

Qualitative and quantitative analysis of pharmaceutical preparations containing acetaminophen available in Portugal and Poland

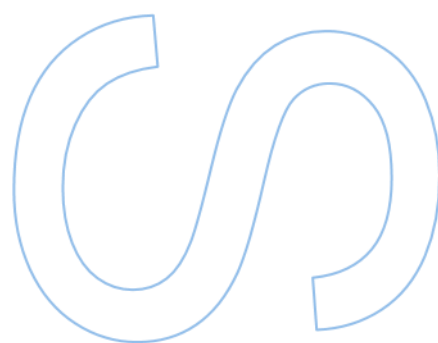
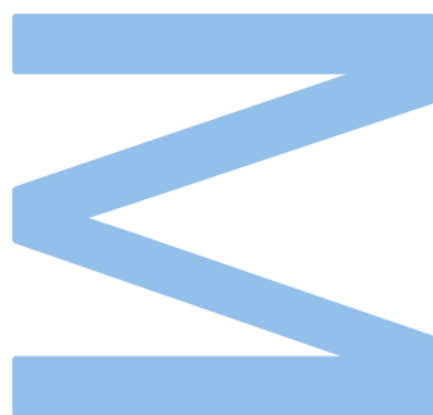
Maria João Polide Lapo

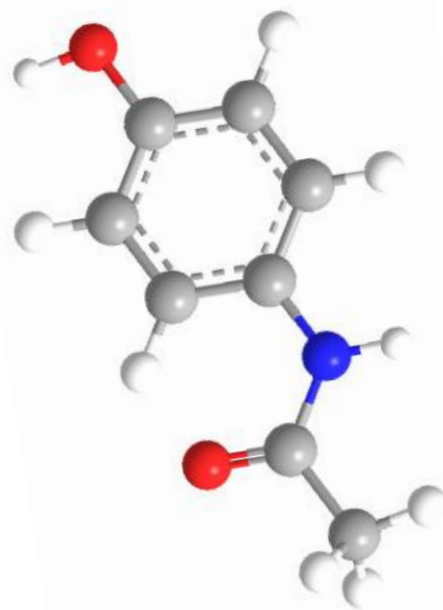
Master in Advanced Methods and Accreditation in Chemical Analysis

Department of Chemistry and Biochemistry

Faculty of Sciences of the University of Porto

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I dedicate this work to my grandparents, whom I miss very much, and I wish they could have seen me get here.

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Resumo

O acetaminofeno, amplamente reconhecido pelas suas propriedades analgésicas, é o princípio ativo de diversos fármacos, e na composição destes podem existir diferentes excipientes. Neste trabalho, propõe-se a análise qualitativa e quantitativa de várias formulações farmacêuticas comercializadas em Portugal e na Polónia (*A1, B2, C3, E5, D4, F6, G7, H8 e I9*), empregando duas técnicas analíticas complementares: espectrofotometria UV-Vis e FTIR-ATR.

A quantificação do acetaminofeno foi realizada com base na construção de uma curva de calibração, através de um método que requereu validação. Foram avaliados parâmetros analíticos cruciais, como exatidão, precisão, linearidade, seletividade, sensibilidade e limites de deteção, com o objetivo de otimizar o procedimento e garantir resultados fiáveis.

A primeira fase do estudo foi conduzida na Universidade de Ciência e Tecnologia de Wroclaw, onde foram preparadas várias soluções padrão de acetaminofeno (0-20 ppm) para delinear a curva de calibração ideal. Com base nesta curva, foram analisadas soluções aquosas das várias formulações através de espectrofotometria UV-Vis, permitindo determinar o teor de acetaminofeno presente em cada medicamento e compará-lo com os valores declarados pelos fabricantes.

Os resultados demonstraram que cinco fármacos (*A1, B2, C3, E5 e I9*) apresentaram variações percentuais inferiores a 3 % em relação às especificações dos fabricantes. No entanto, *D4* e *F6* revelaram discrepâncias significativas de 8,60 % e 10,38 %, respetivamente. As formulações *G7* e *H8* exibiram concentrações de acetaminofeno muito superiores às indicadas nas suas bulas, com desvios de 24 % e 20 %, respetivamente.

A validação do método estabeleceu limites de deteção e quantificação de 0,591 ppm e 1,701 ppm, respetivamente, enquanto a análise de regressão linear confirmou a adequação de um modelo linear, com resultados sólidos em termos de sensibilidade, seletividade e precisão. Os valores de repetibilidade e precisão intermédia, utilizando a absorvância, foram de 0,00164 e 0,000816, respetivamente, com um nível de exatidão de 85 %, perfeitamente enquadrado no intervalo ideal de 80 % a 120 %. Estes resultados atestam a fiabilidade do método desenvolvido para quantificar o acetaminofeno nas formulações analisadas.

Posteriormente, foi realizada uma análise FTIR-ATR na Faculdade de Ciências da Universidade do Porto, visando explorar a complexidade das matrizes farmacêuticas e identificar os excipientes presentes nas suas constituições, que se encontram descritos pelos seus fabricantes, e com base nas suas bandas características de infravermelho. Os resultados obtidos pela técnica revelaram uma grande semelhança entre as formulações em comprimidos, enquanto as formulações de pó solúvel se destacaram pelas suas propriedades hidrofílicas. Excipientes como povidona e diversos tipos de amido foram identificados nos comprimidos, enquanto a sacarose foi predominante nas formulações de pó solúvel.

Os resultados obtidos proporcionam uma compreensão mais aprofundada da composição das formulações farmacêuticas analisadas, além de validar um método analítico eficiente e de baixo custo para a determinação do acetaminofeno.

Palavras-Chave: acetaminofeno, substância ativa, excipiente, espectrofotometria UV-Vis, validação, FTIR-ATR.

Abstract

Acetaminophen, widely recognized for its analgesic properties, serves as the active pharmaceutical ingredient in numerous medications, which may also contain various excipients in their formulations. This study aims to perform both qualitative and quantitative analyses of several pharmaceutical formulations marketed in Portugal and Poland (A1, B2, C3, E5, D4, F6, G7, H8, and I9), employing two complementary analytical techniques: UV-Vis spectrophotometry and FTIR-ATR.

The quantification of acetaminophen was based on the construction of a calibration curve using a method that required validation. Key analytical parameters, such as accuracy, precision, linearity, selectivity, sensitivity, and detection limits, were evaluated to optimize the procedure and ensure reliable results.

The initial phase of the study was conducted at the Wroclaw University of Science and Technology, where several standard solutions of acetaminophen (0–20 ppm) were prepared to establish the ideal calibration curve. Based on this curve, aqueous solutions of the various formulations were analysed via UV-Vis spectrophotometry, allowing for the determination of the acetaminophen content in each medication and comparison with the manufacturer's stated values.

The results indicated that five formulations (A1, B2, C3, E5, and I9) exhibited percentage variations below 3 % relative to the manufacturers' specifications. However, significant discrepancies were observed for D4 and F6, with deviations of 8.60 % and 10.38 %, respectively. Furthermore, formulations G7 and H8 displayed acetaminophen concentrations considerably higher than those specified in their package inserts, with deviations of 24 % and 20 %, respectively.

Method validation established detection and quantification limits of 0.591 ppm and 1.701 ppm, respectively. Linear regression analysis confirmed the suitability of a linear model, demonstrating robust results in terms of sensitivity, selectivity, and precision. Repeatability and intermediate precision values, based on absorbance, were determined to be 0.00164 and 0.000816, respectively, with an accuracy level of 85 %, falling within the ideal range of 80 % to 120 %. These findings confirm the reliability of the developed method for the quantification of acetaminophen in the analysed formulations.

Subsequently, FTIR-ATR analysis was carried out at the Faculty of Sciences of the University of Porto to explore the complexity of pharmaceutical matrices and identify the

excipients present in their compositions, as described by their manufacturers and based on their characteristic infrared absorption bands. The results obtained through this technique revealed substantial similarities among tablet formulations, while soluble powder formulations stood out due to their hydrophilic properties. Excipients such as povidone and various types of starch were identified in tablets, whereas sucrose was predominant in soluble powder formulations.

The findings of this study provide a deeper understanding of the composition of the analysed pharmaceutical formulations and validate an efficient, cost-effective analytical method for the determination of acetaminophen.

Keywords: acetaminophen, active substance, excipient, UV-Vis spectrophotometry, method validation, FTIR-ATR.

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List of Abbreviations

INN	INTERNATIONAL NON-PROPRIETARY NAMES
ATC	ANATOMICAL THERAPEUTIC CHEMICAL
EU	EUROPEAN UNION
EEA	EUROPEAN ECONOMIC AREA
EMA	EUROPEAN PHARMACEUTICALS AGENCY
GMP	GOOD MANUFACTURING PRACTICE
OTC	OVER-THE-COUNTER
NSAID	NONSTEROID ANTI-INFLAMMATORY PHARMACEUTICAL
COX	CYCLOOXYGENASE
USA	UNITED STATES OF AMERICA
IUPAC	INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY
HPLC	HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY
GC	GAS CHROMATOGRAPHY
MS	MASS SPECTROMETRY
LC-MS	LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY
CE	CAPILLARY ELECTROPHORESIS
UV	ULTRA-VIOLET
PXRD	POWDER X-RAY DIFFRACTION
DSC	DIFFERENTIAL SCANNING CALORIMETRY
TGA	THERMALGRAVIMETRIC ANALYSIS
IR	INFRARED
SSNMR	SOLID-STATE NUCLEAR MAGNETIC RESONANCE
FTIR	FOURIER-TRANSFORM INFRARED
ATR	ATTENUATED TOTAL REFLECTANCE
CRM	CERTIFIED REFERENCE MATERIALS
IIL	INTERLABORATORY TESTS
CV	COEFFICIENT OF VARIATION
LOD	LIMIT OF DETECTION
LOQ	LIMIT OF QUANTIFICATION
RSD	RELATIVE STANDARD DEVIATION

1. Introduction

1.1. General definition of pharmaceuticals

A pharmaceutical is any substance or group of substances that has curative or preventive properties, relieves symptoms, and can be administered to a human being with the aim of exerting a pharmacological, immunological, or metabolic action, with the consequence of restoring, correcting, or modifying physiological functions [1].

Characterised as a physical system whose properties depend on the active substance that forms part of its constitution, as well as the excipients and packaging materials, a pharmaceutical must undergo studies that demonstrate its quality, safety, and efficacy [2].

It is a product of high economic interest, whose value is defined by demand in the consumer market, and which will depend on approval by the regulatory authorities. However, it can only be introduced onto a Member State Market if an authorisation is issued by a competent authority, and such a formal application must be accompanied by the following descriptive topics of the medicinal product [3]:

- name of the pharmaceutical;
- qualitative and quantitative composition of its components, mainly of the active substance that is in its composition together with the components of the excipient;
- description of the manufacturing procedure and pharmaceutical form;
- pharmacological properties, clinical information, therapeutic indications and contraindications, and adverse reactions (frequency and severity);
- necessary preventive measures to be adopted in its storage, administration, dosage, among others;
- control methods applied by manufacturers;
- results of tests carried out;
- list of product characteristics;
- manufacturer's production certificate;
- copies of all marketing authorisations for the pharmaceutical from third countries or states.

All these parameters must be updated on a regular basis, and their authorisation to circulate on the market has a short validity of just five years, which is renewable. All these steps and stages are carried out with the main objective of protecting public health and contributing to equal access to quality-assured and controlled pharmaceuticals, which is a fundamental human right for all European citizens [4].

A pharmaceutical is primarily defined by its active substance and excipients. Excipients, also known as inactive substances, encompass any constituents of the medicinal product apart from the active substance and the packaging material [5].

The excipients, although they do not provide the characterising functions of the pharmaceutical, play a role in its appearance and function. They are responsible for giving the tablet its correct shape, making it durable and homogeneous, and they can be non-disintegrating, capsules or in liquid form. They also influence the taste characteristics of the tablet and protect the pharmaceutical from harmful effects from the external environment in which it may be found.

Another function performed by this component is to improve the stability of the pharmaceutical and the bioavailability of the active substance, which, depending on the excipient used, can alter the way it is released, its dosage and localisation in an organism. There has been growing interest in finding excipients, also referred to in this case as absorption promoters, which can be included in new dosage forms that have the ability to perform specific functions and, in a way, directly or indirectly influence the rate of release and absorption of the pharmaceutical [6].

The active substance, by definition, is any substance or mixture thereof, which is intended to be used in the production of a pharmaceutical with the purpose of exerting a pharmacological, immunological, or metabolic effect, with the main aim of restoring, correcting, or modifying physiological functions in a human being, or to make a medical diagnosis [5].

There are countless pharmaceuticals on the market that are fabricated of the same active substance, in the case of the active substance acetaminophen there are numerous pharmaceuticals of distinct brands that share the same constituent and the same therapeutic characteristics. Due to this same issue, the World Health Organisation assigns international non-proprietary names (INN), also known as common names, so that there can be unequivocal identification of pharmaceuticals whose constitution is based on the same active substance [7].

