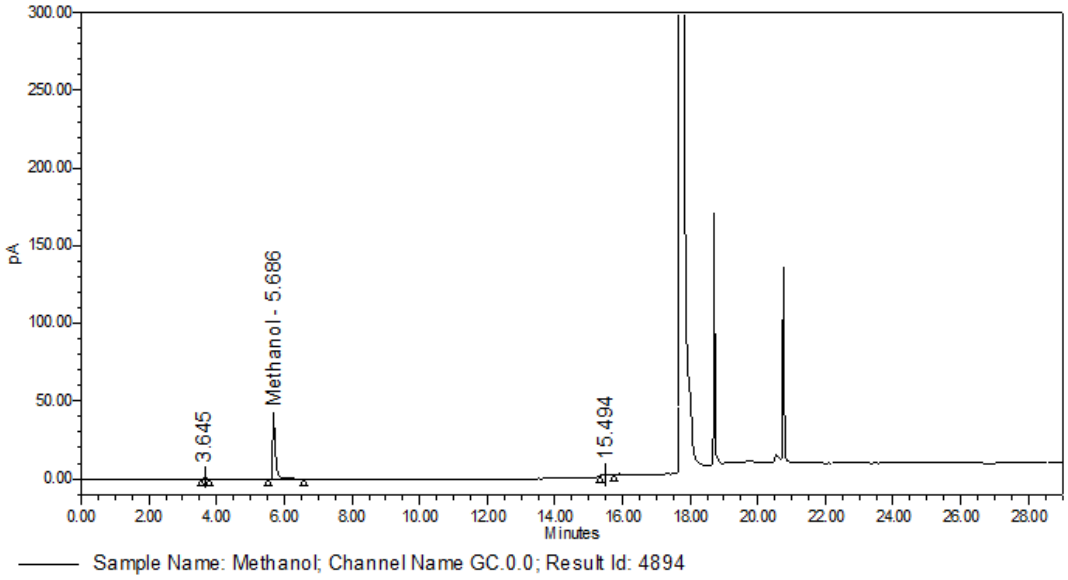


Figure 4.50 - Chromatogram of Methanol, 3000 ppm.

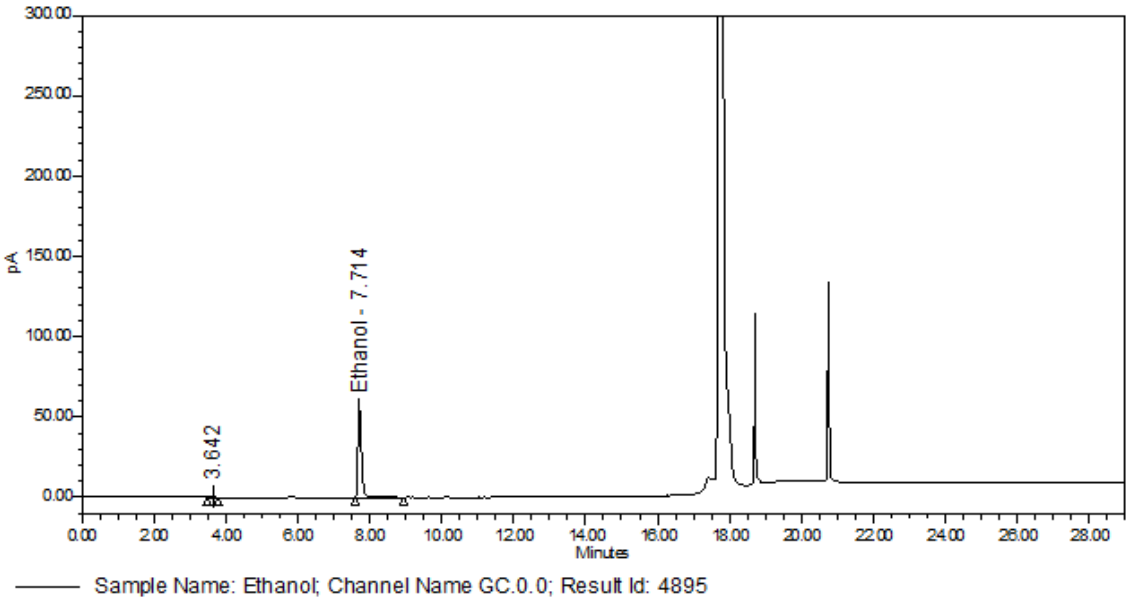


Figure 4.51 - Chromatogram of Ethanol, 5000 ppm.

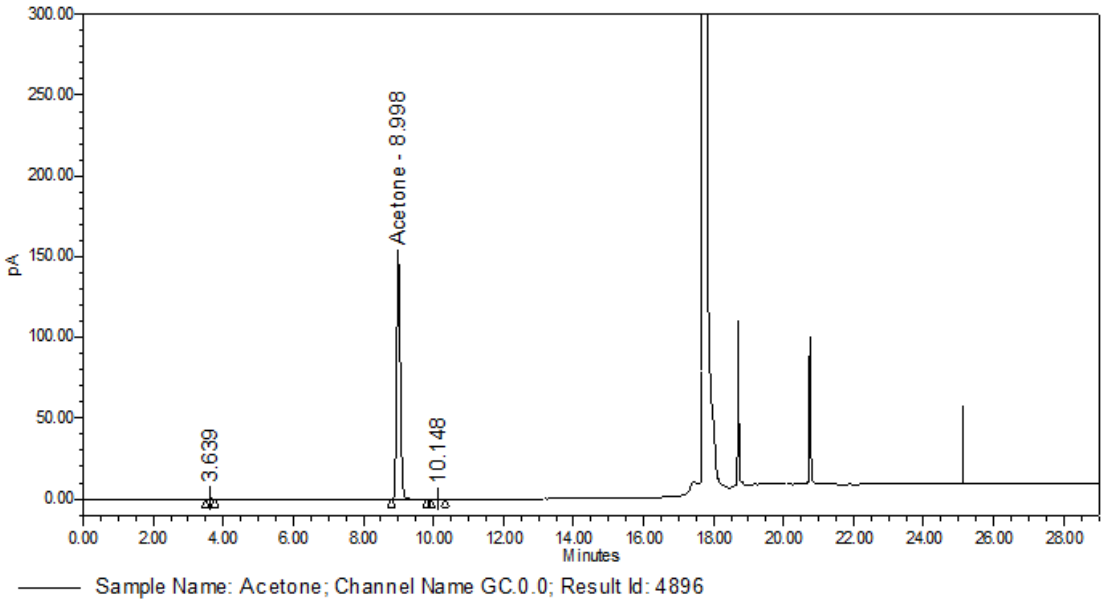


Figure 4.52 - Chromatogram of Acetone, 5000 ppm.

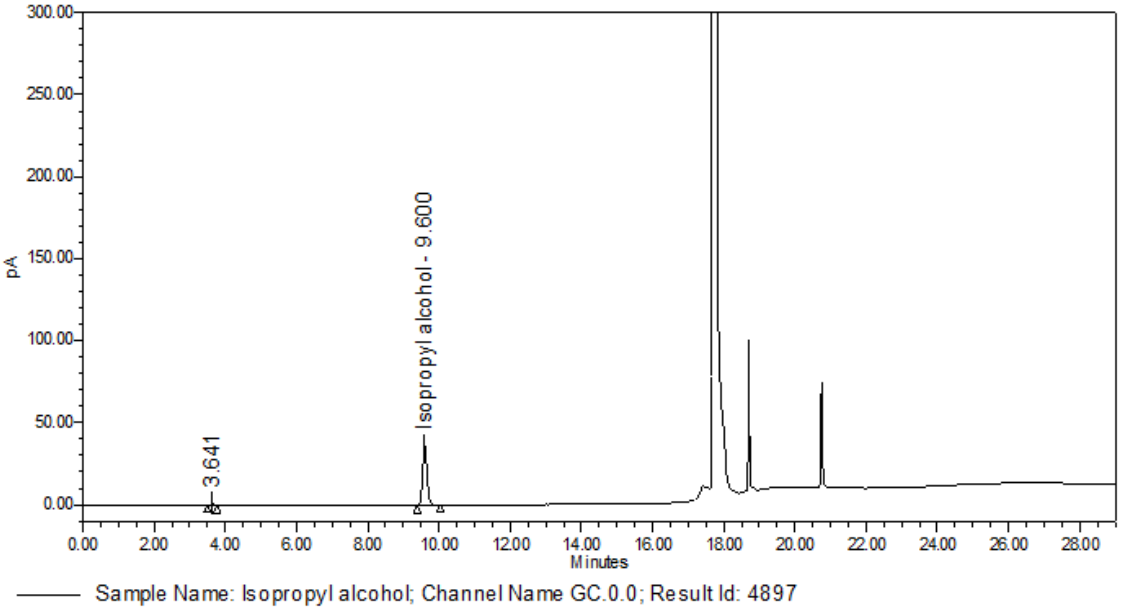


Figure 4.53 - Chromatogram of Isopropyl alcohol, 5000 ppm.