1.1.1. Classification of active substances

In addition to the INNs, the World Health Organisation has also created a system for classifying active substances called Anatomical Therapeutic Chemical (ATC), which divides them into diverse groups according to the organ or system on which they act and also takes into account their therapeutic, pharmacological, and chemical properties. Substances are then categorised based on their therapeutic applications, route of administration and dosage. Pharmaceuticals are categorised into five distinct levels [8]:

ATC 1: this level is subdivided into fourteen anatomical or pharmacological groups;

ATC 2: this is a pharmacological or therapeutic subgroup;

ATC 3 and 4: it is a chemical, pharmacological or therapeutic subgroup;

ATC 5: chemical substance.

Coding is important for obtaining accurate information in epidemiological studies. The five distinct levels allow comparisons to be made at various levels, depending on the purpose of the study. On the other hand, for pharmaceuticals that are made up of more than one active substance, combinations are made in the ATC classification system and codes are assigned that are different from those of a product that only has one active component in its structure.

In the particular case of the active substance under study, acetaminophen, the code N02BE01 is assigned, where N corresponds to anything that affects the nervous system, i.e. the anatomical group under which the active substance exerts its function; N02 represents the group that includes analgesics, which corresponds to the therapeutic group; N02B is the pharmacological group, where acetaminophen falls into the group of analgesics and antipyretics; and finally, N02BE represents the chemical group, in this case anilids [9].

As with acetaminophen, the same classification system is applied to all other existing active substances, making it easier not only to identify one but a mixture of them.

1.1.2. Availability of pharmaceuticals

Accessibility of pharmaceuticals within Europe is contingent upon a multifaceted framework of criteria and regulations meticulously designed to safeguard the safety, efficacy, and quality of medicinal products. Central to this regulatory landscape is the European Union (EU), which assumes a pivotal role in harmonizing pharmaceutical

legislation across its member states. Through comprehensive directives, guidelines, and regulatory mechanisms, the EU establishes robust standards and procedures aimed at ensuring the uniformity and integrity of pharmaceutical practices and products throughout the European Economic Area (EEA). By fostering a cohesive regulatory environment, the EU endeavours to bolster public health outcomes, promote innovation, and engender consumer confidence in the pharmaceutical sector across its diverse member states.

The EU legal framework for human pharmaceuticals establishes standards to safeguard public health and ensure the quality, safety, and effectiveness of approved medications. This framework also aims to facilitate the internal market by encouraging innovation. The fundamental principle is that any medicinal product must undergo evaluation and receive marketing authorization from competent authorities before it can be introduced to the market [10].

Below are some key criteria that contribute to pharmaceuticals availability in Europe [11]:

- marketing authorization: Before a pharmaceutical can be placed on the market in Europe, it must undergo a thorough evaluation by regulatory authorities. The European Pharmaceuticals Agency (EMA) is responsible for the centralized evaluation and authorization of pharmaceuticals in the EU. A marketing authorization is granted only if the pharmaceutical is proven to be safe, effective, and of high quality.
- good manufacturing practice (GMP): Pharmaceutical manufacturers must comply with GMP regulations to ensure that their manufacturing processes meet quality standards. This includes the production, control, storage, and distribution of pharmaceutical products.
- pharmacovigilance: Continuous monitoring of the safety of pharmaceuticals is essential. Pharmaceutical companies are required to have pharmacovigilance systems in place to detect, assess, and prevent adverse reactions to pharmaceuticals.
- batch testing and quality control: Each batch of a pharmaceutical must undergo testing to ensure that it meets specified quality standards. This includes testing for identity, purity, and potency.

- prescription and over-the-counter (OTC) classification: The availability of a pharmaceutical may depend on whether it is classified as prescription-only or available OTC. Some pharmaceuticals may require a prescription from a healthcare professional.
- supply chain and distribution: A well-regulated supply chain is crucial for ensuring that pharmaceuticals reach patients in a timely and secure manner. Distribution practices must comply with regulations to prevent issues such as counterfeiting and tampering.
- reimbursement and pricing: The availability of pharmaceuticals can be influenced by reimbursement policies and pricing strategies in different European countries. These factors can impact the accessibility of certain medications.
- patient access programs: Some pharmaceutical companies may implement patient access programs to facilitate access to certain pharmaceuticals, especially in cases where patients face financial barriers.

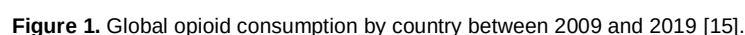
It's important to note that pharmaceutical availability in Europe is a dynamic and multifaceted process, and these criteria may be subject to updates and changes based on evolving regulatory frameworks and healthcare policies.

1.2. Pain medication: analgesics

Categorized as analgesics or painkillers, pain medications represent a pharmacotherapeutic class specifically engineered to mitigate or eradicate pain sensations. Ubiquitously utilized across the globe, they assume a pivotal role in the management of pain induced by a broad spectrum of conditions, ranging from minor traumas to chronic diseases.

The main reason why someone takes an analgesic comes from the discomfort caused by the existence of pain, which is defined as an unpleasant sensory experience associated with actual or potential tissue damage. It is a personal experience that is influenced by factors that surround and are part of the human being, these being biological, psychological, and social [12,13].

Aforementioned issue is raised by the fact that in recent years there has been an exponential increase in the consumption of painkillers around the world, and pain is recognised as a global health problem that affects 20 % of adults. So much so that in the early 2000s there was an opioid crisis that led several countries to develop regulatory measures and limitations on the consumption of analgesics, including dosage and duration limits, removal of certain pharmaceuticals from the market, implementation of prior authorisation requirements and intensified monitoring of prescriptions. Even with these measures and the increasing involvement of the World Health Organisation, consumption levels continue to rise, especially in high-income countries. In Figure 1, we can see a study comparing opioid consumption levels in different countries around the world, and it can be seen that Portugal has a considerably high consumption when compared to Poland but still much smaller than other European countries, such as the UK or Germany. [15].



The world market is full of distinct types of analgesics, including nonsteroidal anti-inflammatory pharmaceuticals (NSAIDs), acetaminophen, and opioids. Each type acts on distinct aspects of the pain process [13].

The first one, the NSAIDs, refer to ibuprofen and naproxen, work by inhibiting the activity of an enzyme called cyclooxygenase (COX), which is responsible for producing chemical messengers that promote inflammation, pain, and fever. By having the capability to blocking COX, NSAIDs reduce the production of those chemical messengers, leading to decreased inflammation and pain. However, it's especially important to notice that NSAIDs have the disadvantage that they also have side effects, such as gastrointestinal irritation and an increased risk of bleeding.

On the other hand, opioids, like morphine, oxycodone, and others, act on a specific receptor in the nervous system called opioid receptors, these are naturally involved in modulating pain signals. Opioids bind to these receptors, reducing the transmission of pain signals and altering the perception of pain in the brain. Although they help a lot of people, especially with chronic pains, they have a huge setback because these can also carry a risk of dependence, tolerance, and side effects such as respiratory depression, making their use a subject of careful consideration and monitoring.

1.3. Acetaminophen

As mentioned above, another type of painkiller is acetaminophen, also called paracetamol, an active substance with an extremely rich history that stretches back to the present day. This is one of the most vastly used OTC pharmaceutical and it was first synthesized by Joseph Von Mering in 1893 [16]. Acetaminophen was developed and recognised as a potent pharmaceutical agent that represents a significant milestone in the field of chemistry and pharmaceutical.

In an initial stage, and because acetaminophen and phenacetin were discovered at the same time, phenacetin gained a lot more popularity, however, it had serious side effects associated and its clinical use declined, and the attention was turned to acetaminophen that was then marketed in 1950. Today, acetaminophen rules the market and is the standard and first-line treatment for fever and acute pain [17].

Even though acetaminophen is part of everyday life for so long, there are many questions about it and about how it works in the human system, and there are doubts as to whether or not this active substance is safe. In the last forty years, acetaminophen toxicity has been primarily responsible for 46 % of all acute liver failure in the United States and between 40 % and 70 % of all cases that have been recorded in Europe [18]. This is

corroborated by the Figure 2, where we can see that paracetamol/acetaminophen is one of the main causes of this disease compared to other factors that lead to liver failure.

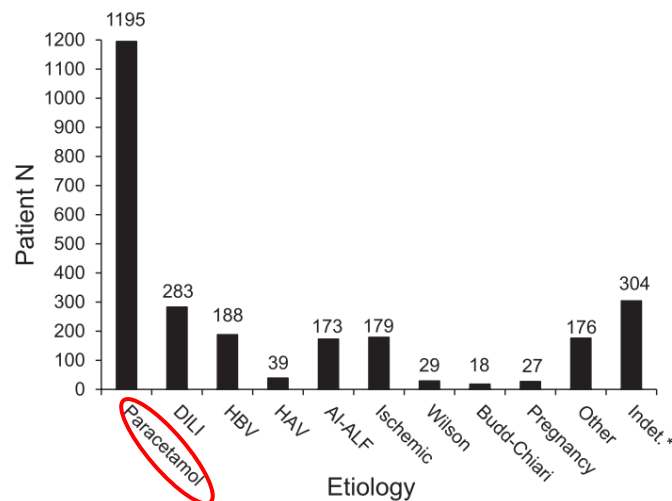


Figure 2. Causes of acute liver failure in US. Data collected between 1998 and 2019 [18].

Following this data, a nationwide surveillance network was established in the USA to monitor instances of acute liver failure, revealing that nearly half of the reported cases were attributed to acetaminophen. They also came to the conclusion that both intentional (suicidal) and unintentional (chronic) poisonings accounted equally for cases of acetaminophen-associated hepatic failure. This highlights the diverse contexts in which acetaminophen toxicity can manifest, encompassing deliberate self-harm as well inadvertent overuse or misuse [19].

1.3.1. Properties and characteristics

Acetaminophen, with the chemical name by IUPAC N-(4-hydroxyphenyl) acetamide, is more commonly known as N-acetyl-p-aminophenol, as its chemical formula is based on a benzene ring replaced by a hydroxyl group and the nitrogen atom of an amide group (Figure 3). This way it can be classified as an acylated aromatic amide.

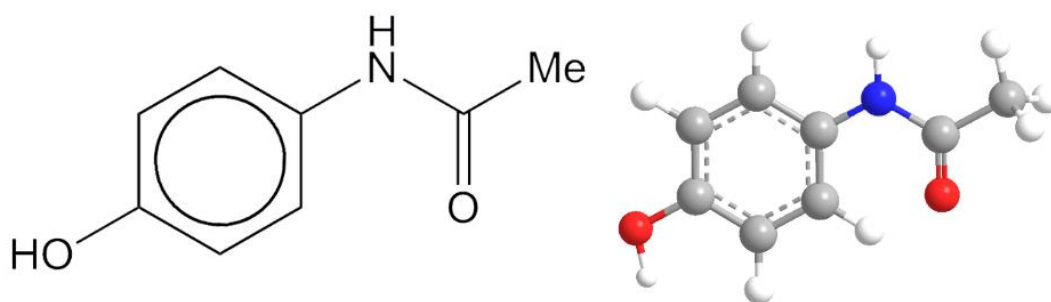


Figure 3. Molecular structure formula of acetaminophen [20].

Along with its chemical formula, the compound has characteristics intrinsic to its existence [21], such as the fact that it is white, has no odour and is a crystalline powder with a characteristic taste. It has a molecular mass of 151.16 g/mole, is extremely soluble in ethanol and chloroform, and moderately soluble in methylene chloride, although it is insoluble in diethyl ether. When handling it, it should be noted that it is an irritant and can cause irritation to the skin and eyes. It is a substance classified as harmful to aquatic environments.

This compound is one of the main ingredients in countless pharmaceuticals manufactured around the world, from cold and flu pharmaceuticals to chronic pain pharmaceuticals, many of which are subject to prescription. It is a constituent that is characterised as safe if administered in normal doses. Unlike other types of painkillers, such as aspirin or ibuprofen, acetaminophen has no anti-inflammatory properties and therefore does not fall into the class of pharmaceuticals known as non-steroidal anti-inflammatories [22].

In adults, can be used for the management of diverse types of painful conditions that can include headaches, period pain, back pain, osteoarthritic pain, musculoskeletal pain, dental pain and so many others. In addition to its numerous applications, it also comes in a variety of forms, including tablets, drops, capsules, injections, and syrup [23].

1.3.2. Mechanism of action

The precise mechanism of action of acetaminophen has not been fully elucidated and it differs from NSAIDs like aspirin or ibuprofen. However, the primary mechanism is believed to involve its interaction with the central nervous system and inhibition of the enzyme COX.

Unlike NSAIDs, which inhibit both COX-1 and COX-2 enzymes, acetaminophen is thought to predominantly inhibit COX-2 in the central nervous system. These enzymes play a crucial role in the synthesis of prostaglandins, which are signalling molecules involved in inflammation, pain, and fever. By inhibiting COX-2 in the brain, acetaminophen reduces the production of prostaglandins, thereby alleviating pain and lowering fever. Besides the central nervous system, there are reasons to believe that this medication also has influential on the peripheral tissues, decreasing the tissue concentration of prostaglandins and pro-inflammatory mediators, whose synthesis is also inhibited by aspirin [24].

Acetaminophen's action is not limited to COX inhibition. Some research suggests that it may affect other neurotransmitters system, such as the endocannabinoid system, which involves pain modulation. But the exact details of these interactions are still under investigation [17].