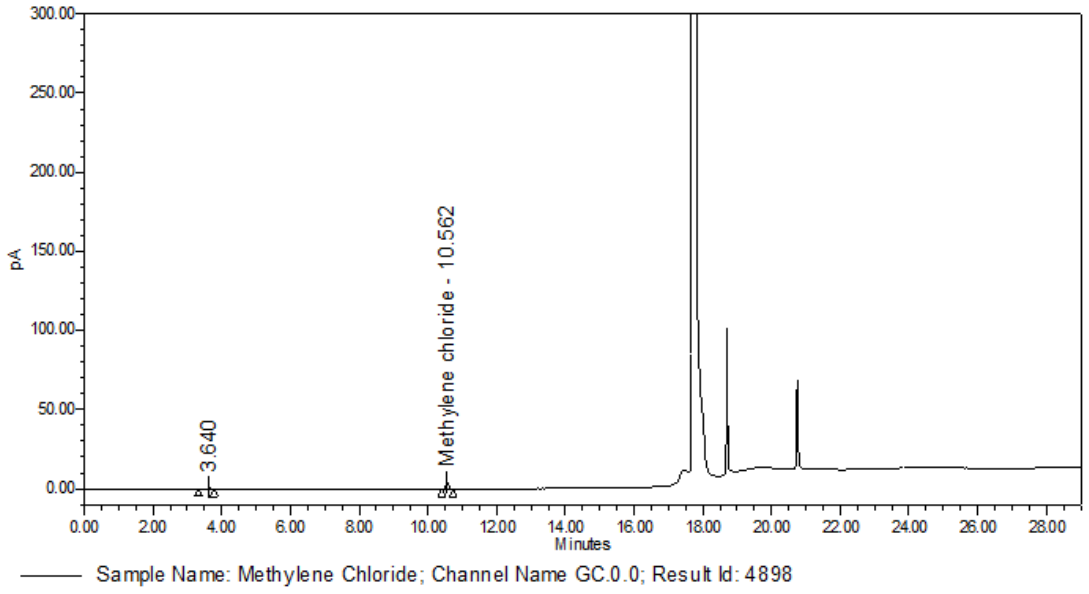


Figure 4.54 - Chromatogram of Methylene chloride, 600 ppm

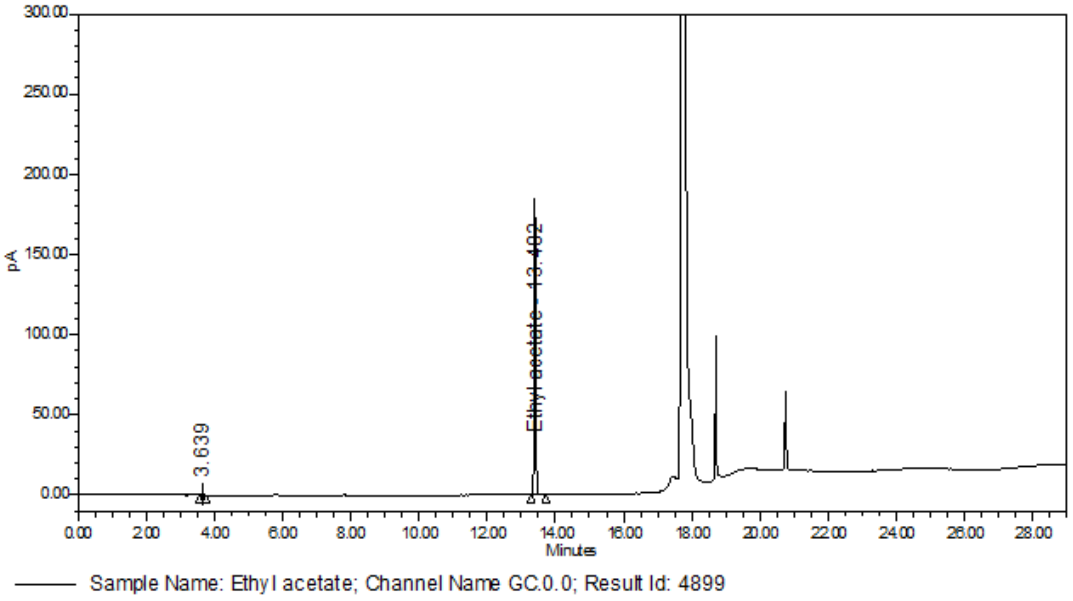


Figure 4.55 - Chromatogram of Ethyl acetate, 5000 ppm

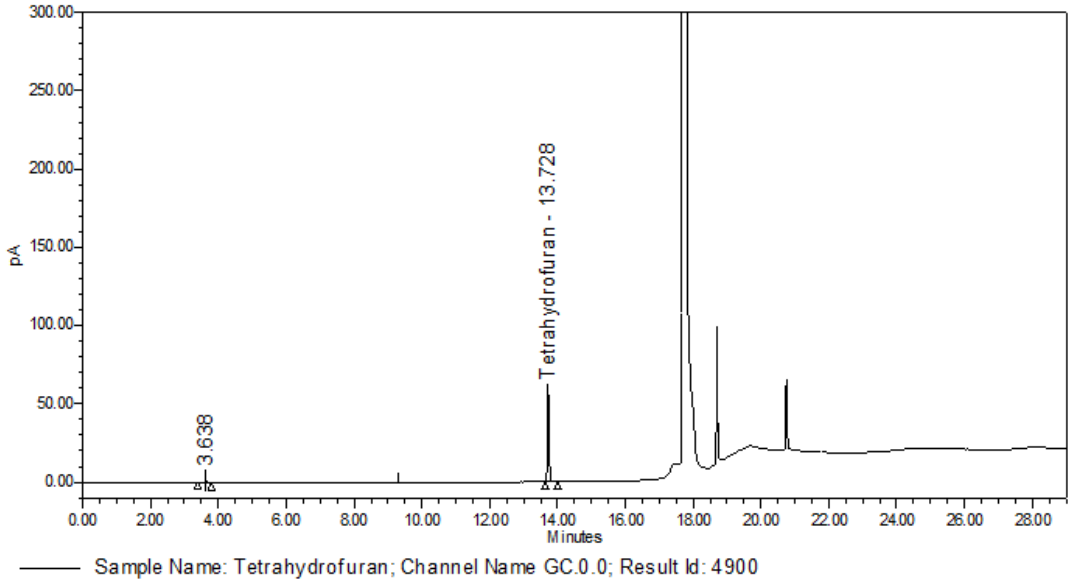


Figure 4.56 - *Chromatogram of Tetrahydrofuran, 720 ppm.*

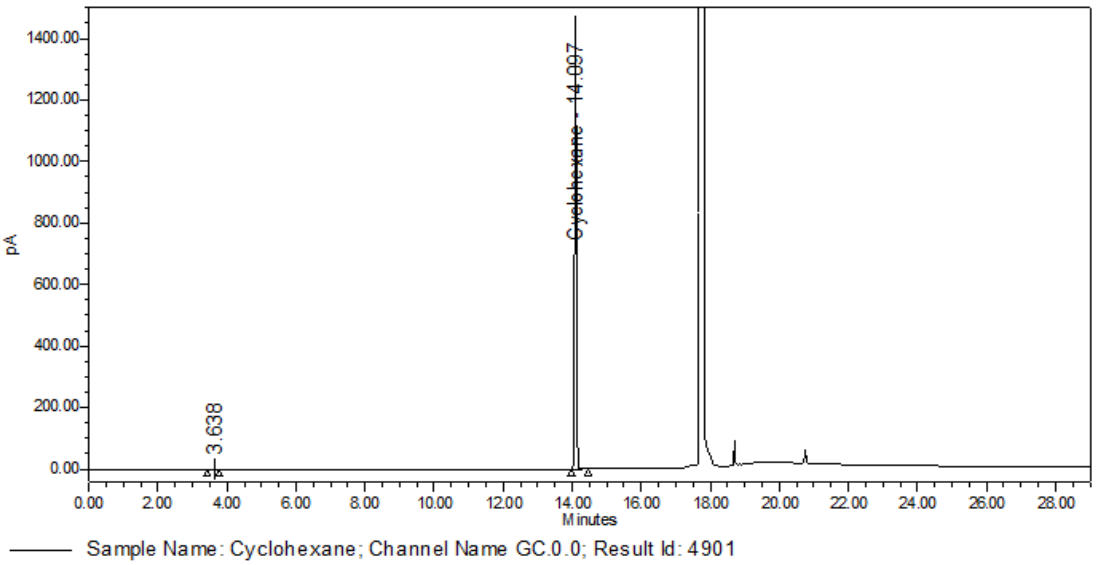


Figure 4.57 - Chromatogram of Cyclohexane, 3880 ppm.

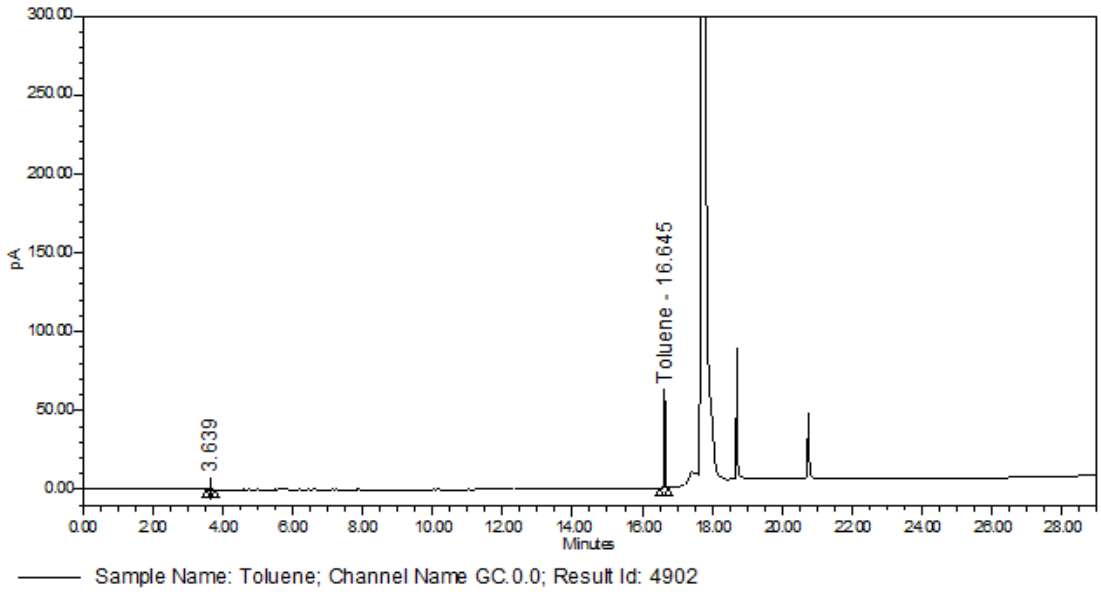


Figure 4.58 - Chromatogram of Toluene, 890 ppm.

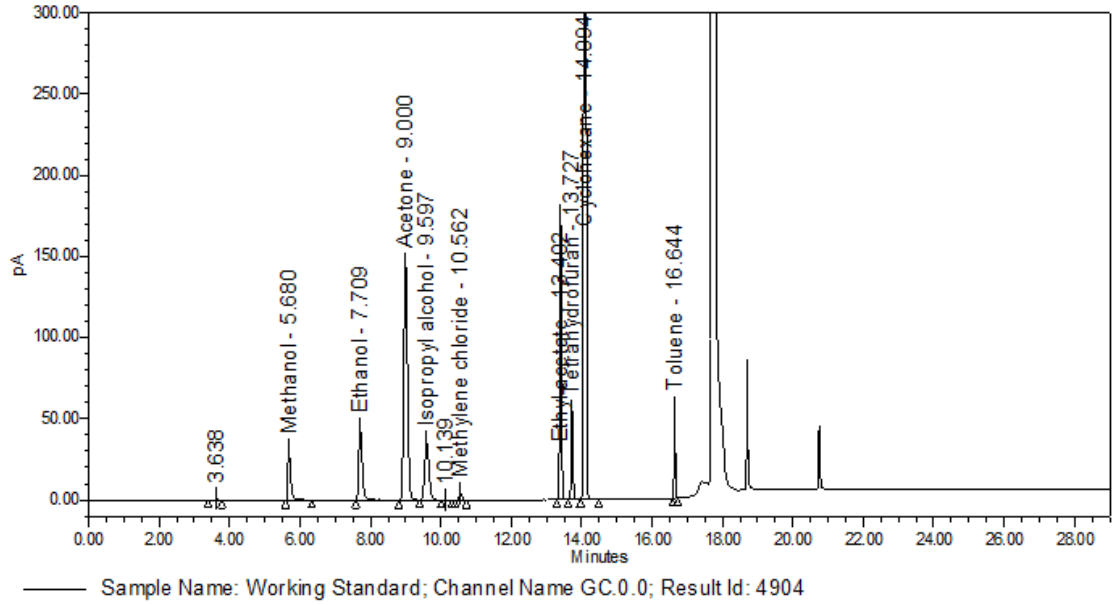


Figure 4.59 - Chromatogram of Residual solvents Standard solution.

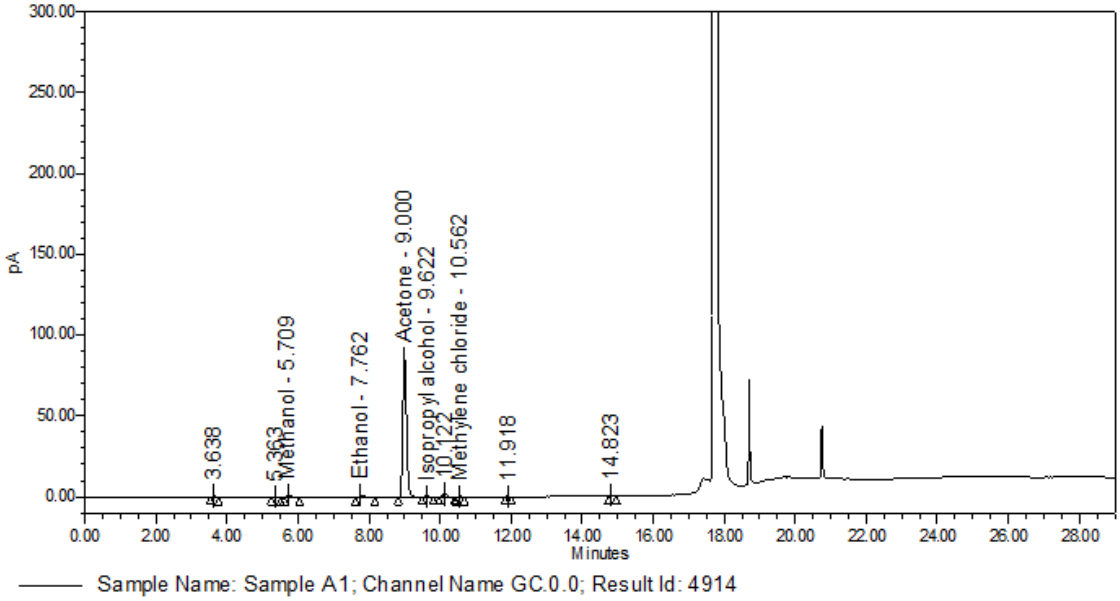


Figure 4.60 - Chromatogram of Sample test solution.