1.3.3. Possible side effects

Acetaminophen is considered to be extremely safe in normal doses, but due to its wide availability on today's markets, deliberate or accidental overdoses are not uncommon. In recommended doses, it does not affect blood clotting, the kidneys, or the arterial duct of the foetus, nor does it irritate the stomach lining. And unlike other medications, it does not cause mood fluctuations or euphoria, and does not present risks of addiction, dependence, tolerance, or withdrawal [16].

What are considered "normal doses", or standard therapeutic doses, for an adult are within a maximum daily range of 3 to 4 g. In tablet form, this pharmaceutical is available in doses of 500 mg and 1 g, and a maximum of eight tablets (500 mg) can be taken within 24 hours. With regard to children's doses, acetaminophen is marketed in different doses depending on the age of the child, ranging from 60 mg for 2-3 months old to 480-750 mg for 12-16 years old [17].

When the recommended therapeutic amounts are taken into account, the active substance is metabolised very quickly and completely, undergoing glucuronidation and sulphation to inactive metabolites which are eliminated in the urine. However, in cases of higher doses there are several studies, such as the one mentioned above, which show that acetaminophen is associated with liver toxicity and kidney failure, and overdose is a frequent cause of these illnesses in the USA and Europe.

1.4. Analytical Methods for quantifying Acetaminophen

There are several analytical techniques commonly used to quantify acetaminophen. These techniques are used to determine the concentration of acetaminophen in pharmaceutical products, biological samples, as well as other matrices.

Among all those described below, there are points in common and points that clearly differ. They all have their advantages and disadvantages and have applications in different areas and for different purposes.

1.4.1. High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) is a widely used analytical technique for the separation, identification, and quantification of various chemical compounds in complex mixtures. HPLC is a powerful tool in the field of analytical chemistry, and it has been used in various applications such as pharmaceuticals, food, environmental, and forensic analysis [22,25].

In the realm of acetaminophen determination, HPLC stands out for several reasons. Firstly, its heightened sensitivity enables the detection of acetaminophen even at low concentrations, a critical attribute for analyses involving biological fluids where acetaminophen concentrations are often minimal. Secondly, HPLC's selectivity is advantageous in effectively separating acetaminophen from complex matrices, such as pharmaceutical formulations and biological fluids. Thirdly, its rapidity and automation capability make it conducive to high-throughput analyses. For example, in this study by Sarah F. Cook et. al [26], the aim was to quantify and study the metabolism of acetaminophen that influences susceptibility to hepatotoxicity induced by the same active substance in experimental clinical trials.

However, despite its merits, HPLC harbours limitations. The apparatus is capital-intensive and necessitates skilled personnel for proficient operation and maintenance. Additionally, relatively substantial sample sizes are requisite, rendering it less suitable for trace compound analyses. The potential for interference from other compounds in the sample matrix may compromise the accuracy and precision of the analysis.

To quantify the active agent, this technique requires the coupling of a detector to form more complete sets, such as high-pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/M), high-pressure liquid chromatography-time-of-flight mass spectrometry (HPLC-MS-TOF), high-pressure liquid chromatography-hybrid quadrupole

orbitrap high-resolution mass spectrometry (HPLC-Q-Orbitrap-HRMS), and many others [27].

1.4.2. Gas Chromatography (GC)

Gas Chromatography (GC) stands out as a potent technique for the quantification of acetaminophen, leveraging its capacity to separate and quantify volatile compounds based on their distinctive physical and chemical attributes [28].

The utility of GC in acetaminophen quantification is underscored by its array of advantages. GC enables the detection of acetaminophen even at low concentrations. Its inherent selectivity is noteworthy, allowing the effective separation of acetaminophen from other components within the sample matrix. Moreover, GC's expeditious nature and automation capability render it amenable to high-throughput analyses.

However, inherent limitations characterize GC. Its destructive nature, necessitating sample vaporization, may result in the loss of volatile compounds. Additionally, GC is confined to the determines of volatile compounds, precluding the examination of non-volatile counterparts [29].

In the realm of acetaminophen quantification, GC methodologies have found application across diverse matrices, encompassing cerebrospinal fluid, urine and plasma [30]. GC methodologies frequently incorporate a derivatization step to convert acetaminophen into a volatile derivative amenable to GC analysis. Rigorous validation protocols attest to the accuracy, precision, and linearity of GC methods in the determination of acetaminophen [31].

To quantify the active agent, this technique requires the coupling of a detector like the flame ionisation detector (FID), electron capture detector (ECD, photo-ionization detector (PID), mass spectrometry (MS), and many others [28].

1.4.3. Liquid Chromatography – Mass Spectrometry (LC-MS)

Another technique used for the quantification of acetaminophen is Liquid Chromatography- Mass Spectrometry (LC-MS), that is a powerful analytical technique that combines liquid chromatography with the detection and identification capabilities of mass spectrometry [27].

LC-MS has several advantages over other analytical techniques, including high sensitivity, selectivity, and specificity. It can detect and identify a wide range of analytes, including charged and neutral molecules, and can be used for both qualitative and quantitative analysis. It is also a fast technique that can be automated, which makes it suitable for high-throughput analysis [32].

LC-MS has been used for the quantification of acetaminophen in various matrices, including plasma [33], urine [26], and cerebrospinal fluid [34]. LC-MS methods for the determination of acetaminophen often involve the use of a mass spectrometer, which detects the mass-to-charge ratio of acetaminophen and its fragments. These methods for the determination of acetaminophen have been validated for accuracy, precision, and linearity.

One of the disadvantages is that the ionization process used for most LC-MS applications is relatively 'soft', which means that little structural information is obtained. Additionally, LC-MS requires specialized equipment and expertise, which can be costly and time-consuming.

1.4.4. Capillary electrophoresis methods

One of the techniques used for the quantification of acetaminophen is Capillary Electrophoresis (CE), that stands as an effective analytical technique for the quantification of acetaminophen, utilizing the separation of molecules within a capillary filled with an electrolyte solution [27].

CE offers several advantages over other analytical methods. It boasts high separation efficiency, characterized by the minimal sample and reagent consumption, and exhibits versatility in analysing a wide range of analytes, encompassing both charged and neutral molecules. The expeditious nature of CE, coupled with its potential for automation, renders it well-suited for high-throughput analyses [35,36].

Nevertheless, CE is not without its limitations. Its sensitivity makes it susceptible to minor changes in experimental conditions, such as alterations in temperature and pH. Additionally, CE is contingent on the availability of suitable capillary coatings, requiring specialized equipment and expertise.

When it comes to acetaminophen quantification, CE has demonstrated efficacy across diverse matrices, including plasma [37], urine [38], and cerebrospinal fluid [39]. CE methodologies for acetaminophen determination frequently incorporate a UV detector,

facilitating absorbance detection at a specific wavelength. The reliability of CE methods is underscored by rigorous validation procedures, ensuring accuracy, precision, and linearity in acetaminophen quantification [22].

1.4.5. Electrochemical methods

In the last few years, electrochemical methods have been a target of numerous studies for the detection of pharmaceutical analytes and environmental pollutants. It is a practical technique that provide a wide linear range, as well as high precision, accuracy, sensitivity, and selectivity. It has the additional advantage of using low-cost instrumentation and being able to give results in a short matter of time [40].

In the realm of acetaminophen quantification, electrochemistry has been employed across diverse matrices, including plasma [41] and urine [42]. Typically, electrochemical methodologies involve a working electrode such as glassy carbon, gold, or platinum, responsible for gauging the sample's electrical properties, a reference electrode such as Ag/AgCl or saturated calomel electrode (SCE) maintaining a constant potential, and a counter electrode such as platinum wire or carbon electrode finalizing the electrical circuit. [22].

Validation procedures have substantiated the accuracy, precision, and linearity of electrochemical methods for acetaminophen determination [43]. Moreover, these methodologies extend to the analysis of acetaminophen in pharmaceutical formulations, exemplified by tablets and capsules.

In brief, electrochemistry emerges as a potent analytical modality for acetaminophen quantification, characterized by its advantageous attributes: high sensitivity, selectivity, and speed. Employing a configuration of electrodes, namely the working, reference, and counter electrodes, electrochemical methods have undergone rigorous validation processes, attesting to their accuracy, precision, and linearity. Notably, these techniques find applicability in diverse matrices, underscoring their versatility, and have proven instrumental in pharmaceutical formulation analysis.

1.4.6. UV-Visible Spectrophotometry

After presenting all the previous techniques, I would like to emphasise the spectroscopic technique, which represents the branch of science that studies the interaction between electromagnetic radiation and matter. This is the technique that will be applied in the

experimental work in question, and it is a tool with immense potential in the study of atomic and molecular structures and can be applied to a wide range of samples. The regions of electromagnetic spectrum are shown in Table 1:

Table 1. Regions of electromagnetic spectrum [44]

Region	Wavelength
Far (or vacuum) ultraviolet	10-200 nm
Near ultraviolet	200-400 nm
Visible	400-750 nm
Near infrared	0.75- 2.2 μm
Mid infrared	2.5-50 μm
Far infrared	50-1000 μm

UV-Visible spectrophotometry is a technique frequently used in pharmaceutical analyses, which consists of measuring the amount of ultraviolet or visible radiation absorbed by a substance in solution. When carrying out a qualitative analysis, organic compounds are identified using a spectrophotometer and, with the data obtained, it is possible to carry out a quantitative analysis aimed at determining the amount of analyte present in the sample under study. This is a simple, fast, moderately specific technique that can be applied to small quantities of compounds [44].

This technique works by passing a beam of light through the sample containing the compound of interest, in this case, acetaminophen. The amount of light absorbed by the analyte is then measured at a specific wavelength using a spectrophotometer. This amount absorbed must be directly proportional to the concentration of the compound of interest in the sample, allowing for the determination of acetaminophen concentration [22]. The scheme can be found in the Figure 4.

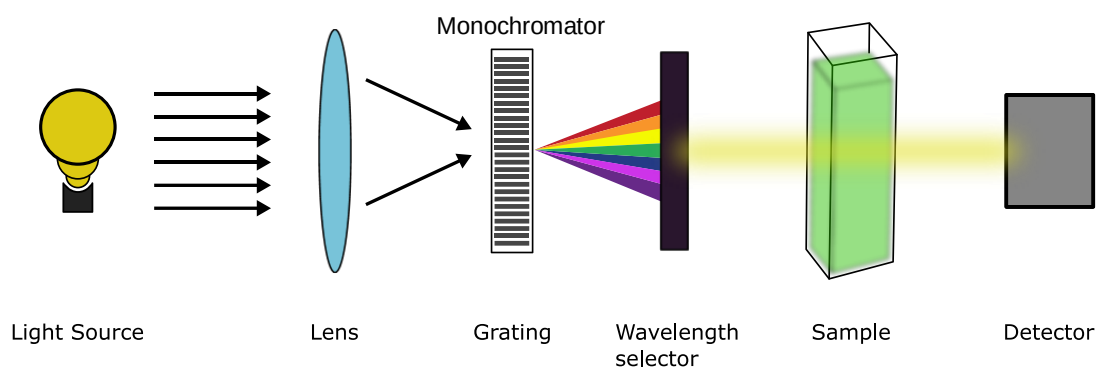


Figure 4. UV-Visible spectrophotometer [45].

The fundamental law that rules the quantitative spectrophotometric analysis is the Beer-Lambert law, which establishes a mathematical relationship between the concentration of the chemical compound of interest and the absorbance at a specific wavelength of light [46]. The simplest mathematical relationship used for this purpose is the linear equation presented below:

$$A = abc$$

Where A represents the absorbance or optical density, a is the absorptivity or extinction coefficient, b corresponds to the path length of radiation through the sample (cm), and c is the concentration of solute in solution. Both a and b are constant, making A directly proportional to the concentration c [44].

UV-Vis spectrophotometry is a widely used technique for the determination and quantification of acetaminophen due to its simplicity, low cost, and high sensitivity. However, it is important to carefully select the appropriate wavelength for measurement and to account any potential interferences. The quantification of a medicinal substance using this kind of technique involves the preparation of solutions and measurements of their absorbance at an appropriate wavelength, in a typical way, the selected wavelength corresponds to the maximum absorption (λ_{max}), where minor errors in wavelength calibration will have a minimal impact to the measured absorbance [47].

This technology has an immensely broad applicability to diverse areas, playing a key role in medical diagnostics, quality control, environmental protection, experimental chemistry, and many others, facilitating the development of simplified models to deal with the complexity of diverse matrices of numerous samples under study [48].

Considering the area of study in question, there are already several studies that use UV-Vis spectrophotometry to quantify the active substances of various pharmaceuticals in their different physical forms, such as the following study by Sayo O. Fakayode et al. [49], where the multicomponent quantification of active substances in pharmaceuticals has been carried out. Also, in work developed by Tadesse Haile Fereja et al. [50], which has applied the analytical technique in question to quantify a synthetic antibacterial compound called Ciprofloxacin in medicinal tablets.

1.5. Techniques for identifying excipients and study the complexity of pharmaceutical matrices

Although pharmacologically inert, excipients can interact with the active substances that make up pharmaceuticals in such a way as to alter the physical and/or chemical stability of the pharmaceutical, which would consequently slow down the pharmaceutical's dissolution process or even its degradation. The choice of excipients in the formulation of a pharmaceutical, as well as the study of their relationship with the active substance are crucial to ensuring the release and bioavailability of the pharmaceutical, in addition to extending its shelf life [51].

The physical characterization of solids pharmaceuticals is a crucial stage of the pharmaceutical development process, ensuring the efficacy, safety, and quality of the product. Historically, this characterization has predominantly relied on powder X-ray diffraction (PXRD) as well as on thermal techniques such as differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). In the 1940s, infrared spectroscopy (IR) emerged as a significant addition to the suite of techniques employed in the multidisciplinary approach to physical characterization. Raman and solid-state nuclear magnetic resonance (ssNMR) spectroscopy were also added to the arsenal of solid-state characterization. Each of the techniques IR, Raman and ssNMR, demonstrated significant success in achieving the overall goal of physical characterization of pharmaceutical substances [52].

1.5.1. Powder X-ray diffraction (PXRD)

Powder X-ray diffraction (PXRD) is a widely utilized analytical technique for the investigation of crystalline structures, applicable across various scientific and industrial fields. This technique is particularly attractive due to its speed and ease of execution,

requiring only lesser amounts of sample, being non-destructive, and capable of providing semi-quantitative analysis of complex mixtures. PXRD is adept at analysing material properties such as phase composition, texture, and structure, and it can be applied to solid samples and even in the liquid state [53,54].

PXRD is integral to numerous fields, from industrial applications to scientific research. It plays a critical role in the pharmaceutical industry, ensuring the consistency and purity of pharmaceutical substances, and in the study of the properties of metals, ceramics, polymers, nanomaterials, and more [55].

Despite its many advantages, including its non-destructive nature, rapid and accurate analysis, and quantitative capabilities, PXRD also has limitations. The complexity of the samples can impact the ease of interpretation of the results. Sample preparation is crucial, as the sample must be a homogeneous powder to obtain reliable results. Additionally, PXRD is less effective for analysing amorphous materials or those with low levels of crystallinity.

1.5.2. Thermoanalytical and gravimetric analysis (DSC and TGA)

Thermoanalytical and gravimetric analysis, particularly Differential Scanning Calorimetry (DSC) and Thermalgravimetric Analysis (TGA), are essential techniques for the characterization of material properties, widely utilized in various scientific and industrial applications. These methods provide valuable insights into the thermal behaviour and compositional stability of materials, making them indispensable in fields such as pharmaceuticals, polymers, and materials science.

DSC is a thermoanalytical technique that measures the heat flow associated with phase transitions and chemical reactions as a function of temperature. It is especially beneficial in the characterization of active pharmaceutical substances and excipients, ensuring proper formulation and stability of the final product [56,57].

TGA, on the other hand, measures the change in the mass of a sample as it is heated or cooled in a controlled atmosphere. This technique is highly effective in determining moisture content, volatile components, and decomposition temperatures of materials [58].

Both DSC and TGA have their unique advantages and limitations. DSC is extremely sensitive and capable of detecting small thermal events, making it ideal for studying phase transitions and purity analysis; however, it may not provide detailed compositional

information of a sample. By the contrary, TGA offers precise compositional information but is less effective in detecting thermal transitions. Being aware of this points, it's clear that this techniques complement each other and provide a more complete understanding of a sample in study.

1.5.3. Raman spectroscopy

Raman spectroscopy is a powerful analytical technique used to investigate the vibrational, rotational, and other low-frequency modes in a system. It provides detailed information about the molecular composition, structure, and interactions of a sample, making it an invaluable tool in various fields such as chemistry, materials science, pharmaceuticals, and biochemistry [59,60].

One of the primary advantages of Raman spectroscopy is its ability to analyse a wide range of samples, including solids, liquids, and gases, without the need for extensive sample preparation. This non-destructive technique can provide real-time, in situ analysis, making it particularly useful for studying dynamic processes and monitoring reactions. In addition, Raman spectroscopy can be used to study samples through transparent containers, allowing for analysis in harsh or controlled environments. Despite its many advantages, Raman spectroscopy has some limitations. The technique is generally less sensitive than other spectroscopic methods, such as infrared (IR) spectroscopy, and can be affected by fluorescence interference from the sample. Additionally, Raman scattering is inherently weak, requiring sensitive detectors and powerful laser sources [61].

1.5.4. Solid-State Nuclear Magnetic Resonance (ssNMR)

Solid-state nuclear magnetic resonance (ssNMR) is a powerful technique used to investigate the structural and dynamic properties of solid materials at the atomic level. Designed to study solids, it provides invaluable information about molecular arrangements, interactions, and motions that are often inaccessible through other analytical methods. This technique is widely utilized in various fields, including chemistry, materials science, pharmaceuticals, and biochemistry [52].

In the pharmaceutical industry, ssNMR is employed to characterize active pharmaceutical ingredients and excipients, identify polymorphs, and study pharmaceutical delivery systems. For instance, ssNMR can distinguish between different

polymorphic forms of a pharmaceutical, which may have significant implications for its bioavailability, stability, and efficacy. It can also monitor the interactions between pharmaceuticals and excipients in formulations, ensuring consistency and quality control in pharmaceutical products [62,63].

Despite its many advantages, ssNMR also has limitations. The technique typically requires high magnetic fields and sophisticated instrumentation, making it relatively expensive and less accessible than other analytical methods. Additionally, ssNMR experiments can be time-consuming, and the interpretation of spectra often requires a high level of expertise.

1.5.5. Fourier-Transform Infrared (FTIR)

Fourier Transform Infrared (FTIR) spectroscopy is a widely utilized analytical technique in various scientific fields, including chemistry, materials science, pharmaceuticals, and environmental science. It provides comprehensive information about the molecular composition, structure, and interactions of a sample by measuring the absorption of infrared radiation. This technique is particularly valued for its ability to identify functional groups and elucidate molecular structures with high precision and accuracy [64].

FTIR spectroscopy operates on the principle of measuring the absorption of infrared radiation by a sample as a function of wave number. The sample is exposed to a broad spectrum of infrared light, and the resulting absorption spectrum is recorded. Each peak in the spectrum corresponds to a specific vibrational mode of the molecules in the sample, providing a unique molecular fingerprint that can be used for identification and characterization. The technique involves the use of an interferometer, which modulates the infrared light and generates an interferogram. This interferogram is then transformed into an absorption spectrum using Fourier Transform algorithms, hence the name Fourier Transform Infrared spectroscopy [65].

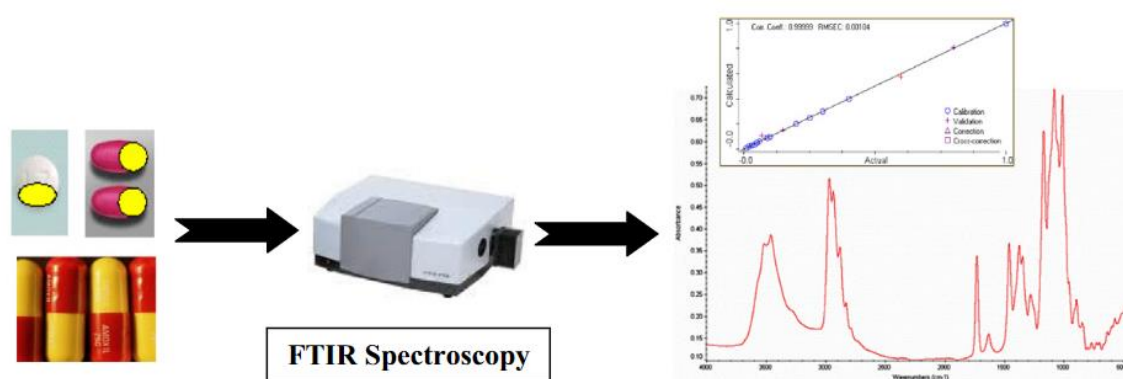


Figure 5. Graphic abstract of sample processing in the FTIR equipment [64].

Fourier Transform Infrared Spectroscopy coupled with Attenuated Total Reflectance (FTIR-ATR) is a powerful and highly sensitive analytical technique that significantly enhances the capabilities of traditional FTIR spectroscopy. This combined method is particularly valuable for analysing a wide range of samples with minimal preparation and improved ease of use. FTIR-ATR has become a staple in many scientific and industrial laboratories due to its versatility and efficiency. It operates on the same basic principle as FTIR spectroscopy; however, the key difference lies in the incorporation of the ATR accessory. The ATR technique involves a crystal with a high refractive index, such as diamond, where the sample is placed in direct contact. Infrared radiation is directed into the crystal at a specific angle, causing it to reflect internally within the crystal multiple times. This reflection creates an evanescent wave that penetrates a few micrometers into the sample. The evanescent wave interacts with the sample, and some of the infrared radiation is absorbed by the sample's molecular vibrations. The absorbed wave numbers correspond to the specific vibrational modes of the sample molecules, producing an absorption spectrum [66,67].

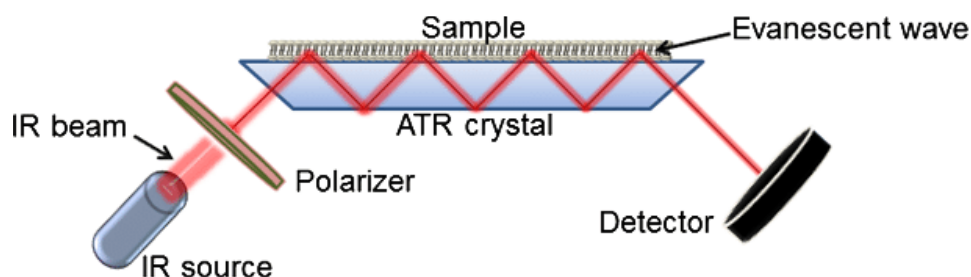


Figure 6. Schematic representation of how FTIR-ATR works [68].

Like in FTIR spectroscopy, one of its primary advantages is its ability to analyse a wide range of samples, including gases, liquids, and solids, with minimal sample preparation. Unlike traditional FTIR, which often requires samples to be ground into fine powders or dissolved in solvents, FTIR-ATR can analyse samples directly. It is a non-destructive technique, allowing for the preservation of the sample for further analysis. It can be used in both qualitative and quantitative analysis, making it versatile for various applications.

2. Method validation

Method validation is a process whose purpose is to confirm that the analytical procedure used for a specific test is the most appropriate. A laboratory should validate non-standardised methods developed by the laboratory itself and standardised methods applied in new contexts, and this should be as extensive as necessary, including procedures for sampling, handling, and transport [69].

Following the same line of thought, for a result to be considered validated it must fulfil specific quality requirements as demanded of it, and for this to be possible to achieve, three assessments are applied to determine the quality of the result in question, these being accuracy, precision, and uncertainty [70].

The results from the validation of a method are then suitable for the intended use and can be applied to study the quality, reliability, and consistency of analytical results.

The validation of a method must be carried out prior to its introduction into routine use, whenever the conditions for which the method was validated change, and whenever the method is altered, or the alteration is outside the original scope of the method. [44].

2.1. Accuracy

Accuracy is defined as the agreement between the value obtained and the value conventionally accepted as true [70]. This parameter is essentially obtained by carrying out a direct assessment, involving a combination of random (assessed by precision) and systematic (assessed by trueness) error components.

$$(x_i - \mu) = (x_i - \bar{x}) + (\bar{x} - \mu)$$

$$\text{measurement error (accuracy)} = \text{random error (precision)} + \text{systematic error (trueness)}$$

The processes used to make this assessment possible include the use of certified reference materials (CRM), inter-laboratory tests (IIL) or recovery tests. [71].

A CRM is characterised by having a concentration value, or other quantity, and an associated uncertainty, from which different processes are applied to evaluate the results obtained by analysing a CRM:

- relative error;
- hypothesis test (t-test);

- performance factor (Z-Score);
- normalised error;
- recovery percentage (%Re).

Within this set of possible processes, recovery tests are extremely important in qualitatively analysing the analytical response. This form of evaluation is concerned with the relationship between the amount of analyte recovered in the process and the actual amount present in the sample, with the main aim of identifying the viability of the method [72].

The associated procedure involves adding a known quantity of a standard solution to a real sample, which is then called a fortified sample, and the quantity of analyte obtained from this is compared to an unfortified sample and the %Re is calculated. This checks for systematic effects that may exist due to unknown causes, such as the matrix effect (characteristic interferences that cause an increase/decrease in the analytical response).

The equation that connects all these concepts is shown below, where $c_f(V + V_1)$ corresponds to the concentration and volume after fortification, $c_i V_1$ corresponds to the concentration and volume of the actual sample and, $c_a V$ corresponds to the concentration and volume of the standard solution used:

$$\%Re = \frac{|c_f(V + V_1) - c_i V_1|}{c_a V} \times 100$$

Regarding acceptance criteria, recovery should, for instance, fall within the range of 80 to 120 % [72,73]. Nevertheless, the laboratory has the flexibility to establish alternative criteria depending on the performance and/or complexity of the method. In other words, acceptance criteria for recovery may vary based on method performance and complexity, and the previously mentioned range serves as a general guideline but is not strictly fixed.

2.2. Precision

This parameter is one of the minimum requirements to be studied for the validation of internal test methods. It is a general term used to evaluate the dispersion of results obtained between independent tests, repeated on the same sample or standard, under previously defined conditions. [71]

Precision can initially be portrayed by the standard deviation or coefficient of variation (CV), which corresponds to dividing the standard deviation of precision by the average of the results obtained:

$$s = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n - 1}}$$

$$\%CV = \frac{s}{\bar{x}} \times 100$$

Broadly speaking, research studies can be categorized into three distinct tiers of precision: repeatability, intermediate precision, and reproducibility. These tiers delineate the extent to which a study's findings can be consistently replicated and relied upon.