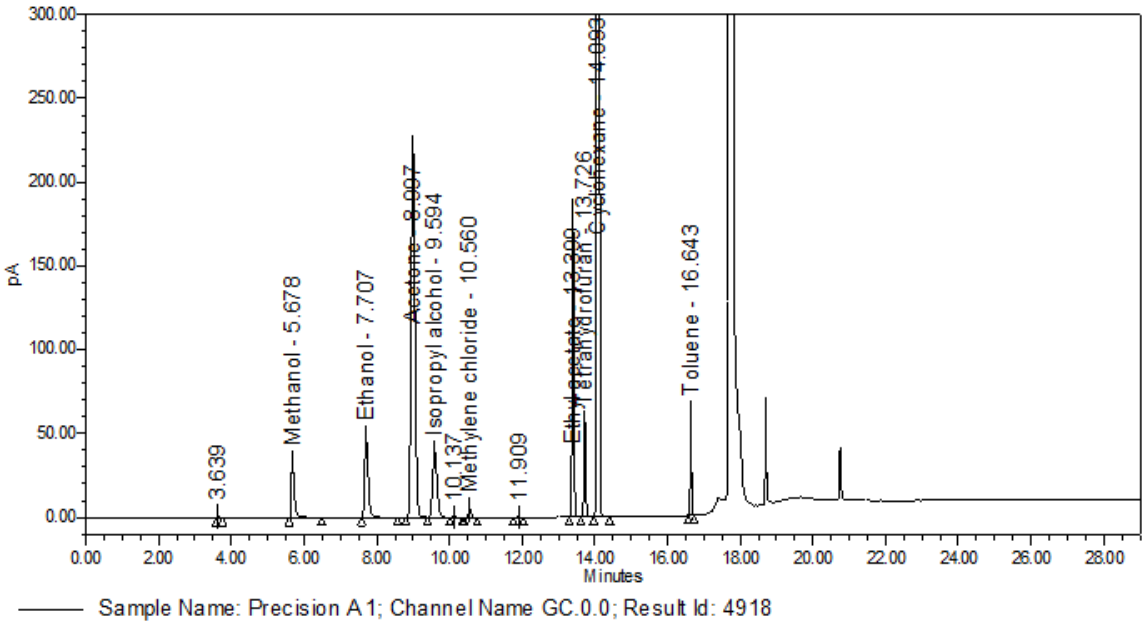


Figure 4.61 - Chromatogram of sample solution, spiked with residual solvents at specification limits.

Table 4.46 – Elution order of Residual Solvents in individual and standard solutions.

Solvent	Individual solution rt (min)	Standard solution rt (min)
Methanol	5.69	5.68
Ethanol	7.71	7.71
Acetone	9.00	9.00
Isopropyl alcohol	9.60	9.60
Methylene chloride	10.56	10.56
Ethyl acetate	13.40	13.40
Tetrahydrofuran	13.73	13.72
Cyclohexane	14.10	14.09
Toluene	16.65	16.64

The system met the specificity requirements since there is no blank interference at the retention times corresponding to any of the solvents tested; the elution order and retention times obtained from individual standard solutions and standard (SST) solution are comparable for each solvent (i.e. ± 0,2 min) and sample preparation met the specification criteria.

4.7.3 Quantitation Limit

The limit of quantitation for residual solvents was experimentally confirmed through six replicate injections of a solution at QL concentration. Results are shown in the *Table 4.47*.

Table 4.47 - QL concentration confirmations.

Solvent name	QL (µg/mL)	QL (in ppm w.r.t. sample)	% RSD Peak area (n=6)
Methanol	4.8	120	7.96
Ethanol	5.2	130	4.95
Acetone	1.7	42	2.92
Isopropyl alcohol	5.0	126	6.60
Methylene chloride	4.2	105	3.90
Ethyl acetate	2.2	54	3.86
Tetrahydrofuran	1.3	33	1.09
Cyclohexane	0.2	6	1.68
Toluene	2.4	60	4.00

QL concentrations described by the supplier were confirmed. The RSD of the peak area response of each solvent is ≤ 15 %.

4.7.4 Linearity

Linearity test study the proportionality between analyte concentration and instrument response.

To evaluate the linearity of this method, standard solutions of residual solvents were prepared at a minimum of five different concentrations, ranging from QL, 50% to 150% of the specification limits for each one of the solvents. Linearity test was done in triplicate. Results of the linearity study for residual solvents are shown in the *Tables 4.48- 4.56* and the regression plots in *Figures 4.62-4.78*.

Table 4.48 - Results of Methanol linearity study.

Level	Con centration in % (relative to test)	Concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	4.8	4.1	3	4.33
2	50.0	59.3	90	1.01
3	75.0	89.0	135	0.89
4	100.0	118.6	189	4.43
5	125.0	148.3	232	2.07
6	150.0	177.9	273	0.41

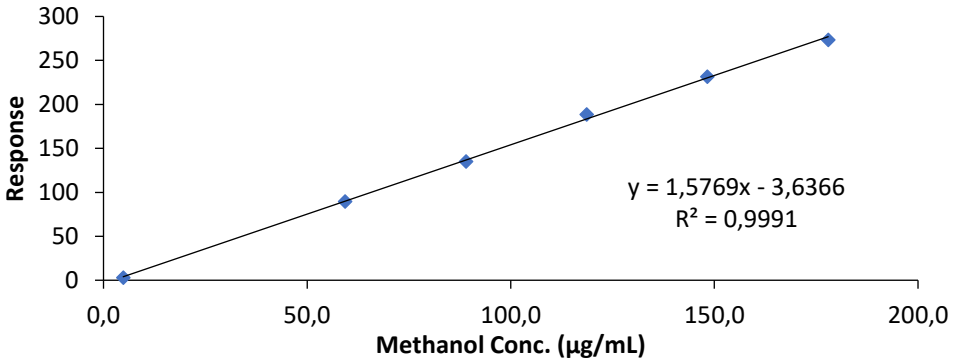


Figure 4.62 – Linear regression plot of Methanol.

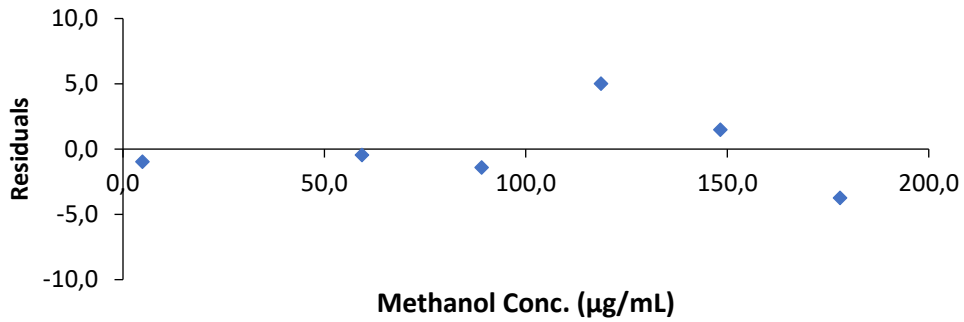


Figure 4.63 – Residual plot of Methanol.

Table 4.49 - Results of Ethanol linearity study.

Level	Con centration in % (relative to test)	Concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	2.6	5.2	5	7.39
2	50.0	100.6	150	1.30
3	75.0	150.9	225	1.45
4	100.0	201.2	315	5.48
5	125.0	251.5	389	2.22
6	150.0	301.8	454	0.21

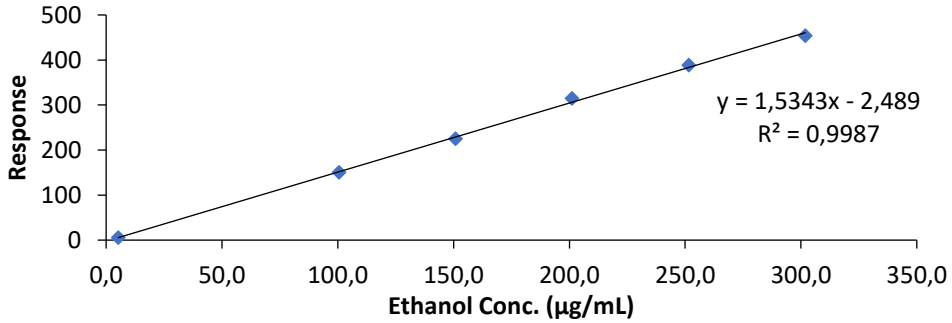


Figure 4.64 – Linear regression plot of Ethanol.

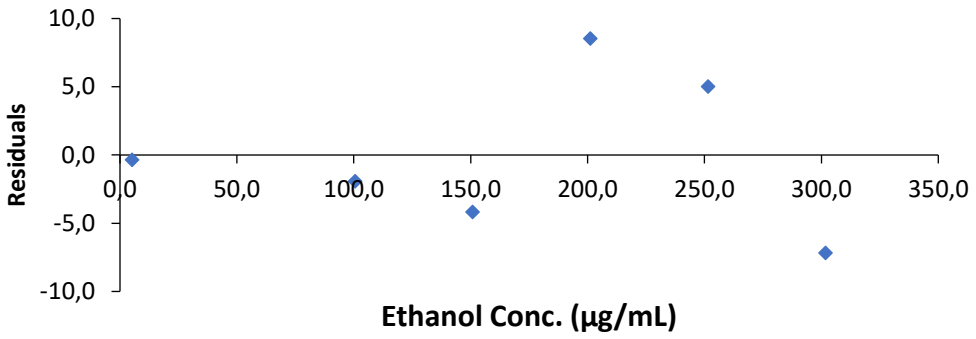


Figure 4.65 – Residual plot of Ethanol.

Table 4.50 - Results of Acetone linearity study.

Level	Con centration in % (relative to test)	Concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	0.8	1.7	2	2.35
2	50.0	100.8	521	0.28
3	75.0	151.2	787	0.64
4	100.0	201.6	1055	2.48
5	125.0	252.0	1315	1.15
6	150.0	302.4	1562	0.19

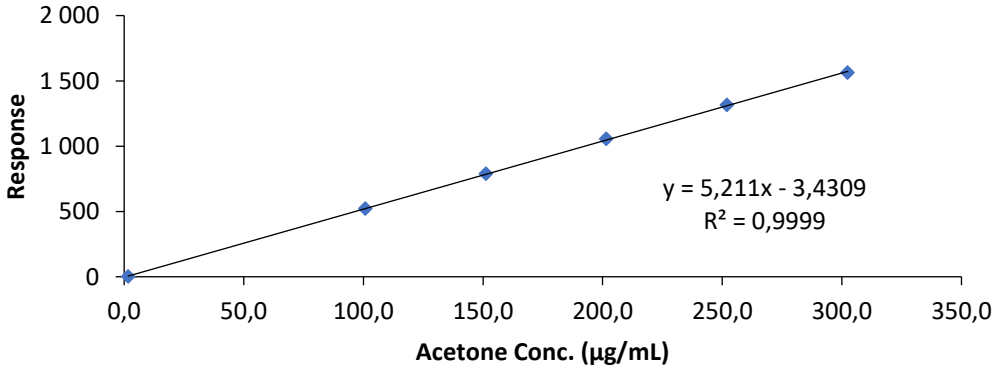


Figure 4.66 – *Linear regression plot of Acetone.*

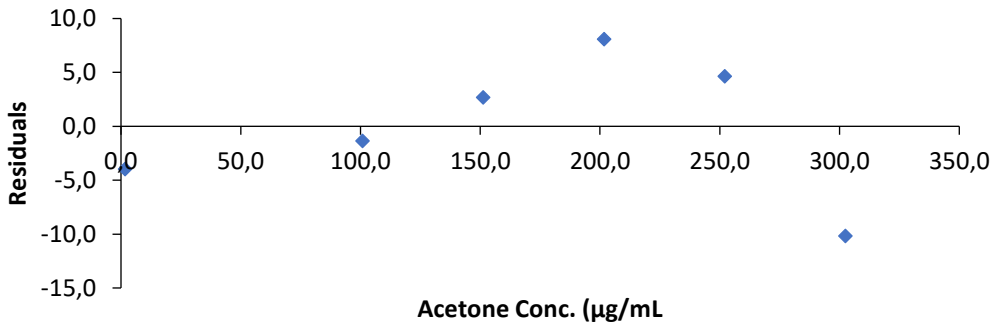


Figure 4.67 – Residual plot of Acetone.

Table 4.51 - Results of Isopropyl alcohol linearity study.

Level	Con centration in % (relative to test)	Concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	2.5	5.0	2	4.20
2	50.0	100.0	149	1.19
3	75.0	150.1	224	1.16
4	100.0	200.1	315	5.20
5	125.0	250.1	387	2.35
6	150.0	300.1	450	0.52

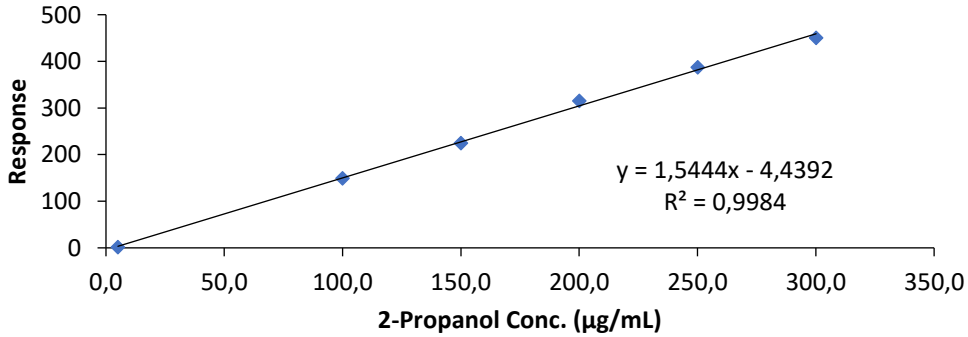


Figure 4.68 – Linear regression plot of Isopropyl alcohol.

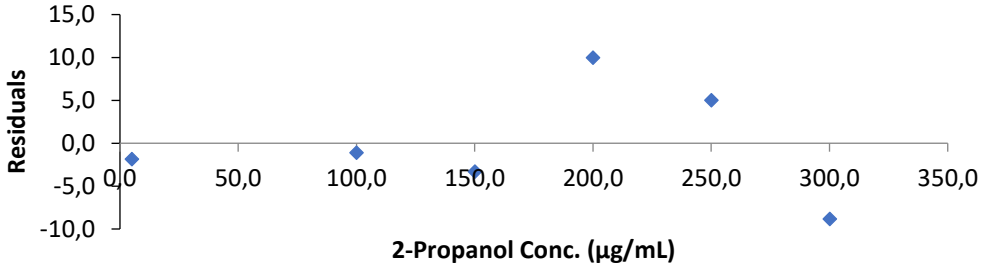


Figure 4.69 – Residual plot of Isopropyl alcohol.

Table 4.52 - Results of Methylene Chloride linearity study.

Level	Con centration in % (relative to test)	Concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	17.6	4.2	5	3.04
2	50.0	11.9	13	0.73
3	75.0	17.9	19	0.48
4	100.0	23.9	26	3.03
5	125.0	29.8	32	1.20
6	150.0	35.8	38	0.66

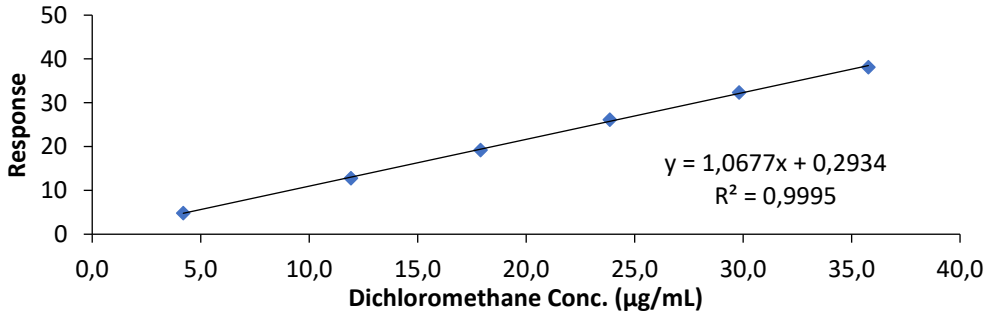


Figure 4.70 – Linear regression plot of Methylene Chloride.

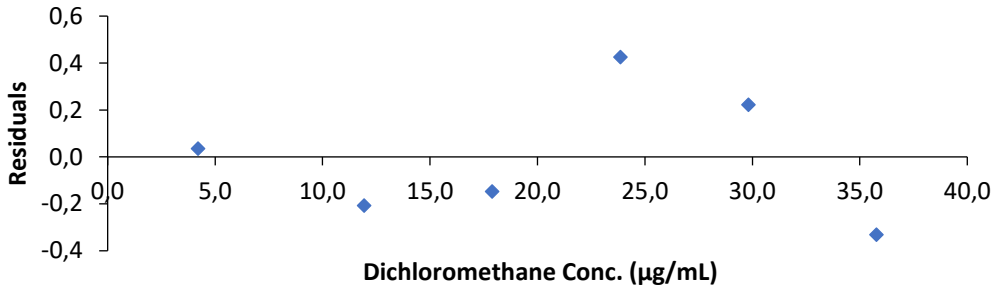


Figure 4.71 – Residual plot of linearity of Methylene Chloride.

Table 4.53 – Results of Ethyl acetate linearity study.

Level	Con centration in % (relative to test)	Concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	1.1	2.2	2	2.90
2	50.0	99.2	283	0.29
3	75.0	148.8	427	0.73
4	100.0	198.4	579	2.85
5	125.0	248.1	716	1.45
6	150.0	297.7	850	0.21

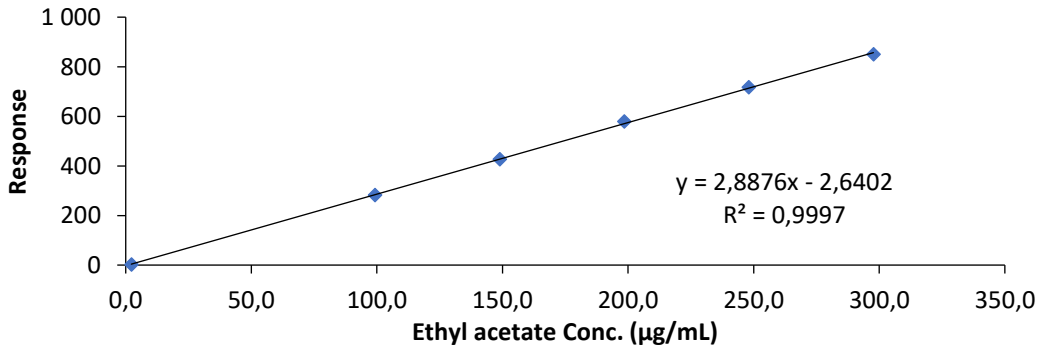


Figure 4.72 – Linear regression plot of Ethyl acetate

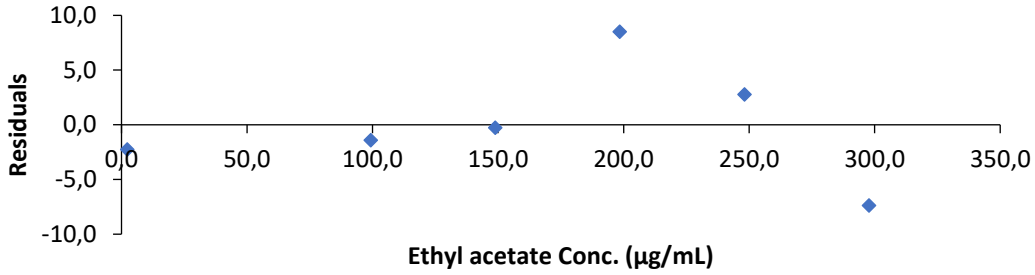


Figure 4.73 – Residual plot of linearity of Ethyl acetate.

Table 4.54 – Results of Tetrahydrofuran linearity study.

Level	Con centration in % (relative to test)	Concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	4.56	1.3	11	1.54
2	50.0	14.4	90	0.23
3	75.0	21.7	137	0.62
4	100.0	28.9	185	2.26
5	125.0	36.1	230	1.09
6	150.0	43.3	272	0.17

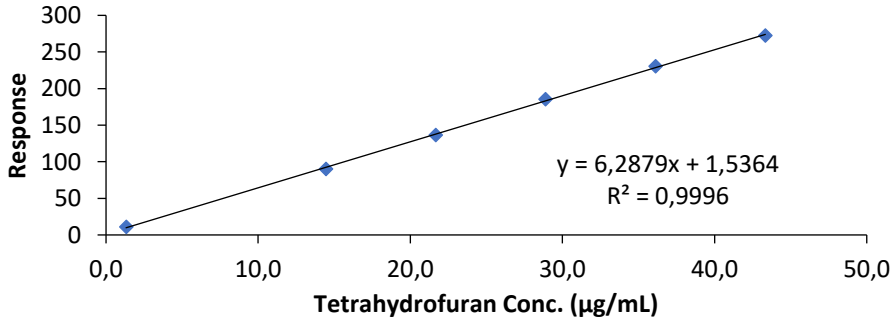


Figure 4.74 – Linear regression plot of Tetrahydrofuran

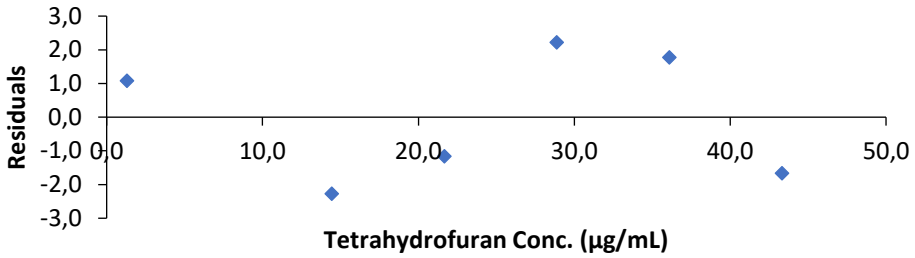


Figure 4.75 – *Residual plot of linearity of Tetrahydrofuran.*

Table 4.55 – Results of Cyclohexane linearity study.

Level	Con centration in % (relative to test)	Concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	0.16	0.3	3	1.06
2	50.0	77.8	2161	0.37
3	75.0	116.7	3280	0.46
4	100.0	155.6	4337	0.82
5	125.0	194.6	5412	0.77
6	150.0	233.5	6398	0.10

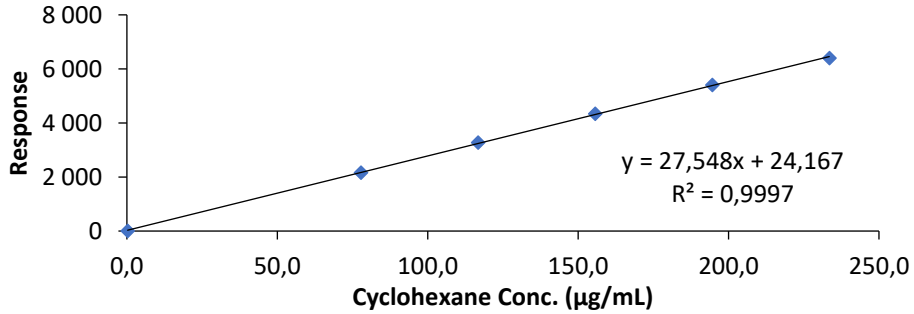


Figure 4.76 – *Linear regression plot of Cyclohexane.*

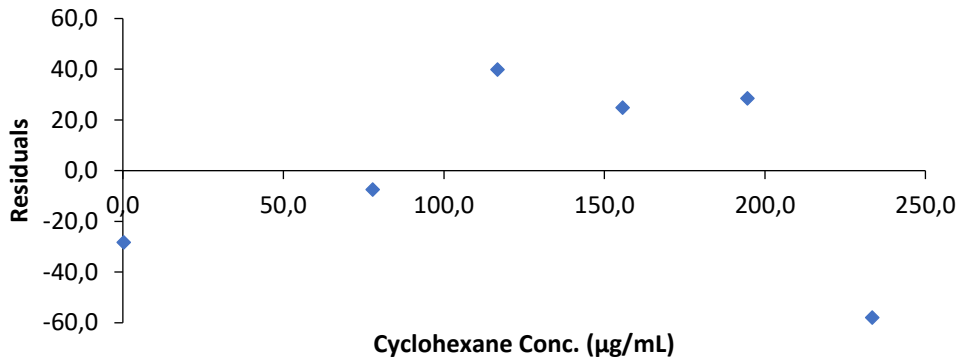


Figure 4.77 – Residual plot of linearity of Cyclohexane.

Table 4.56 – Results of Toluene linearity study.

Level	Con centration in % (relative to test)	Concentration (µg/mL)	Average Peak Area (n=3)	RSD (%)
1	6.80	2.4	10	2.13
2	50.0	17.7	63	1.06
3	75.0	26.6	95	1.13
4	100.0	35.4	131	4.69
5	125.0	44.3	162	2.23
6	150.0	53.2	188	0.44

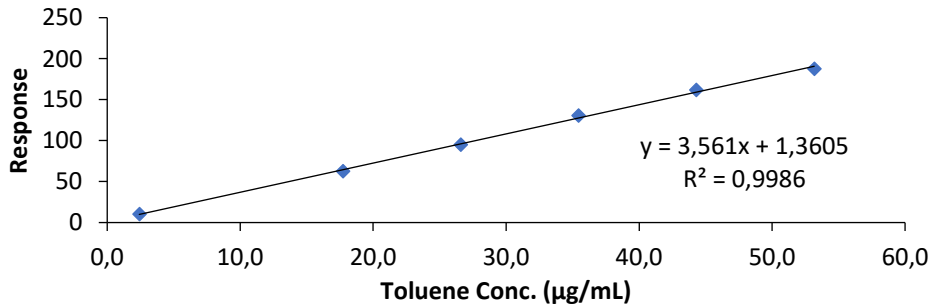


Figure 4.78 – Linear regression plot of Toluene.