The variability observed in measurements can be primarily attributed to four key factors. First, there's the element of time, which pertains to whether measurements are taken on the same day or across multiple days. Secondly, the calibration of the measurement instrument plays a significant role in influencing the accuracy of results. Thirdly, the human factor is critical, as measurements can vary depending on different operators conducting them. Finally, even the equipment used for measurements can introduce variations; it may be identical but not correctly calibrated, or it may differ from the equipment utilized in prior measurements, further impacting the study's outcome. These factors collectively contribute to the overall variability of measurements in a study.

However, since the concept of reproducibility is related to the fact that measurements are carried out in different laboratories, this level of precision will not be addressed in the work in question.

2.2.1. Repeatability

The precision of a test method carried out under identical conditions is expressed through repeatability, provided that the tests in question were carried out in the same laboratory, by the same analyst, using the same equipment, using the same reagents and within brief time intervals [71].

Determining the repeatability of an analytical method is based on assessing the dispersion of a certain set of results obtained under the previously defined conditions, and this is carried out using the following equation for when the number of repetitions is constant for the tests carried out on different days, where s represents the standard

deviation for each group and k represents the number of standards used for the calculations [74]:

$$s_{r\text{pooled}} = \sqrt{\frac{s_1^2 + s_2^2 + \dots + s_k^2}{k}}$$

It is also important to note that the repeatability limit (r) plays a fundamental role in defining the lowest value at which the absolute difference between two test results should lie, with a probability usually set at 95 %. This is calculated using the following equation:

$$r = t \times \sqrt{2} \times s_{r\text{pooled}} = 1,96 \times \sqrt{2} \times s_{r\text{pooled}} = 2,8 \sqrt{s_{r\text{pooled}}^2}$$

Another parameter related to the topic in question is the coefficient of variation of repeatability (CV_r), which is expressed as a percentage:

$$\%CV_r = \frac{s_{r\text{pooled}}}{\bar{x}} \times 100$$

Where CV_r is numerically identical to the standard deviation of repeatability divided by the average of all the values considered in the calculation.

2.2.2. Intermediate precision

Intermediate precision, also known as intra-laboratory reproducibility, refers to tests that are only carried out within the same laboratory, i.e. intra-laboratory tests. On the other hand, and in order to clarify terminology, in the case of reproducibility, different laboratories are involved, thus assessing a certain level of inter-laboratory precision [74].

This parameter assesses precision on the same sample, which has been subjected to several tests with the same analytical method, in the same laboratory or not, and by varying one or more of the following conditions [71]:

- different analysts;
- different equipment;
- separate times;
- with or without calibration verification.

In the work in question, this parameter was assessed taking into account that only the time factor varied, and the sample was subjected to various tests over an extended period of time. Within this framework of conditions, the intermediate precision is

quantified by the following equation, which is taken as usual when calculating the standard deviation:

$$S_I = \sqrt{\frac{\sum_{i=1}^n (y_i - \bar{y})^2}{n - 1}}$$

Where n represents the number of tests that will be carried out experimentally and, $\bar{y}$ represents the arithmetic mean of the individual results obtained.

However, when the analyte is not stable enough over time, as will be seen in the work in question that the acetaminophen solution will not be stable, intermediate precision is obtained from several samples of similar compositions and with similar concentrations of the analyte. In this specific case, the procedure for determining the validation parameter in question is based on the selection of t samples, each of which is subjected to a fixed number n of readings, which must be constant for all the different solutions. Therefore, the formula that will allow access to the intermediate precision value will be:

$$S_I = \sqrt{\frac{\sum_{j=1}^t \sum_{k=1}^n (y_{jk} - \bar{y}_i)^2}{t(n - 1)}}$$

2.2.3. Reproducibility

Reproducibility represents the precision of a testing method carried out under different experimental conditions. It involves applying the same testing method to the same sample while varying certain measurement conditions, such as different operators; different laboratories; different equipment; and different time intervals [71].

This parameter reflects the extent of random quantification errors on a transnational scale and can only be estimated through interlaboratory trials. For this purpose, a set of samples is sent to participating laboratories, which then perform tests on the same sample. The variance associated with reproducibility is calculated using the following equation, where S_{Ri}^2 represents the variance of reproducibility; S_{Li}^2 is the interlaboratory variance; represents the S_{ri}^2 repeatability variance:

$$S_{Ri}^2 = S_{Li}^2 + S_{ri}^2$$

When implementing a new analytical method, studying the reproducibility is essential. This ensures that the method's precision is evaluated under varying working conditions. However, reproducibility is less critical when validating a method within a single

laboratory, as it is intended to be executed using the same equipment and under experimental conditions that vary minimally over time.

The reproducibility limit, ΔR is the maximum acceptable value for the absolute difference between tests conducted under reproducibility conditions, determined at a 95% confidence level and is calculated following the formula:

$$\Delta R = t_{0.05(m-1)} \times u \times \sqrt{2} \times S_{Ri}$$

Additionally, the coefficient of variation for reproducibility (CV_r) should also be considered. It is numerically defined as the ratio between the standard deviation of reproducibility (S_{Ri}) and the mean of the obtained results ($\bar{x}$), expressed as:

$$\%CV_r = \frac{S_{Ri}}{\bar{x}} \times 100$$

However, in the study in question, the same operator, the same facilities and the same conditions were always used, so there was no need to apply the reproducibility study.

2.3. Uncertainty

Uncertainty is a parameter that is presented along with the result and is intended to characterise the dispersion of the results. It makes it possible to assess the performance of a method when it is subjected to variations in experimental conditions, and a laboratory must identify any and all contributions that are significant to measurement uncertainty [69].

Some of the components that contribute to the increase in the uncertainty of the result are generally defined by the experimental standard deviation or can be evaluated from the statistical distribution of results from various measurements. Other components, as well as being characterised by the standard deviation, are assessed by probability distributions [75]. So, the uncertainty is given by calculating the standard deviation multiply by the t -student, under conditions of intermediate precision, and the result is then presented as follows:

$$Final\ result = \bar{x} \pm (t \times s)$$

The calculation of the standard deviation is obtained assuming that there is propagation of the error, where for each value of x_0 there is a confidence interval for the true value of

y_0 , so the following equation is applied to calculate the standard deviation of a given concentration x_0 :

$$S_{x_0} = \frac{S_{y/x}}{b} \times \sqrt{\frac{1}{N} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \times \sum_{i=1}^N (x_i - \bar{x})^2}}$$

In the equation shown above $S_{y/x}$ is the residual standard deviation, b represents the slope of the calibration curve, N is the number of standards carried out in the calibration, n corresponds to the number of independent tests, or replicates, carried out on the sample, y_0 corresponds to the instrumental signal, $\bar{y}$ is the average of the instrumental signal values, $\bar{x}$ is the average of the concentration values of all the standards used in the calibration, and finally, x_i represents the individual concentration values.

2.4. Linearity

This parameter is an important property in an analytical method, which is related to the method's response to the concentration/amount of analyte of interest [76].

It refers to the analytical method's ability to produce responses that are directly proportional to the concentration of analyte present in the sample, within a predefined working range. In other words, a linear analytical method is one that follows a simple mathematical function, constituting a straight line when representing the calibration graph, thus translating into a linear response.

The initial step in the linearity study is to construct the calibration curve within a given working range. Here, the vertical axis (y-axis) should always represent the instrumental response of the equipment, and the horizontal axis (x-axis) should correspond to the concentrations of the standards, assuming that the errors associated with the values on the abscissa axis are negligible compared to the values on the ordinate axis. [71]. In this way, the algebraic equation that represents the line in question will be of the type:

$$y = bx + a$$

Where b corresponds to the slope of the line and a represents the ordinate at the origin. Together with this equation, the linear correlation coefficient (R) is calculated, which indicates whether or not the line is appropriate for the model in question, and for it to be considered ideal the value of r must be as close to 1 as possible.

This property of studying the method is extremely important in the sense that it allows us to assess whether it is reliable and accurate in quantifying the analyte at different concentrations. There are various methods for determining linearity, such as the Madel test (ISO 8466-1), the normalised area test and the Rikilt's test.

2.4.1. Test for homogeneity of variances

When running a calibration curve that represents a first-degree polynomial function, in other words, that represents a line whose linear regression uses the least squares method, it is assumed that the errors have a normal distribution and that there is homogeneity of variances along the line [71].

However, although it is assumed that there is homogeneity of variances over the working range, this needs to be checked. It is recommended that there are ten calibration points, which should be equally distributed throughout the concentration range. Of these standards, the first and last should be subjected to more extensive readings and can be analysed in ten independent replicates [77]. Starting from this scenario, the variances of the values of the first and last standards are calculated, and the following formula is applied:

$$PG = \frac{S_1^2}{S_2^2} \quad , \quad S_1^2 > S_2^2$$

When the variances between the groups are tested to see if there is a significant difference between the calculated variances.

The PG value obtained is then compared with the tabulated value of the F distribution, for an established degree of confidence, with a degree of freedom of $n - 1$ for the nominator and denominator, whereby if $PG \leq F$, the differences in variances are not significant, the working range is well adjusted; or, if $PG > F$, the differences between variances are significant and the working range must be readjusted.

2.4.2. Test for linearity

2.4.2.1. Mandel test

As mentioned above, linearity can be assessed using the value of the linear correlation coefficient. However, this is a crude assessment and is not sufficient on its own to evaluate the linearity of a calibration curve.

Linearity can then be assessed using a statistical model where, from a set of ordered pairs, two calibration functions are calculated, one linear and the other non-linear, and then a comparison is made between both to assess which model best fits the case in question. This process is called the Mandel test, which compares the variances calculated for each of the models, creating two hypotheses: the null hypothesis states that the two variances are not significantly different, which leads to the linear model being accepted as the best fit; on the other hand, the alternative hypothesis states that the two variances are significantly different, which leads to the conclusion that the linear model is not the one that leads to the best fit, but rather the non-linear model.

To carry out this test, the two linear and non-linear calibration functions are calculated, along with their respective residual standard deviations ($S_{y/x}$ e S_{y2}), and the difference between the variances (DS^2) is obtained using the following formula [71]:

$$DS^2 = (N - 2)S_{y/x}^2 - (N - 3)S_{y2}^2$$

Where N corresponds to the number of standards that are run in the procedure being carried out in the study.

$$PG = \frac{DS^2}{S_{y2}^2}$$

Similar to the test for homogeneity of variances, PG is calculated and its value is then compared with the tabulated value of the F distribution, for a given degree of confidence, and if PG is higher the calibration function that fits best is the non-linear function, i.e. the second degree polynomial, whereas if PG is lower than or equal to the tabulated F value, the function with a significant fit is the linear calibration function.

2.4.2.2. Normalized area test

The main objective and applicability of this linearity test is to compare the experimental values with the values calculated using the equation obtained by constructing the calibration curve.

The need to carry out this test arises from the fact that in most situations, not all the points fall exactly under the regression line, but are scattered around it, so it is necessary to know the exact response areas for the concentrations in question [78].

In this test, for each point on the calibration curve, the ratios of experimental areas and areas predicted by the calibration line, y predicted, are calculated, and it is then assessed which of the values obtained is closest to one that will be considered a reference value. Based on this process, the various normalised areas corresponding to each point are then calculated using the following formula, where R_{ref} represents the concentration with best correlation, being used as a reference, and R_i corresponds to the ratio closest to one :

$$Normalized\ area\ (\%) = \frac{R_i}{R_{ref}} \times 100$$

If all the values are within the acceptable range of 85 to 115 %, it can be concluded that the linear function is the one that best fits the model.

2.4.2.3. Rikilt's test

In addition to the tests presented above, the Rikilt's test is another test aimed at studying the linearity of the calibration curve and examining whether the linear function is the best fit.

This test has the particularity of making it possible to analyse the linearity of each point on the calibration line, by calculating the ratio between the instrumental signal and the concentration of the corresponding standard ($R_{y/x}$), then the average of all the ratios is calculated $((R_{y/x})_i)$, and this value is considered a reference for the rest of the calculations. The ratio is then calculated ($R_i(\%)$), in percentage between the reference value and the signal/concentration ratios.

$$R_i(\%) = \frac{(R_{y/x})_i}{R_{y/x}} \times 100$$

Once all the necessary components have been obtained, a graph is constructed that will allow you to visualise the values of $R_i(\%)$ that are within the limits considered acceptable, 85 to 115 %. If any value is outside this range, it should be rejected, and the test should be run again considering the new working range.

2.5. Method Selectivity/Specificity

The main purpose of the selectivity and specificity parameters is to make the analyst realise whether there are any interferences and what influence they have on the results obtained by the analytical method. These interferences can come from constituent components of the matrix that lead to possible changes in measurement performance [72].

Selectivity means the ability of an analytical method to identify and distinguish a specific analyte in a complex mixture, without interference from other components, impurities, or similar substances.

A method is only classified as specific when it can discriminate between the analyte to be determined and the other substances present in the sample, making it possible to guarantee that the quantity being measured and potentially quantified is only the desired analyte.

Recovery tests are conducted to assess the applicability of an analytical method. This method is deemed applicable only if the recovery percentages fall within the predefined acceptance criteria established by the laboratory performing the analysis [71].

2.6. Sensitivity of the Method

The so-called sensitivity of the analytical method is the ability of the method to distinguish minimal differences in the concentration of the analyte and is defined as the first-order derivative of the calibration curve in the concentration zone in question.

Sensitivity can be calculated using the following formula, which corresponds to the quotient of the increase in the read value (ΔL) and the change in concentration (ΔC) corresponding to the aforementioned increase [71]:

$$Sensit. = \frac{\Delta L}{\Delta C}$$

However, if it is a linear calibration curve, the sensitivity will remain constant over the working range and equal the slope of the calibration line [72]:

$$Sensit.(b) = \frac{\Delta y}{\Delta x}$$

This parameter is commonly confused with the detection limit, but they are vastly different concepts, since sensitivity is associated with the magnitude of the signal, while the detection limit is associated with the signal's resolution capacity.

In the evaluation of analytical methods, the sensitivity of the method plays an extremely significant role, in the sense that it allows comparisons to be made between various methods, based on linear models, for the same analyte; it also allows the sensitivity of various analytes to be compared.

2.7. Analytical Thresholds

The analytical thresholds of the test method comprise two types of limits, the limit of quantification and the limit of detection, and there are several ways of calculating them.

These are extremely important because they make it possible to define the analytical thresholds that the method is capable of reaching, and to define a semi-quantitative detection zone [72].

The aforementioned limits must be recalculated whenever there are changes in the influencing factors/experimental conditions and whenever a new calibration curve is made. In the latter case, a study can be carried out over time and an arithmetic mean of all the limits obtained for all the calibration curves made can be used as the method's limits.

2.7.1. Limit of Detection (LOD)

The limit of detection (*LOD*) corresponds to the minimum level at which it is possible to identify the presence of the analyte with acceptable uncertainty, i.e., it corresponds to the smallest amount of analyte that is detectable in the sample, but not necessarily quantified [71]. In other words, it is the minimum concentration value that is distinguishable from the blank and the sample without analyte.

Any reading below this calculated limit does not mean that there is no analyte in the sample, but rather that its concentration is below a certain value.

In order to correctly define the detection limit, it is necessary to introduce two statistical concepts [71]:

- error type I (risk α): the probability of detecting the presence of the analyte when it does not exist in the sample under study;
- error type II (risk β): the probability of not detecting the presence of the analyte when it is present in the sample under study.

In order to minimise these two errors throughout the study, a degree of significance of 5 % is considered so that a correct analysis can be conducted.

In a general case, to calculate this limit under study, the detection limit, the following equation is applied, where X_0 corresponds to the average values of the blank obtained over an extended period of time and s_0 represents the standard deviation of these same values:

$$LOD = X_0 + k \times s_0$$

The value of k , for a confidence level of 99.7 %, is a known quantitative value of 3.3:

$$LOD = X_0 + 3,3 \times s_0$$

However, when using a method that involves the application of a linear calibration, as is the case in the work in question, the formula for calculating the detection limit undergoes some modifications, becoming the following:

$$LOD = \frac{[3,3 \times S_{y/x}]}{b}$$

In this formula, b stands for the slope of the linear calibration line and $S_{y/x}$ is the residual standard deviation of the calibration curve that follows the least squares method.

2.7.2. Limit of Quantification (LOQ)

In turn, the limit of quantification (LOQ) is defined as the lowest quantity that can be quantified with a given accuracy and precision by the analytical method applied. In other words, it corresponds to the lowest measured concentration from which quantification of

the analyte can be carried out; in practical terms, it is the concentration from the calibration standard with the lowest analyte concentration.

After obtaining the value corresponding to the limit of quantification, it is advisable and widely recommended to carry out a test to ascertain whether or not the accuracy and precision that the method achieves can be considered acceptable.

As with the limit of detection, there is a formula that is applied in a general way to calculate the limit of quantification, which once again involves the arithmetic mean of a series of blanks together obtained over an extend period of time with their standard deviation:

$$LOQ = X_0 + 10 \times s_0$$

As in the work in question, in cases where the analytical method and study involve the construction of a linear calibration curve, the equation for calculating the limit of quantification can be adjusted for the case in question:

$$LOQ = \frac{[10 \times S_{y/x}]}{b}$$

In this formula, b represents the slope of the linear calibration line and $S_{y/x}$ is the residual standard deviation of the calibration curve that follows the least squares method.

3. Main objective

The primary objective of this study was to quantify the active substance, acetaminophen, in nine selected pharmaceutical preparations (one from Portugal and the others from Poland), six in tablet form and three in soluble powder form, using UV-Vis spectrophotometry as the detection and quantification method. In addition, the study aimed to compare the results obtained with these pharmaceuticals with the values declared by the respective manufacturers. The validation and optimisation of the method were also essential components of the work plan. In addition, FTIR-ATR was used to identify the excipients listed on the leaflets of the pharmaceutical formulations and to carry out a qualitative analysis of the various matrices under investigation.

It should be noted that all the experimental work carried out was based on characteristics of the active substance and the analytical methods previously validated.

4. Materials

4.1. Reagents and equipment

Below are the various pieces of equipment and reagents that were used throughout the distinct stages of the experimental study.

Equipment and materials:

- Analytical balance - Mettler Toledo ME204/M
- Moisture analyser - Mettler Toledo HE73/01
- Single-use Aluminium Sample Pans for moisture analysers - Mettler Toledo
- UV-VIS spectrophotometer - HACH LANGE DR 6000
- 10 mm high precision quartz cell - Hellma Analytics
- FTIR-ATR – Perkin Elmer, Spectrum two
- Automatic pipettes 20-200 µl, 100-1000 µl, 0.5-5 mL and 1-10 mL -Transferpette
- Porcelain mortar and pestle
- syringe filters - PTFE Ø25 mm 0.45 µm
- syringes 10 mL - KD-JECT, Germany
- Parafilm "M" - Bemis
- Plastic and glass laboratory utensils

Reagents:

- Redistilled and deionised water obtained in the laboratory
- Acetaminophen, A5000, 98.0-102.0 % - Sigma-Aldrich
- Methanol pure – CHEMPUR
- Ethanol pure (degree of purity unknown)
- hydrochloric acid 35-38 %, purity: for analysis - Merck KGaA

Research material:

Table 2. Information on the pharmaceuticals being studied in this experimental study

Product name	Active substance and dosage	Excipients	Format
A1	Acetaminophen 1000 mg	povidone, corn starch, talc, stearic acid, hydrated colloidal silica, sodium carboxymethyl starch	tablets
B2	Acetaminophen 500 mg	carnauba wax, hypromellose, macrogol, povidone, pregelatinised starch, croscarmellose sodium, stearic acid	tablets
C3	Acetaminophen 500 mg	Povidone K30, Pregelatinised maize starch, Sodium amidoglycolate (type A), Stearic acid (type 50)	tablets
D4	Acetaminophen 500 mg + Hyoscine butylbromide 10 mg	microcrystalline cellulose, potato starch, povidone K30, sorbitol, magnesium stearate, colloidal silica anhydrous, sodium carboxymethyl starch, hypromellose, macrogol 6000	tablets
E5	Acetaminophen 500 mg + Caffeine 65 mg	pregelatinised starch, maize starch, povidone (K 25), potassium sorbate, talc, stearic acid, croscarmellose sodium; triacetin	tablets
F6	Acetaminophen 325 mg + Ibuprofen 200 mg	povidone, pregelatinised starch, microcrystalline cellulose, magnesium stearate	tablets
G7	Acetaminophen 500 mg + Ascorbic Acid 200 mg + Pheniramine 25 mg	sucrose, anhydrous citric acid, acacia gum, saccharin sodium, natural lemon flavour and rum	soluble powder
H8	Acetaminophen 1000 mg + Ascorbic Acid 100 mg + Phenylephrine Hydrochloride 12.2 mg	sucrose, citric acid, sodium citrate, acesulfame potassium (E 950), lemon flavours, aspartame (E 951), quinoline sunset yellow (E 104)	soluble powder

19	Acetaminophen 500 mg + Guaifenesin 200 mg + Phenylephrine Hydrochloride 10 mg	sucrose, citric acid, tartaric acid, sodium cyclamate, sodium citrate, aspartame (E951), acesulfame potassium (E950), menthol powder, lemon flavouring, lemon juice flavouring, quinoline yellow (E104)	soluble powder
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5. Experimental procedures and Results

5.1. Preparation of pharmaceutical samples

5.1.1. Determining the average mass of the tablets

All the pharmaceutical products that will be studied in this experimental work are shown in Table 2 (previous section). The packs corresponding to each pharmaceutical contain different numbers of tablets, and in the case of the portuguese pharmaceutical A1, a box consists of eighteen tablets. A total of twenty-seven tablets corresponding to this pharmaceutical were used for the study. All the tablets were weighed using an analytical balance before being crushed using a mortar (Figure 7).



Figure 7. Mortar used and result of crushing the sample.

The masses obtained were used to determine statistical parameters such as the mean and respective standard deviation, the minimum and maximum of the data set. These parameters are given in Table 3, where n represents the number of tablets, $\bar{m}_{tab}$ the average mass of the tablets and s the respective deviation, RSD (%) corresponds to the relative standard deviation, m_{min} and m_{max} are the minimum and maximum values of the set and, finally, Gap is the difference between these values:

Table 3. Statistical parameters for the mass of A1 tablets

n	$\bar{m}_{tab}$ (g)	s (g)	RSD (%)	m_{min} (g)	m_{max} (g)	Gap (g)
27	1.2093	0.0136	1.1225	1.1883	1.2357	0.0474

The following formulae were used to calculate the mean and standard deviation:

$$\bar{m}_{tab} = \frac{m_1 + m_2 + \dots + m_n}{n}$$

$$s = \sqrt{\frac{(m_1 - \bar{m}_{tab})^2 + \dots + (m_n - \bar{m}_{tab})^2}{n - 1}}$$

The following formula was applied to calculate relative standard deviation:

$$RSD (\%) = \frac{s}{\bar{m}_{tab}} \times 100$$

With regard to the remaining pharmaceuticals, seven tablets were randomly selected from their respective packs and weighed on an analytical balance, giving the values shown in Table 4:

Table 4. Values corresponding to the mean and standard deviation of the masses of each pharmaceutical product

Product name	nº	$\bar{m}_{tab} \pm s$ (g)
A1	27	1.2093 $\pm$ 0.0136
B2	7	0.5594 $\pm$ 0.0154
C3	7	0.6008 $\pm$ 0.0044
D4	7	0.7327 $\pm$ 0.0020
E5	7	0.6836 $\pm$ 0.0028
F6	7	0.7004 $\pm$ 0.0034
G7	1	12.9793
H8	1	4.9733
I9	1	4.3331

The next step in sample preparation was to determine the moisture content, in the case of the pharmaceutical product A1. The procedure described was also applied to the remaining pharmaceuticals. After grinding, aliquots of 1 g were measured approximately,

placed in a disposable aluminium tray, and placed in the Moisture analyser - Mettler Toledo HE73/01 (Figure 8).



Figure 8. Moisture analyser - Mettler Toledo HE73/01.

In this equipment, the samples were weighed, and when the equipment is closed, the heating process is started until a temperature of 105 °C is reached, which simultaneously dries the sample and continuously measures the weight. The process is terminated when the dry sample weight stabilises. This measurement was carried out a total of 11 times, the masses and moisture contents of which are shown in the following Table 5.

Table 5. Values for the various weighing's of the sample and their respective moisture indices, together with associated statistical parameters, in the case of the A1 pharmaceutical

Primary sample (g)	Dry sample (g)	Moisture (%)	Test Q-Dixon (95 %)		Average (%)	s (%)	RSD (%)
1.118	1.100	1.25	Q ₁	0.0833	1.393	0.109	7.84
1.063	1.045	1.27					
1.024	1.008	1.27					
1.015	0.996	1.32					
1.074	1.056	1.37					
1.072	1.009	1.38	Q _{crit}	0.444			
1.013	0.995	1.44	Q ₁₀	0.313			
1.242	1.221	1.46					
1.102	1.084	1.48					
1.183	1.162	1.49					
1.071	1.051	1.59					

In the Table 5, shown above, we can see that a statistical test, the Q-Dixon test, was carried out at a 95 % confidence level in order to ensure that none of the values obtained could be considered an outlier. Statistical parameters such as the mean, standard deviation and relative standard deviation were also calculated taking into account the formulas presented above.

The Q-Dixon statistical test was calculated using the following formulae:

$$\text{Lower extremes: } Q_n = \frac{X_2 - X_1}{X_{n-1} - X_1}$$

$$\text{Higher extremes: } Q_n = \frac{X_n - X_{n-1}}{X_n - X_2}$$

Given that the critical value corresponding to the number of samples in question, 11, is 0.444 (Q_{crit}) at a confidence level of 95 %, and as can be seen, after applying the statistical test no value is considered an outlier. The mean and standard deviation parameters therefore take into account all the values obtained in the study.

During the experimental procedure designed to quantify the active agent, only the dry masses of the samples, which were obtained following a thorough dehumidification process, were utilized. This step ensured that any moisture present in the samples was completely removed, allowing for accurate and consistent measurements of the active agent's concentration in all samples.

5.2. Calibration curve study

The calibration process is characterised as a descriptive element of a method. It is a tool implemented in a study in order to interpret the information gathered by the tests carried out within a study.