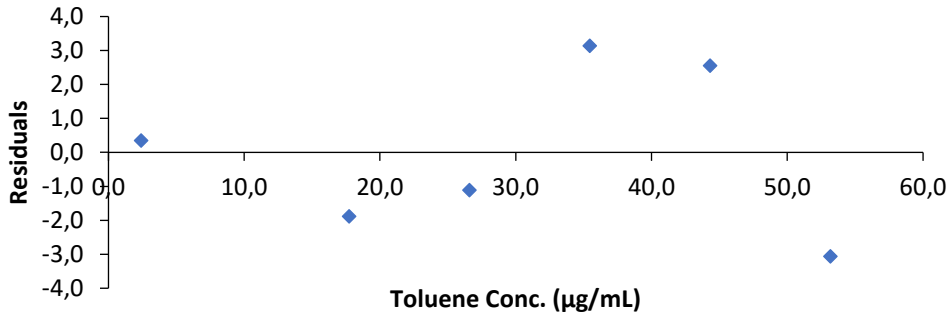


Figure 4.79 – Residual plot of Toluene linearity.

Table 4.57 – Parameters of the Residual solvents linearity study

Parameter	Methanol	Ethanol	Acetone	Isopropyl alcohol	Methylene chloride	Ethyl acetate	THF	Cyclohexane	Toluene
Correlation coefficient	1.000	0.999	1.000	0.999	1.000	1.000	1.000	1.000	0.999
R Squared	0,999	0.999	1.000	0.998	0.999	1.000	1.000	1.000	0.999
Interception	-3.6366	-2.4890	-3.4309	-4.4392	0.2934	-2.6402	1.5364	24.1673	1.3605
Slope	1.5769	1.5343	5.2110	1.5444	1.0677	2.8876	6.2879	27.5482	3.5610

The results met the requirements. As shown in *Table 4.57* the correlation coefficient for each solvent is ≥ 0,99 and there is no systematic trend in residuals.

One can conclude that the method is linear in the concentrations range tested, for each of the components tested.

4.7.5 Accuracy

Accuracy can be inferred from linearity. In this case, a new straight line shall be calculated in which the calculated concentration is reported, by interpolation of the straight line obtained in the linearity study, as a function of the experimentally prepared concentration.

Besides that, also recovery was calculated to demonstrate how close the results are from theoretical values. The results are present in *Tables 4.58*- 4.66 and the regression plots in *Figures 4.80-4.96*.

Table 4.58 – Results of Methanol accuracy study.

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
4,8	4,2	114,4
59,3	59,0	100,5
89,0	88,1	101,0
118,6	121,8	97,4
148,3	149,2	99,4
177,9	175,6	101,4

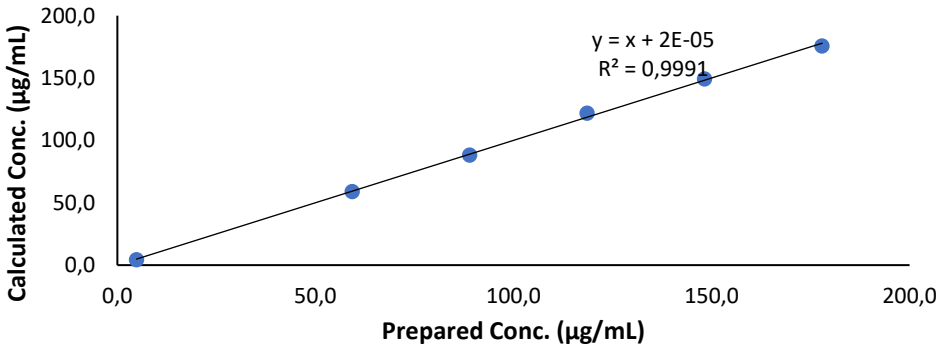


Figure 4.80 - Linear regression plot of Methanol

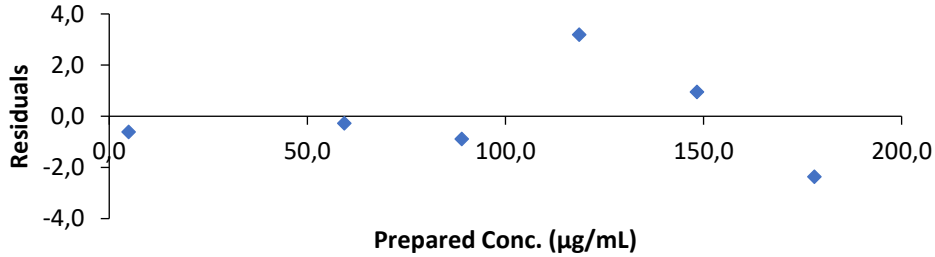


Figure 4.81 – Residual plot of Methanol accuracy

Table 4.59 – Results of Ethanol accuracy study.

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
5,2	5,6	93,3
100,6	97,4	103,3
150,9	144,9	104,1
201,2	201,9	99,7
251,5	248,7	101,2
301,8	289,9	104,1

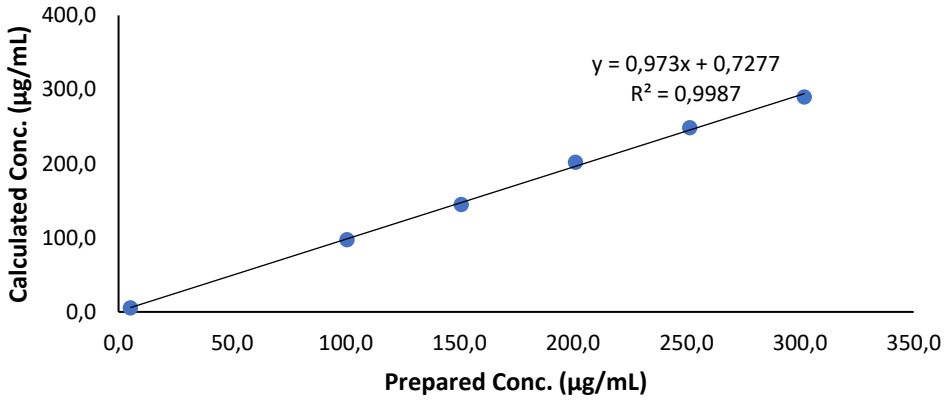


Figure 4.82 – Linear regression plot of Ethanol

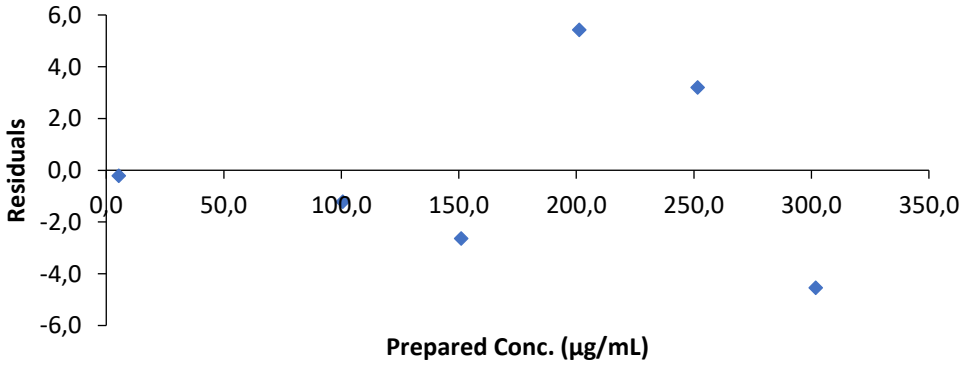


Figure 4.83 – Residual plot of Ethanol accuracy

Table 4.60 – Results of Acetone accuracy study

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
1,7	0,9	180,9
100,8	100,6	100,3
151,2	151,7	99,7
201,6	203,2	99,2
252,0	252,9	99,7
302,4	300,5	100,7

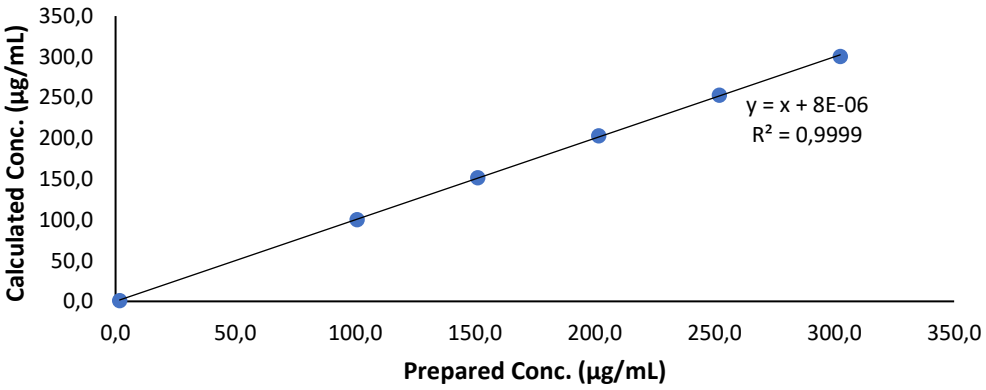


Figure 4.84 – Linear regression plot of Acetone

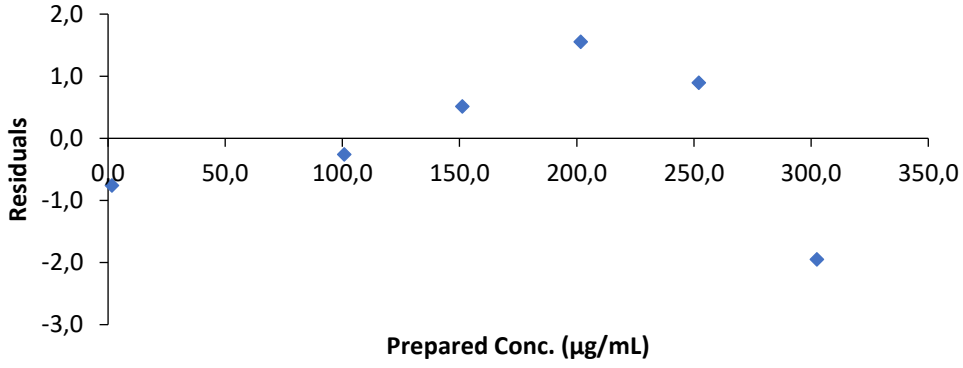


Figure 4.85 – Residual plot of Acetone accuracy.

Table 4.61 – Results of Isopropyl alcohol accuracy study

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
5,0	3,9	130,8
100,0	99,3	100,7
150,1	147,9	101,4
200,1	206,6	96,9
250,1	253,4	98,7
300,1	294,4	101,9

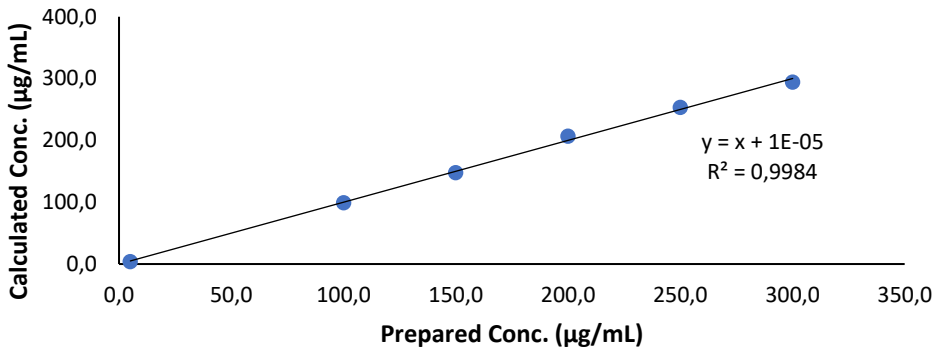


Figure 4.86 – Linear regression plot of Isopropyl alcohol

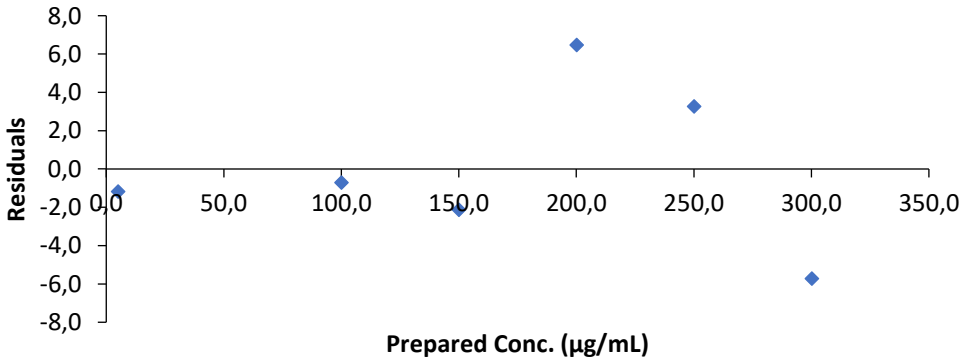


Figure 4.87 – Residual plot of Isopropyl Alcohol accuracy.