When portraying a quantitative analysis, as in the work in question, calibration indicates the relationship between the response of a measuring system and a concentration/amount of substance being analysed [71]. Generally speaking, the procedure associated with this type of quantitative analysis is as follows:

- initially, standard solutions are prepared, where the concentration of the parameters to be dosed is known;
- the solutions are subjected to readings in an analytical apparatus, all subjected to the same conditions of analysis;

- based on the readings taken and the concentrations of the standard solutions, a graphical relationship is established from which the concentration of the analyte under study can be determined by interpolation.

When carrying out this type of study, there are some considerations to be taken into account, including the fact that it is advisable to carry out the calibration at the time of the analysis, i.e. the calibration should be carried out on the same day as the analysis of the desired sample. Acceptance criteria should also be applied to this study, mainly linearity and stability between calibrations.

When using the least squares method, the vertical axis (y) must always represent the instrumental response of the equipment, while the horizontal axis (x) must correspond to the concentrations of the standards, due to the fact that it is always assumed that the errors associated with the x values are negligible when compared to the y values.

The representative algebraic form of the equation of a straight line is given by:

$$y = b x + a$$

Where b represents the slope of the line, and a corresponds to the ordinate at the origin. Another characteristic parameter of a calibration line is the determination coefficient R^2 , given by the following equation:

$$R^2 = \frac{\sum_{i=1}^N \{(x_i - \bar{x}) \times (y_i - \bar{y})\}}{\sqrt{[\sum_{i=1}^N (x_i - \bar{x})^2 \times \{\sum_{i=1}^N (y_i - \bar{y})^2\}]}}$$

This last parameter, characterising a calibration curve, indicates whether it has a positive correlation (positive slope) or a negative correlation (negative slope). In a chemical analysis, calibration curves must have determination coefficient values equal to or greater than 0.995.

5.2.1. Preliminary tests

To ascertain an optimal range of values aligning with the calibration curve tailored to the requirements of the experimental study at hand, a series of tests were conducted. These tests involved varying the working range and the concentrations of both maximum and minimum standards. Additionally, preliminary experimentation entailed exploring diverse volumes of standard solutions and employing different solvents. This comprehensive

approach facilitated the assessment of which curve best adhered to the linearity validation parameters.

The experimental procedure for running the calibration curve was similar for all the tests carried out.

5.2.2. Preparation of standard solutions

Before preparing the standard solutions that will make up the quantification method, it is necessary to prepare the stock solution, a working solution and the solvent that will make up all the solutions.

The stock solution, with a theoretical concentration of 1000 ppm, is made by adding 0.0500 g of standard acetaminophen to a 50 mL container, which is filled with solvent consisting of 15 % pure methanol and 85 % redistilled and deionised water. From this solution, the 100 ppm working solution is made by adding 5 mL of the stock solution to a 50 mL container. These solutions are prepared on the day the calibration curve is to be carried out and are disposed of safely after use.

The masses weighed during the preparation of these two solutions and the calculation of the actual concentration of the working solution are showed in Table 6.

Table 6. Weights for the preparation of the stock and working solutions

Stock solution		Working solution		
m_{ACT} (g)	$m_{final\ ms}$ (g)	m_{added} (g)	$m_{final\ w}$ (g)	C_w (ppm)
0.0561	48.991	5.185	48.696	96.564

In Table 6, m_{ACT} corresponds to the weight of standard acetaminophen that was added to the stock solution and $m_{final\ ms}$ is the final weight after the volume was filled with solvent. The preparation of the working solution involves adding 5 mL of the stock solution, whose mass value is represented by m_{added} , and the final weight of the working solution is represented by $m_{final\ w}$.

All values contribute to the calculation of the actual concentration of the working solution (C_w) which is calculated using the following formula:

$$C_w = \frac{\frac{m_{ACT} \times \rho}{m_{final\ ms}} \times 10^6 \times m_{added}}{m_{final\ w}}$$

To carry out this calculation, all the masses involved in preparing the solution are taken into account in order to eliminate random errors that may be made by the operator, because as you can see, an exact concentration of 100 ppm is not obtained. The density of the methanol used for the solvent is also taken into account, which corresponds to a value of 0.792 g/mL.

5.2.3. Determining the calibration curve with the best fit

Taking into account all the characteristics of a calibration curve mentioned above, and with the aim of matching all the parameters required, the range with the best fit was that comprising concentrations of 0.5 to 20 ppm.

Starting from the working solution mentioned above, the standards for the calibration curve were made. Depending on the desired concentration for each standard, aliquots of the working solution were added to 10 mL containers and 0.05 mL of hydrochloric acid was added (in order to preserve the solutions until their absorbance was read). The volumes were filled with the solvent.

Similarly to the data given in Table 6, when making the standards, the initial and final masses of the solution were also weighed in order to calculate the real concentrations of each standard solution, using the following formula:

$$C = \frac{C_w \times m_{added}}{m_{final}}$$

In the following formula, m_{added} corresponds to the initial mass of the solution, i.e. the mass corresponding to the volume of working solution added to the standard, and m_{final} represents the final weight of the solution, after preface the volume.

The values are shown in Table 7, referring to the weighed masses and actual concentrations of each standard, as well as the absorbances measured using the UV-VIS spectrophotometer - HACH LANGE DR 6000 (Figure 9).

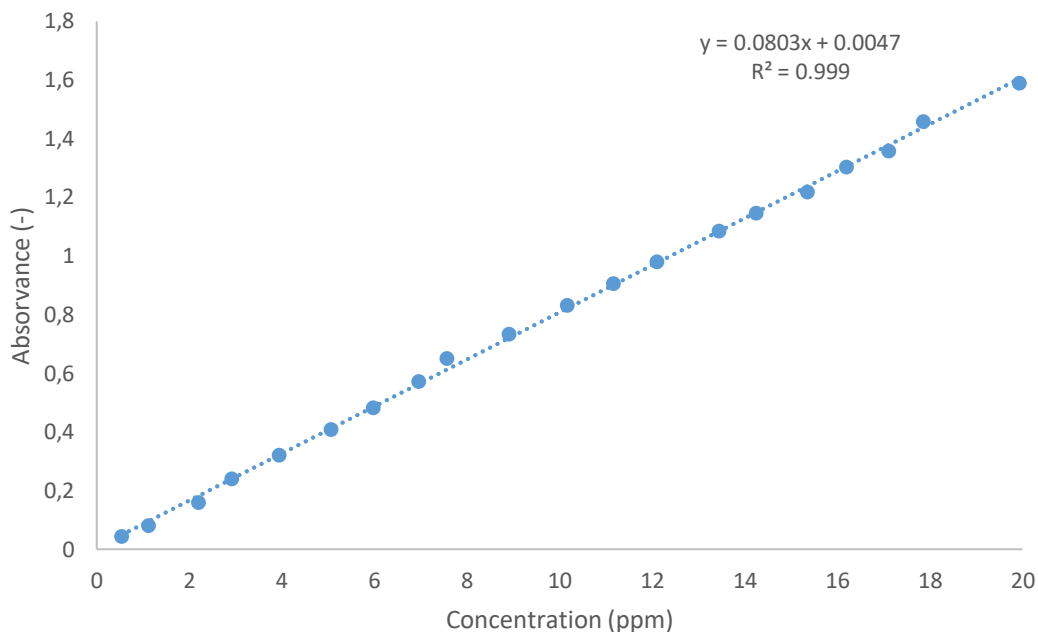


Figure 9. UV-VIS spectrophotometer - HACH LANGE DR 6000 and reading standards.

Table 7. Values of the masses weighed for each standard solution, their actual concentration and absorbance reading

Standard	Final Volume (mL)	Volume added (mL)	m_{added} (g)	m_{final} (g)	C (ppm)	A
1	25	0.129	0.134	24.374	0.531	0.043
2	25	0.259	0.280	24.105	1.122	0.082
3	10	0.207	0.223	9.817	2.197	0.160
4	10	0.311	0.295	9.754	2.922	0.240
5	10	0.414	0.397	9.715	3.944	0.321
6	10	0.518	0.508	9.702	5.057	0.409
7	10	0.621	0.600	9.716	5.967	0.483
8	10	0.725	0.700	9.709	6.960	0.572
9	10	0.828	0.757	9.669	7.563	0.650
10	10	0.932	0.894	9.691	8.909	0.733
11	10	1.036	1.021	9.706	10.162	0.832
12	10	1.139	1.120	9.689	11.159	0.907
13	10	1.243	1.212	9.675	12.097	0.981
14	10	1.346	1.356	9.738	13.444	1.085
15	10	1.450	1.429	9.688	14.246	1.147
16	10	1.553	1.542	9.695	15.357	1.219
17	10	1.657	1.628	9.706	16.200	1.303
18	10	1.760	1.713	9.667	17.113	1.359
19	10	1.864	1.781	9.632	17.857	1.458
20	25	5.178	5.058	24.503	19.933	1.589

Based on the values shown in Table 7, the calibration curve was run, relating the actual calculated concentrations (x-axis) to the absorbances corresponding to each standard (y-axis). In this way, the straight line equation with the best fit to the calibration curve and the associated determination coefficient are obtained (see Graph 1).



Graph 1. Calibration curve for acetaminophen using a solvent consisting of 15 % pure methanol and 85 % redistilled and deionised water.

The following table shows the statistical parameters associated with the calibration curve obtained in the study, where b represents the slope of the line and a the ordinate at the origin; $S_{y/x}$ is the residual standard deviation of the regression line, which expresses the dispersion of the instrumental signal values; and S_b and S_a are the standard deviations of the slope and the origin ordinate, respectively:

Table 8. Statistical parameters of the calibration curve

b	0.0803	0.0047	a
s_b	0.00052	0.0057	s_a
R^2	0.999	0.014	$S_{y/x}$
		19	gL

To make the calculations and obtain the values shown in Table 8, the following formulae were used :

$$b = \frac{\sum_{i=1}^N [(x_i - \bar{x}) \times (y_i - \bar{y})]}{\sum_{i=1}^N (x_i - \bar{x})^2}$$

$$a = \bar{y} - b \times \bar{x}$$

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

$$S_b = \frac{S_{y/x}}{\sqrt{\sum_{i=1}^N (x_i - \bar{x})^2}}$$

$$S_a = S_{y/x} \times \sqrt{\frac{\sum_{i=1}^N x_i^2}{N \times \sum_{i=1}^N (x_i - \bar{x})^2}}$$

Considering the values obtained in Table 8, it is possible to calculate the confidence limits, with a 95 % degree of confidence, of the parameters b and a , bearing in mind that t corresponds to the value of the Student variable for the respective confidence level and degrees of freedom:

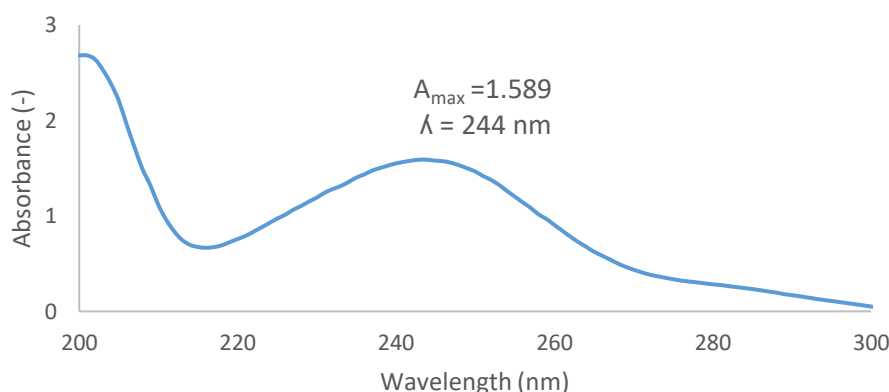
$$t_{(n-2)} = 2.101$$

$$a \pm t \times S_a = (0.005 \pm 0.012) \text{ ppm}$$

$$b \pm t \times S_b = (0.008 \pm 0.011) \text{ ppm}$$

5.2.4. Definition of maximum absorbance

Starting from the last standard on the calibration curve, i.e. the standard with the highest concentration, it is possible to define the maximum absorption for the readings to be taken. The absorption spectrum is shown in Graph 2.



Graph 2. Absorption spectrum of the standard solution with a concentration of 19.93 ppm.

As can be seen, the value defined as the absorption maximum ($A_{\max}$) is found at the wavelength $\lambda = 244$ nm. Based on this value, all subsequent absorbance measurements were carried out at this established wavelength. This absorption band is characteristic of the electronic transition $n - \pi^*$ of the group $C = O$ corresponding to the active substance acetaminophen [61].

5.3. Stability study test on acetaminophen solutions

One of the main disadvantages of the active substance acetaminophen is that it is slightly sensitive when exposed to light in solution form [79], therefore the absorbance measurement should be carried out immediately after the preparation of the solutions.

Given the nature of the experimental work carried out in this dissertation, it is necessary to take into account how this characteristic of the active substance under study might affect the results eventually obtained. To this end, and in order to test this information, an experimental test was carried out taking into account three different storage conditions in order to investigate how the acetaminophen solution behaves under different conditions.

As with the preparation of the working solution, as previously described in section 5.2.2., the solution for the stability test followed the same preparation guidelines, with a solution being made and then a working solution. Table 9 shows the weights of the two solutions and the final concentration of the working solution.