Table 4.62 – Results of methylene chloride accuracy study

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
4,2	4,2	99,2
11,9	11,7	101,7
17,9	17,8	100,8
23,9	24,3	98,4
29,8	30,0	99,3
35,8	35,5	100,9

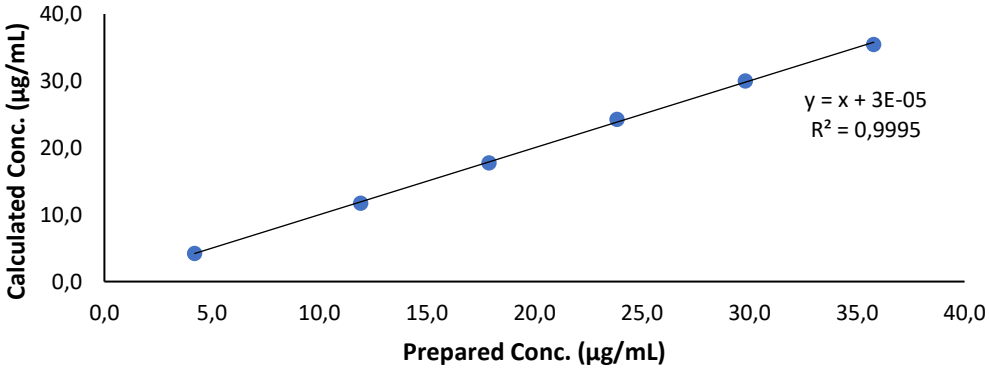


Figure 4.88 – Linear regression plot of Methylene Chloride

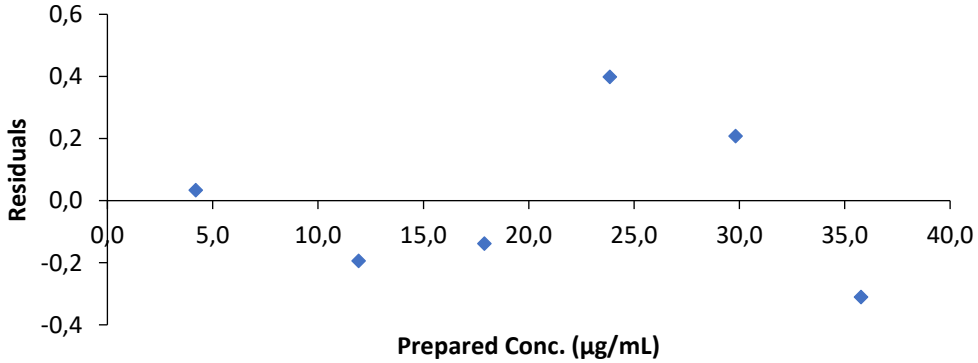


Figure 4.89 – Residual plot of Methylene Chloride accuracy.

Table 4.63 – Results of ethyl acetate accuracy study

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
2,2	1,4	154,1
99,2	98,7	100,5
148,8	148,7	100,1
198,4	201,4	98,5
248,1	249,0	99,6
297,7	295,1	100,9

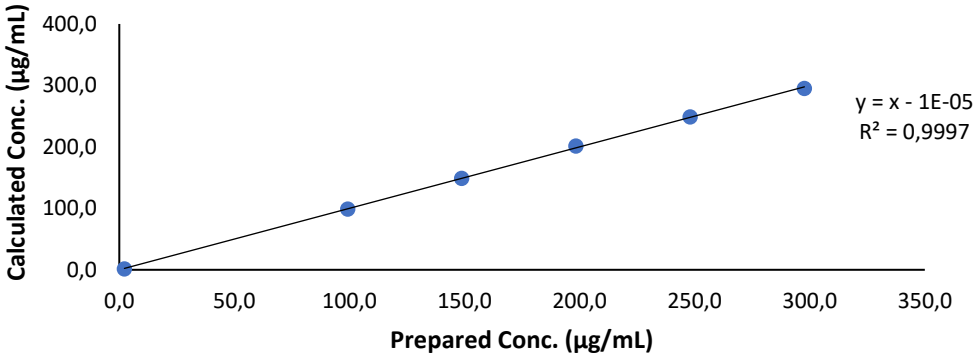


Figure 4.90 - Linear regression plot of Ethyl Acetate

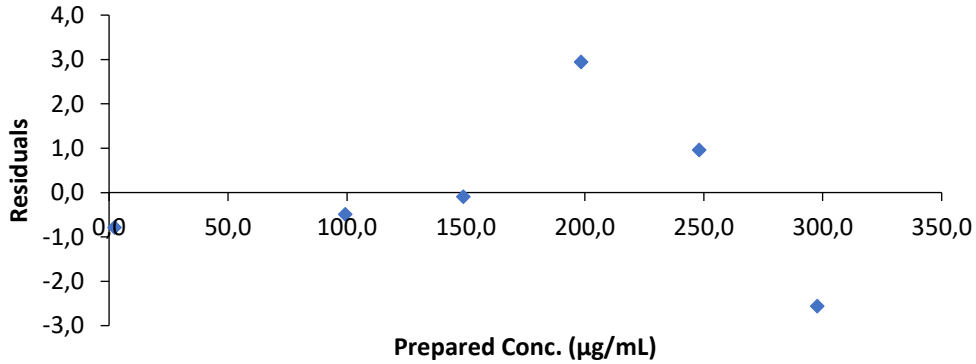


Figure 4.91 - Residual plot of Ethyl Acetate accuracy.

Table 4.64 – Results of tetrahydrofuran accuracy study

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
1,3	1,5	88,4
14,4	14,1	102,6
21,7	21,5	100,9
28,9	29,2	98,8
36,1	36,4	99,2
43,3	43,1	100,6

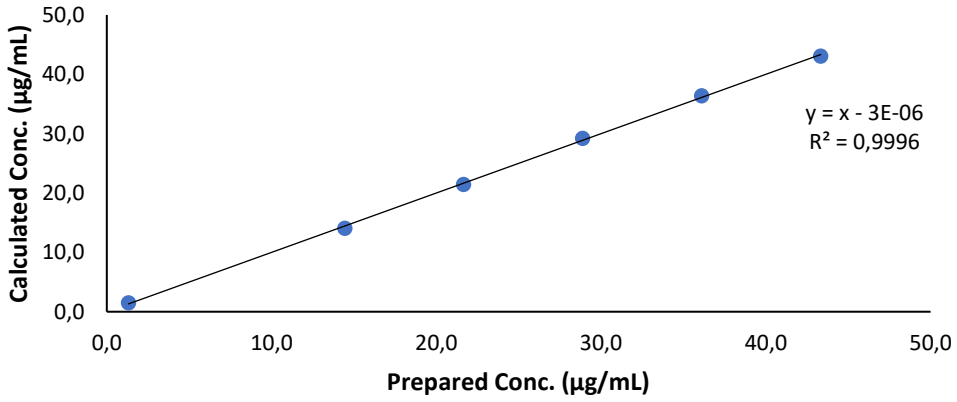


Figure 4.92 – Linear regression plot of Tetrahydrofuran

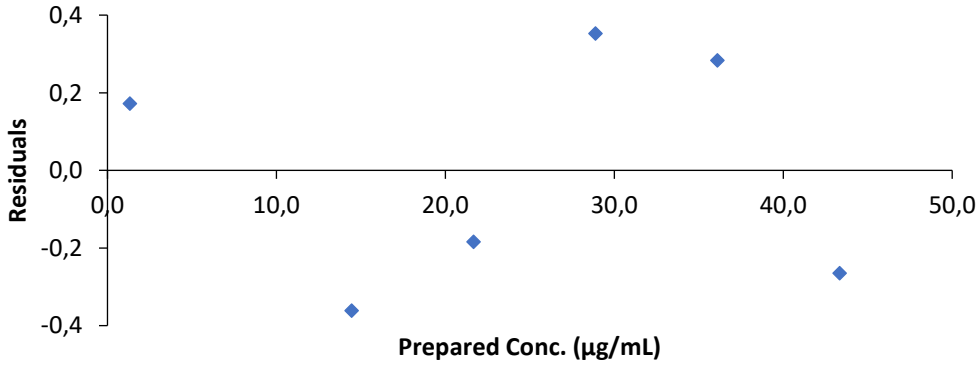


Figure 4.93 – Residual plot of Tetrahydrofuran accuracy.

Table 4.65 – Results of cyclohexane accuracy study

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
77,8	77,6	100,3
116,7	118,2	98,8
155,6	156,6	99,4
194,6	195,6	99,5
233,5	231,4	100,9

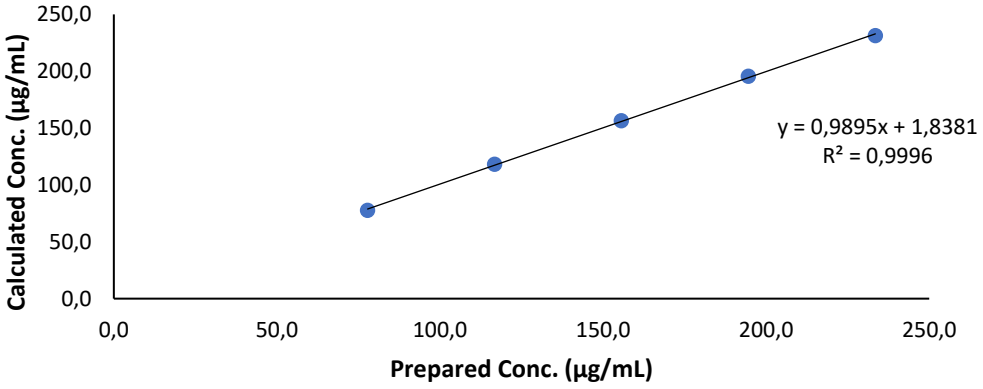


Figure 4.94 – Linear regression plot of Cyclohexane

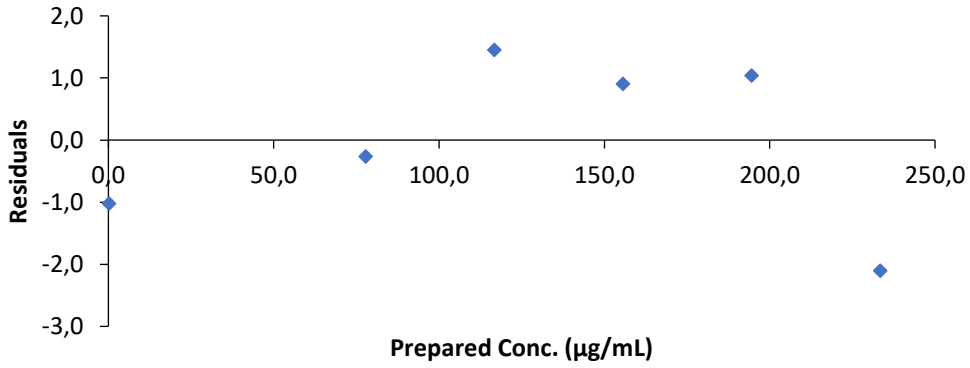


Figure 4.95 – Residual plot of Cyclohexane accuracy.

Table 4.66 – Results of toluene accuracy study

Prepared Concentration (µg/mL)	Theoretical Concentration (µg/mL)	Recovery (%)
2,4	2,5	96,0
17,7	17,2	103,1
26,6	26,3	101,2
35,4	36,3	97,6
44,3	45,0	98,4
53,2	52,3	101,6

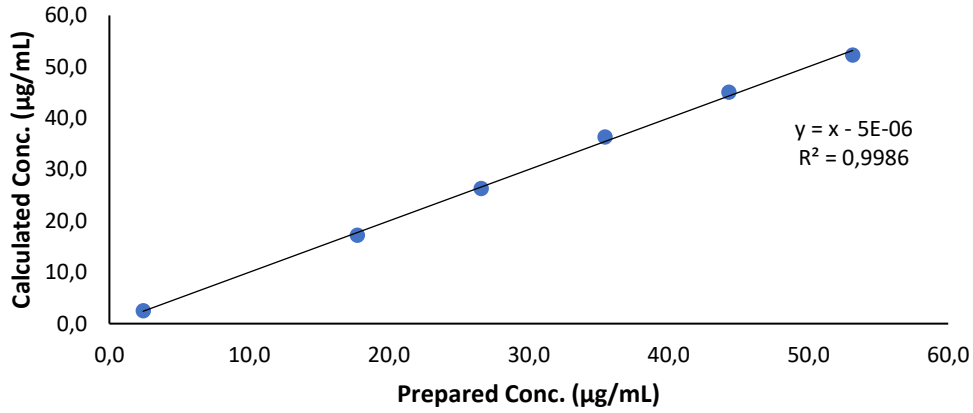


Figure 4.96 – Linear regression plot of Toluene.

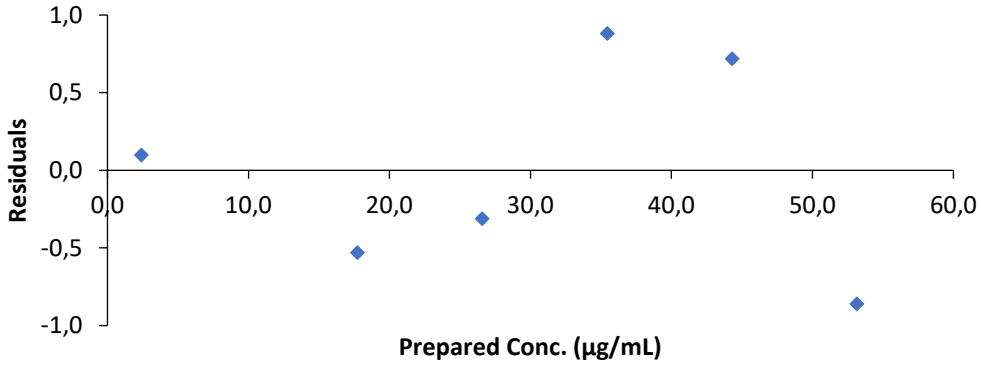


Figure 4.97 – Residual plot of Toluene accuracy.

Table 4.67 – Parameters of the Residual Solvents accuracy study

Parameter	Methanol	Ethanol	Acetone	Isopropyl alcohol	Methylene chloride	Ethyl acetate	THF	Cyclohexane	Toluene
Correlation coefficient	0,999	0,999	0,999	0,999	0,999	0,999	0,999	0,999	0,999
R Squared	0,999	0,999	0,999	0,998	0,999	0,999	0,999	0,999	0,999
Interception	2,3E-05	0,728	8,0E-06	1,0E-05	3,2E-05	-9,7E-06	-3,1E-06	1,1E-05	-4,8E-06
Slope	0,999	0,973	1,000	1,000	1,000	0,999	0,999	1,000	1,000

The results met the requirements. As shown in *Table 4.67* the correlation coefficient for each solvent is ≥ 0,99 and there is no systematic trend in residuals.

One can conclude that the method is accurate in the concentrations range tested, for each of the components tested.

4.7.6 Precision (System Repeatability)

This parameter tests the variability due only to the instrument under the chromatographic conditions indicated in the method. Residual solvents standard solution was injected six times.

The results shown in the *Tables 4.68-4.69* are expressed in terms of average and RSD of areas and retention times.

Table 4.68 – System repeatability of API Residual solvents.

Identification		1	2	3	4	5	6	Average	RSD (%)
Methanol	**R_t**	5.68	5.68	5.68	5.68	5.68	5.68	5.68	**0.01**
	Area	180	187	185	193	190	186	187	**2.26**
Ethanol	**R_t**	7.71	7.71	7.71	7.71	7.71	7.71	7.71	**0.01**
	Area	305	311	306	324	320	309	312	**2.43**

Table 4.69 – System repeatability of API Residual solvents (continuation).

Identification		1	2	3	4	5	6	Average	RSD (%)
Acetone	**R_t**	9.00	9.00	9.00	9.00	9.00	9.00	9.00	**0.01**
	Area	1066	1064	1060	1081	1081	1063	1069	**0.88**
Isopropyl alcohol	**R_t**	9.60	9.60	9.60	9.60	9.60	9.60	9.60	**0.02**
	Area	305	310	307	324	319	310	312	**2.42**
Methylene chloride	**R_t**	10.56	10.57	10.56	10.56	10.56	10.56	10.56	**0.01**
	Area	26	26	26	27	27	26	26	**1.17**
Ethyl acetate	**R_t**	13.40	13.40	13.40	13.40	13.40	13.40	13.40	**0.00**
	Area	574	574	571	586	584	574	5767	**1.08**
THF	**R_t**	13.73	13.73	13.73	13.73	13.73	13.73	13.73	**0.01**
	Area	184	183	183	186	186	183	184	**0.79**
Cyclohexane	**R_t**	14.09	14.09	14.10	14.10	14.10	14.09	14.10	**8.39**
	Area	4377	4313	4305	4333	4345	4309	4330	**0.63**
Toluene	**R_t**	16.64	16.64	16.64	16.64	16.64	16.64	16.64	**0.00**
	Area	127	129	128	134	132	128	130	**2.23**

The system met the repeatability requirements. The RSD of the peak area response is ≤ 15,0 %, for each solvent.

4.7.7 Precision (Method Repeatability)

For evaluating the method repeatability, six sample solutions were spiked with residual solvents at their specification limit concentration and analysed. Three non-spiked sample solutions were also prepared to determine the residual solvent content in the matrix. The amount of solvents present in the matrix was considering for the calculation of the recovery percentage.

The results are expressed in terms of average, SD and RSD of the peak area responses and recovered percentage in *Table 4.70*.

Table 4.70 – Precision results (%recovery) of API residual solvents.

Solvent	Methanol	Ethanol	Acetone	Isopropyl alcohol	Dichloromethane	Ethyl Acetate	THF	Cyclohexane	Toluene
% Recovery	99,3	103,0	94,8	106,1	97,6	104,3	102,8	101,3	106,4
	103,7	105,6	98,4	109,7	100,0	107,0	105,1	103,3	109,5
	100,5	103,7	95,2	106,6	97,7	104,7	103,0	101,3	106,8
	96,3	98,1	91,4	101,6	94,4	101,6	100,5	100,8	102,4
	98,6	100,1	92,3	102,4	95,6	102,5	101,3	101,2	102,9
	102,4	104,7	95,9	107,7	98,2	105,2	103,2	100,7	107,8
Average	**100,1**	**102,7**	**94,7**	**105,7**	**97,3**	**104,2**	**102,7**	**101,2**	**106,0**
RSD (%)	**2.67**	**3.01**	**2.68**	**2.96**	**2.05**	**1.87**	**1.55**	**0.58**	**2.62**

The results have showed that there are no significant differences. The RSD values obtained are lower than 15,0% and the % of recovery obtained for each substance is within 90% and 110%.

The system met the precision requirements.

4.8 Content of N-methyl D-glucamine

The method transfer includes the evaluation of following parameters: specificity, linearity and precision (method repeatability).

4.8.1 Specificity

The method is considered to be specific if it is capable of accurately quantifying the analyte with no interference from excipients or other substances present in the sample matrix.

Specificity of the method was confirmed by testing blank and sample solution, *Table 4.71*.

Table 4.71 – Specificity results of N-methyl D-glucamine content

Solution	Volume of 0,1N Perchloric acid solution consumed (mL)
Blank	0,12
Sample	3.95

4.8.2 Linearity

Linearity test study the proportionality between analyte concentration and instrument response.

To evaluate the linearity of this method, API sample solutions were tested at five different concentrations, ranging from 50% to 150% of sample concentration. Linearity test was done in triplicate. Results of the linearity study for N-methyl D-glucamine are shown in the *Table 4.72* and the regression plot in *Figure 4.98*.

Table 4.72 – N-methyl D-glucamine content - Linearity results.

Level	% Nominal Concentration	API weight (mg), in anhydrous basis	Average API weight (mg), in anhydrous basis	Volume of 0,1N Perchloric acid solution consumed (mL) - Blank	Average Volume of 0,1N Perchloric acid solution consumed (mL)	RSD (%)
1	48.5	98.3 95,2 97.6	97.1	1.6 1.6 1.7	1.7	5.60
2	71.1	145.1 142.0 139.5	142.3	2.6 2.6 2.5	2.6	3.13
3	100.1	207.4 197.6 195.3	200.1	4.0 3.8 3.8	3.9	3.34
4	120.5	239.7 243.1 239.9	240.9	4.4 4.5 4.6	4.5	2.76
5	146.5	294.2 293.9 291.1	293.0	5.6 5.5 5.3	5.4	2.66

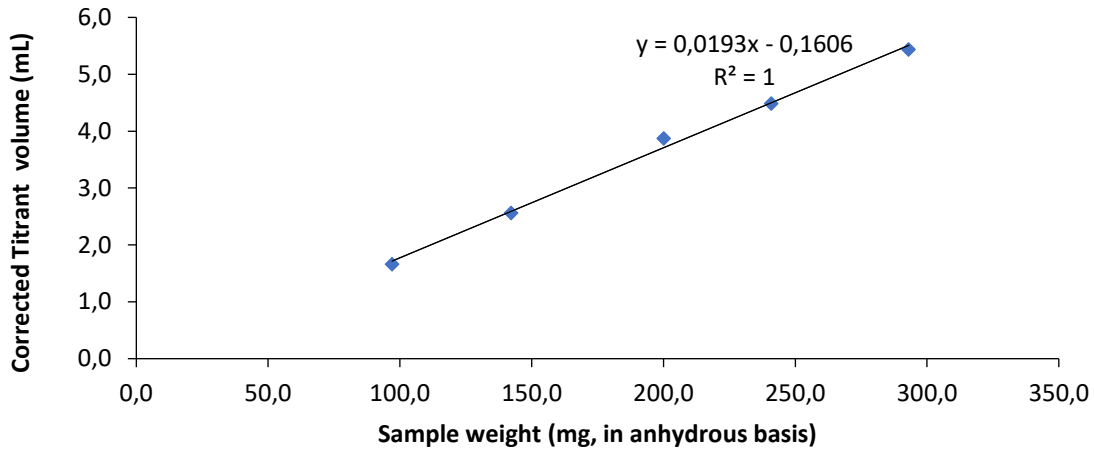


Figure 4.98 – Linear regression plot of N-methyl D-glucamine.

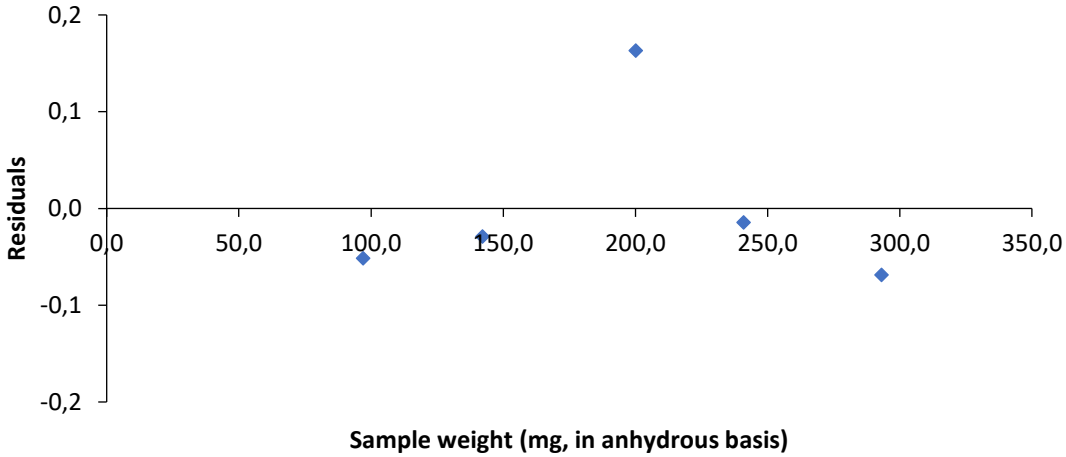


Figure 4.99 – Residual plot of low range linearity of N-methyl D-glucamine.

Table 4.73 – Parameters of the N-methyl D-glucamine linearity study.

Parameter	Value	Result
Correlation coefficient	0.998	Complies
R Squared	0.996	---
Interception	-0.1606	---
Slope	0.0193	---

Analysing the *Figure 4.99*, there is no systematic trend in residual plot and the correlation coefficient is ≥ 0,99. Besides that, all of parameters present in *Table 4.73* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is linear in the concentration range tested.

4.8.3 Accuracy

Accuracy can be inferred from linearity. In this case, a new straight line shall be calculated in which the calculated concentration is reported, by interpolation of the straight line obtained in the linearity study, as a function of the experimentally prepared concentration.

Besides that, also recovery was calculated to demonstrate how close the results are from theoretical values. The results are present in *Table 4.74* and the regression plot in *Figure 4.100.*

Table 4.74 – Results of N-methyl D-glucamine accuracy study.

API weight (mg), in anhydrous basis	API theoretical weight (mg), in anhydrous basis	Recovery (%)
97,1	94,6	102,6
142,2	141,1	100,8
200,1	209,0	95,7
240,9	240,7	100,1
293,1	290,2	101

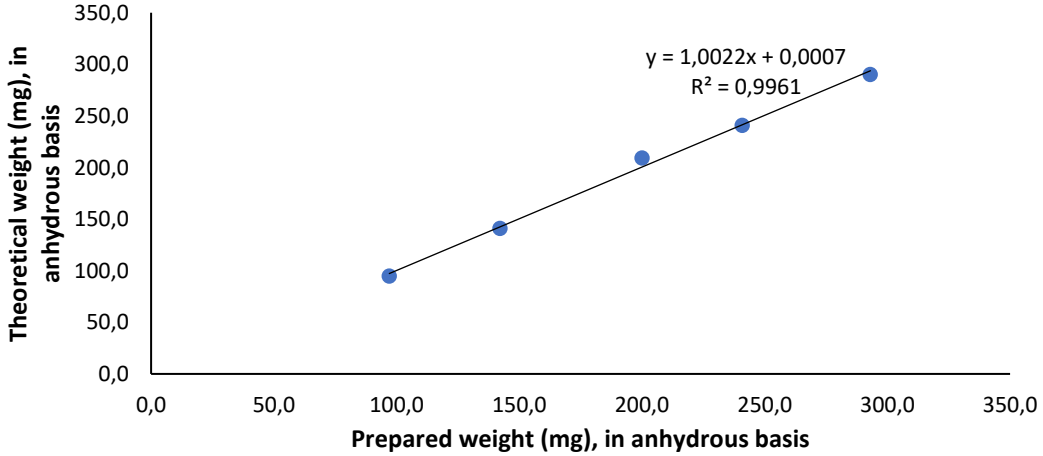


Figure 4.100 – Linear regression plot of N-methyl D-glucamine for Accuracy study.

Table 4.75 – Parameters of the N-methyl D-glucamine accuracy study

Parameter	Value	Result
Correlation coefficient	0,998	Complies
R Squared	0,996	Complies
Interception	0,0007	---
Slope	1,002	---

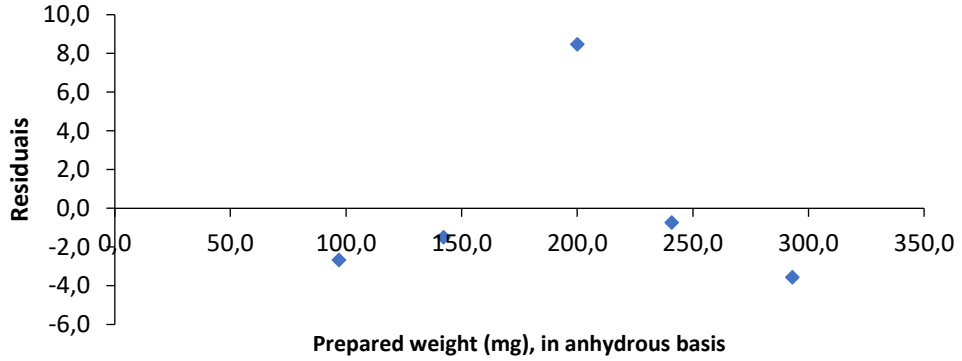


Figure 4.101 – Residual plot of N-methyl D-glucamine accuracy.

Analysing the *Figure 4.101*, there is no systematic trend in residual plot. Besides that, all of parameters present in *Table 4.75* complies with acceptance criteria, so the results met the requirements. One can conclude that the method is accurate in the concentration range tested

4.8.4 Precision (Method Repeatability)

The method repeatability tests the variability of the method by means of a series of tests on the same homogeneous sample. To evaluate the repeatability of the method, 6 independent sample solutions were prepared and titrated, as explained in the protocol. The results are shown in *Table 4.76.*

Table 4.76 – N-methyl D-glucamine content – Method repeatability results.

API weight (mg)	Volume of 0,1N Perchloric acid solution consumed (mL)	N-methyl D-glucamine content (on anhydrous basis) %
211.6	4.1	37.8
201.6	3.9	37.8
193.0	3.5	35.3
199.2	3.9	37.7
194.0	3.7	37.0
198.58	4.0	38.9
Average		**37.4 %**
RSD		**3.18 %**

The system met the requirements. The % RSD of N-methyl D-glucamine content on anhydrous basis results obtained from six replicates is not more than 5,0.

4.9 Water Content: Semi-Micro.determination (KF)

The parameters considered for the method transfer included system suitability.

4.9.1 System Suitability

The values obtained for the water content determination are reported in the *Table 4.77*.

The percentage recovery (r) after each addition was determined using the following *Equation 4.1*:

$$r = 100 \times \frac{W_2}{W_1} \qquad (4.1)$$

where, W_1 is the amount of water added, in milligrams and W_2 is the amount of water found, in milligrams.

Table 4.77 – API water content – Suitability.

Determination	Added water (mg)	Cumulative water added (mg)	M + Cumulative water determined (mg)	Recovered water, *r* (%)
1	2,5	2,5	4,8	100,5
2	2,4	5,0	7,3	103,2
3	2,5	7,5	9,8	102,8
4	2,5	10,0	12,4	101,8
5	2,5	12,5	15,0	104,9
6	2,5	15,0	17,5	97,6
7	2,5	17,5	20,0	99,2
8	2,5	20,0	22,5	100,1
9	2,5	22,5	25,1	104,6
		Average		**101.6**
		RSD (%)		**2.42**

The regression line was calculated between the cumulative water added (*x*-axis) and the sum of the initial water content determined for the substance (*M = 2.2 mg*) and the cumulative water determined after each addition (*y*-axis), *Figure 4.102*.

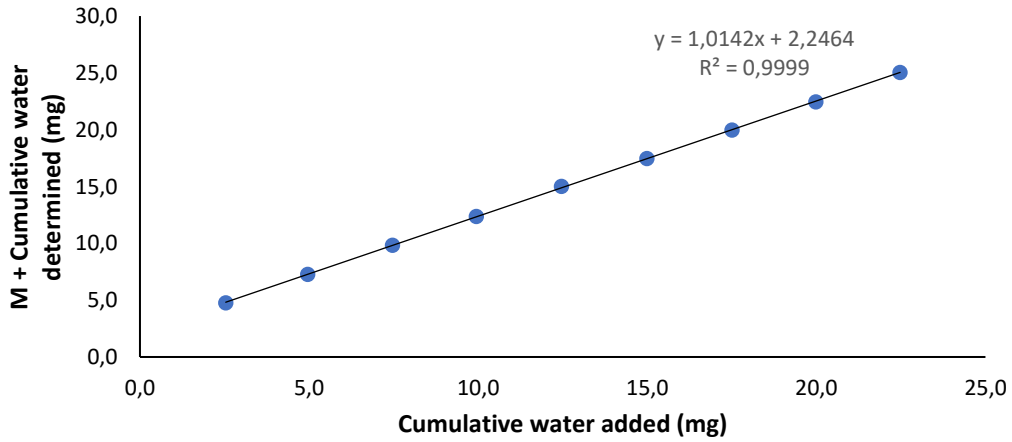


Figure 4.102 - Linear regression plot for water content determination.

The percentage errors (e_1 and e_2) were calculated using the following expressions:

$$e_1 = 100 \times \frac{a-M}{M} \quad (4.2)$$

$$e_2 = 100 \times \frac{|d|-M}{M} \quad (4.3)$$

where *a* is the y-axis intercept, in milligrams of water, *d* is the x-axis intercept, in milligrams of water and *M* is the water content of the substance, in milligrams of water.

The results are presented in the *Table 4.78*.

Table 4.78 – Linear regression parameters and results.

Parameter	Result
M	2.2 mg
a (y-axis intercept)	2.2464
b(slope)	1.0142
d (=a/b)	2.2151
Correlation coefficient	1,000
e_1	1.69
e_2	0.27
$\bar{r}$	101.6%

The results obtained confirmed that the reagent/solvent system is considered to be acceptable to determine the water content of API drug substance, since || and || are not greater than 2,5% and *b* is between 0,975 and 1,025.

5. Conclusions

The main goal of this work was achieved: the analytical methodology used to control the drug substance is considered transferred. The transferred methods: Drug substance identification and Assay by HPLC, Content of N-methyl D-glucamine, Water content, Related substances by HPLC (Tests 1 and 2), Stereochemical purity by HPLC, Content of acetic acid by HPLC and Residual solvents by GC met the requirements and established criteria.

All the parameters evaluated in the analytical transfer, such as specificity, linearity, precision, accuracy and quantitation limit comply with the defined acceptance criteria.

The analytical method for Drug substance identification proved to be specific, since there are no peaks interfering with the peaks of the substances to quantify should be detected.

The analytical method for Drug substance assay proved to be specific, it proves to be linear and accurate for a concentration range between 152.3 µg/mL and 456.8 µg/mL and has repeatability (system and method) as the relative standard deviation for both was less than 2.0%, thus demonstrating precision.

The analytical method for related substances determination (test 1) proved to be specific. The method proved to be linear and accurate in quantitative determination of API impurities with correlation coefficients greater than 0.99 and randomly distributed residues, for a concentration range of ≈ 0.6 – 4.7 µg/mL. The method allows quantifying the API from 0.6 µg/mL, impurity A from 0.5 µg/mL, impurity B from 0.8 µg/mL, impurity C from 0.6 µg/mL, impurity D from 0.6 µg/mL and impurity F from 0.2 µg/mL, confirming the quantitation limits defined by the API supplier. In addition, both API and impurities showed system repeatability with relative standard deviation below 5.0% and method repeatability with relative standard deviation below 10.0%.

The analytical method for related substances determination (test 2) proved to be specific, it proves to be linear and accurate for quantitative determination of Impurity E over the concentration range of ≈ 1.4 – 4.2 µg/mL and has repeatability (system and method) as the coefficient of variation for both was less than 5.0% and 10.0%, respectively. Besides, the quantitation limit defined by the API supplier was experimentally checked validated at a concentration of 0.2 µg/mL. However, as no impurity peak was observed E, it was decided to confirm the reporting threshold at 1.4 µg/mL (0.07% relative to nominal concentration).

The analytical method for stereochemical purity demonstrated to be specific, precise showing system repeatability with relative standard deviation below 5.0% and method repeatability with

relative standard deviation below 10.0%. It proves to be accurate and linear, for a concentration range of ≈ 0.1 – 1.4 µg/mL. In addition, the method allows quantifying the analyte from 0.1 µg/mL.

The analytical method to determine the content by acetic acid proved to be specific, precise showing system repeatability with relative standard deviation below 5.0% and method repeatability with relative standard deviation below 10.0%. It proves to be accurate and linear, for a concentration range of ≈ 4.2– 187.2 µg/mL. In addition, the method allows quantifying the analyte from 4.3 µg/mL.

The analytical methods for residual solvents determination demonstrated to be specific. The methods proved to be linear and accurate with correlation coefficients greater than 0.995 and randomly distributed residues. The quantitation limit was confirmed with all coefficients of variation less than 15.0%, and has repeatability (system and method) as the relative standard deviation for both was less than 15.0%, thus demonstrating precision.

The analytical method to determine the content of N-methyl D-glucamine proved to be specific, linear and accurate to a concentration range from 50% to 150% of sample concentration and precise, with relative standard deviation for method repeatability below 5.0%.

The analytical method to determine water content by semi-micro determination proved to be suitable and linear with less than 5.0 w/w of water content and an average of percentage of recovery of 101.6%.

In summary, the analytical method for Drug substance analysis was considered transferred successfully since the analytical method transfer validation performed during this thesis, reached the same conclusions obtained by the API manufacturer which is responsible for the analytical method development. The validation of analytical methods is extremely important because it allows guaranteeing mechanisms in quality control, allows identifying critical variables that ensure viable results so that routine analyzes reproduce consistent values compared to a reference value.

5.2 Future Work

After the transfer of analytical procedures of the active substance are concluded and guarantee the ability to perform the entire procedure properly, development of the proposed drug product can be established. In this case, drug product analytical procedures will be developed and validated, along with the development of a formula for the drug product and the manufacture of several batches for analysis.

Likewise, the transfer of analytical procedures, some methods are required to be validated: water content – semi-micro determination, identification and assay by HPLC, related substances by HPLC

(test 1 and 2) and stereochemical purity. In addition to parameters evaluated during this work (specificity, linearity, precision, accuracy and quantitation limit), also intermediate precision, detection limit, relative response factor (RRF), range, intra-daily stability, robustness and evidence of the suitability of the method as stability indicator are evaluated. Concerning methods that do not require validation: description, pH, osmolality, reconstitution time, completeness and clarity of solutions, clarity and degree of opalescence, uniformity of mass of single-dose preparations and dosage units and particulate particles (sub-visible and visible particles), some have already been tested as pH, osmolality, reconstitution time and particulate particles (sub-visible and visible particles).

After the validation of analytical procedures of the proposed drug product is concluded, large scale production can be performed.

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