Table 9. Weights of the stock and working solution preparations

Stock solution		Working solution		
m_{ACT} (g)	$m_{final\ ms}$ (g)	m_{added} (g)	$m_{final\ w}$ (g)	C_w (ppm)
0.0585	48.959	4.925	48.630	95.847

Starting with the working solution, a standard solution was prepared by diluting 5 mL of the working solution into a final volume of 50 mL, which was topped up with the solvent. A volume of 0.25 mL of hydrochloric acid was also added to help preserve the solution.

After reading the solution on the UV-Vis spectrophotometer, considering zero time, the solution was divided equally into three different containers. Each container was subjected to a single storage condition: one was left on the bench exposed to sunlight (light), another was protected by being wrapped and placed inside a cupboard (dark) and finally, the third and last condition, placed in a refrigerator, i.e. in a chilled environment (refrigerated).

Absorbance readings were taken of the different solutions at three separate times: 24, 48 and 120 hours; the solution that was in the refrigerated environment was brought to room temperature before being read. The readings for each condition and the respective concentrations are shown in Table 10.

For each day that the absorbance of the solutions were read, which consisted of three readings each, a calibration curve was run on the same day, from which calculations were made to obtain the concentration values corresponding to each absorbance value obtained. This calculation was carried out using the following formula:

$$C = \frac{\bar{A}}{b} + a$$

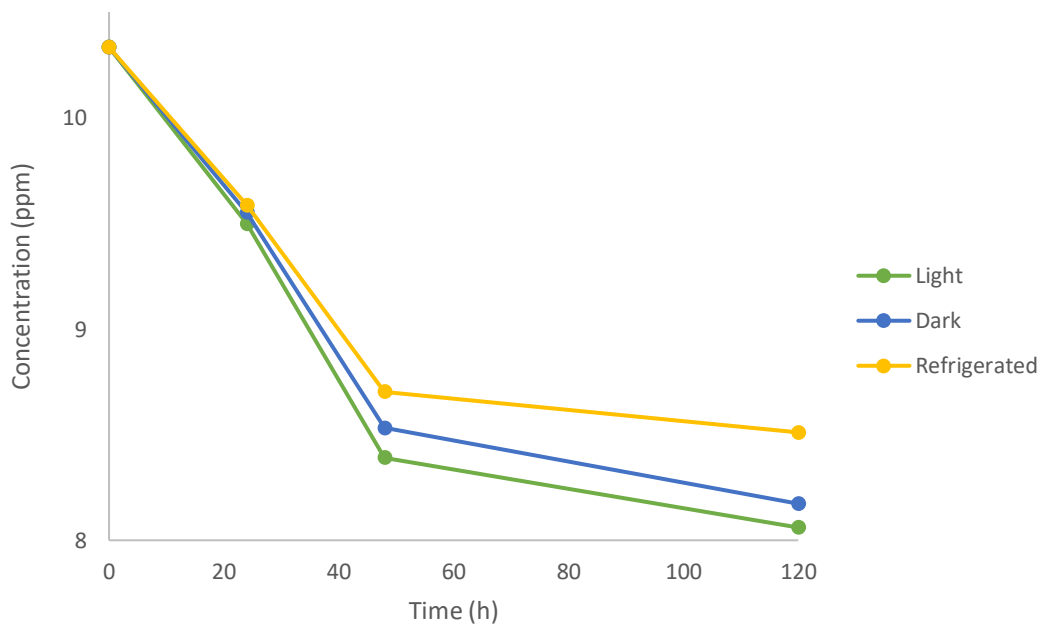
Based on the concentrations obtained, the values were compared with the value calculated for time zero in order to understand the difference in concentrations over time. The calculation was based on the following formula:

$$\Delta C(\%) = \frac{C - C_0}{C_0} \times 100$$

Table 10. Absorbance measurements corresponding to the different storage conditions and times, along with their concentrations and the percentage decrease in their concentration

time (h)	Calibration Curve		Storage conditions	$\bar{A}$	s	C (ppm)	$\Delta C(\%)$
	b	R ²					
0	0.07993	0.9991	-	0.8260	0.0036	10.330	0.000
24	0.08303	0.9992	Light	0.7886	0.0005	9.498	-8.079
			Dark	0.7930	0.0000	9.551	-7.574
			Refrigerated	0.7960	0.0052	9.587	-7.225
48	0.08029	0.9992	Light	0.6736	0.0005	8.391	-18.79
			Dark	0.6850	0.0000	8.532	-17.43
			Refrigerated	0.6986	0.0006	8.702	-15.79
120	0.07997	0.999	Light	0.6446	0.0006	8.061	-21.99
			Dark	0.6536	0.0006	8.173	-20.91
			Refrigerated	0.6806	0.0006	8.511	-17.64

Based on the values shown in Table 10., a visual graphical representation was made to better see the percentage decrease in concentrations for the different storage conditions over time (see Graph 3.).



Graph 3. Representation of concentrations for the different storage conditions over time.

Analysis of the graphical representation depicted in Graph 3 reveals intriguing insights into the stability of acetaminophen solutions under varying environmental conditions. Notably, a discernible trend emerges, with the solution exposed to sunlight exhibiting a sharper decrease in percentile over time. This observation aligns with the initial hypothesis positing the inherent instability of acetaminophen solutions to light exposure, substantiating the notion that prolonged exposure to such conditions renders the solution non-viable over time.

Conversely, the solution stored in a refrigerated environment demonstrates the least change in concentration over time, albeit with a significant deviation compared to measurements at zero time. Despite this variation, the refrigerated condition emerges as the most favourable for solution storage, indicating its efficacy in preserving acetaminophen stability over extended durations.

These findings underscore the critical importance of environmental factors in influencing the stability and viability of pharmaceutical solutions, with implications for formulation design and storage protocols. Furthermore, they highlight the need for meticulous consideration of storage conditions to ensure product integrity and efficacy throughout its shelf life.

5.4. Acetaminophen content of the various pharmaceutical preparations under study

5.4.1. Methodology

In order to quantify acetaminophen in the pharmaceutical preparations under study, solutions were prepared in a final volume of 25 mL, known as primary solutions, consisting of 100 mg of acetaminophen equivalent of pharmaceutical preparations (taking into account the labelled value shown in Tables 2 and 11). These solutions were weighed using an analytical balance at the beginning and end of their preparation.

The primary solutions of each sample were diluted in two stages. In the first stage, a 40-fold dilution was carried out (intermediate solution), removing 0.625 mL of the primary solution into a final volume of 25 mL, the volume being perfected with the solvent. In the second stage, a 10-fold dilution was carried out, using a syringe and a filter, 1 mL of the intermediate solution was removed to a 10 mL container and 0.05 mL of hydrochloric

acid was added, making three solutions a, b and c. All the solutions were weighed before and after preparation.

As mentioned, masses of pharmaceutical preparations equivalent to 100 mg of acetaminophen were used. These were determined on the basis of the dosage described by the respective manufacturers and on the basis of the average weights available in Table 4, taking into account the following equation:

$$m_{to\ add} = \frac{\bar{m}_{tab} \times 100\ mg}{m_{prod}}$$

In the equation $\bar{m}_{tab}$ corresponds to the average mass of the tablets/soluble powders of the pharmaceutical preparations, and m_{prod} the amount of acetaminophen presents in them according to the manufacturer.

Table 11 shows the quantities to be added to the different solutions, taking into account the experimental parameters outlined and defined:

Table 11. Weights of the various pharmaceutical preparations equivalent to 100 mg of acetaminophen

Product name	m_{prod} (mg)	nº	$\bar{m}_{tab} \pm s$ (g)	$m_{to\ add}$ (g)
A1	1000	27	1.209 ± 0.014	0.1209
B2	500	7	0.559 ± 0.015	0.1118
C3	500	7	0.601 ± 0.004	0.1202
D4	500	7	0.733 ± 0.002	0.1465
E5	500	7	0.684 ± 0.003	0.1367
F6	325	7	0.700 ± 0.003	0.2155
G7	500	1	12.98	2.5959
H8	1000	1	4.97	0.4973
I9	500	1	4.33	0.8666

5.4.2. Results obtained and calculations performed

In general, for each pharmaceutical preparation, three 10-fold dilution solutions, labelled a, b and c, were made. For all the solutions analysed three readings were taken at a wavelength of 244 nm.

The following tables (12, 14 to 19) show the values of the weighed masses of each solution at the beginning and end of its preparation, as well as the measured absorbance values and their standard deviation.

Based on the absorbance values obtained, the following equation was applied, which takes into account the parameters obtained from the calibration curve carried out on the same day as the measurements were taken.

$$c = \frac{\bar{A}}{b} \times \frac{m_{f40}}{m_{i40}} \times \frac{m_{f10}}{m_{i10}}$$

Using this equation, where m_i corresponds to the initial mass weighed and m_f to the final mass (after adding the solvent), acetaminophen was quantified in the pharmaceutical preparations under study.

Table 12. Data corresponds to the pharmaceutical preparation called A1, where the weights of the respective solutions and the absorbance values measured at 244 nm are shown

Product name	number	Starting solution		Dilution (40x)		Dilution (10x)			A	$\bar{A}$	s
		m_{added} (g)	m_{final} (g)	$m_{initial\ 40}$ (g)	$m_{final\ 40}$ (g)	id	$m_{initial\ 10}$ (g)	$m_{final\ 10}$ (g)			
A1	1	0.1220	23.5166	0.5925	24.5665	a	1.0055	9.4757	0.716	0.716	0.001
									0.716		
									0.715		
						b	0.9886	9.5655	0.726	0.726	0.000
									0.726		
									0.726		
						c	1.1176	10.2197	0.761	0.761	0.001
									0.76		
									0.761		
	2	0.1261	24.5447	0.5925	24.4256	a	1.1781	9.4863	0.793	0.793	0.001
									0.793		
									0.792		
						b	0.9685	9.2174	0.814	0.814	0.000
									0.814		
									0.814		
						c	1.2082	9.5948	0.812	0.811	0.001
									0.811		
									0.811		

Table 13. Calculated values for the quantification of acetaminophen in the pharmaceutical preparation A1

Product name	number	id	Curve of calibration		c (ppm)	$\bar{c}$ (ppm)	s (ppm)	$m_{tab\ calc.}$ (mg)
			b	R ²				
A1	1	a	0.08303	0.999	3299	3381	73	995
		b			3437			
		c			3406			
	2	a			3110	3343	376	993
		b			3777			
		c			3141			

The average concentration obtained for each solution was first calculated and then the average weight of the primary substance was calculated using the following equation:

$$m_{tab\ calc.} = \bar{c} \times \frac{m_{final}}{\rho} \times 10^{-3} \times \frac{\overline{m_{tab}}}{m_{added}}$$

Where ρ corresponds to the density of the methanol used in the solvent mixture, which takes a value of 0.792 g/mL, and $\overline{m_{tab}}$ represents the average weight of the pharmaceuticals shown in Table 11.

The values shown in Table 13 take into account that a Grubbs statistical test was carried out to check for outliers before proceeding with the rest of the calculations. At a significance level of 95 %, none of the values obtained were found to be outliers, and they were all used for the average concentration.

The same calculations were applied to the other absorbance values obtained for the pharmaceutical preparations under study, for the same wavelength of 244 nm. A calibration curve was carried out on the same day as each set of measurements.

Table 19 shows the values for the three pharmaceutical preparations under study in soluble powder form, G7, H8 and I9, where only one solution was prepared for each and the content equivalent to one sachet was evaluated.

Table 14. Data corresponds to the pharmaceutical preparation called B2, where the weights of the respective solutions and the absorbance values measured at 244 nm are shown

Product name	number	Starting solution		Dilution (40x)		Dilution (10x)			A	$\bar{A}$	s
		m_{added} (g)	m_{final} (g)	$m_{initial\ 40}$ (g)	$m_{final\ 40}$ (g)	id	$m_{initial\ 10}$ (g)	$m_{final\ 10}$ (g)			
B2	1	0.1126	22.6899	0.5658	23.7725	a	1.1561	9.601	0.799 0.800 0.800	0.799	0.001
						b	0.9891	9.2763	0.826 0.825 0.825	0.825	0.001
						c	1.1604	8.9796	0.920 0.920 0.920	0.920	0.000
						a	1.0765	9.3742	0.814 0.813 0.813	0.813	0.001
						b	1.3972	9.2406	0.828 0.828 0.828	0.828	0.000
						c	1.2142	9.4147	0.817 0.817 0.817	0.817	1.359E-16
	2	0.1076	22.6060	0.6155	24.6053	a	1.0765	9.3742	0.814 0.813 0.813	0.813	0.001
						b	1.3972	9.2406	0.828 0.828 0.828	0.828	0.000
						c	1.2142	9.4147	0.817 0.817 0.817	0.817	1.359E-16
						a	1.0765	9.3742	0.814 0.813 0.813	0.813	0.001
						b	1.3972	9.2406	0.828 0.828 0.828	0.828	0.000
						c	1.2142	9.4147	0.817 0.817 0.817	0.817	1.359E-16

Table 15. Data corresponds to the pharmaceutical preparation called C3, where the weights of the respective solutions and the absorbance values measured at 244 nm are shown

Product name	number	Starting solution		Dilution (40x)		id	Dilution (10x)		A	$\bar{A}$	s
		m_{added} (g)	m_{final} (g)	$m_{initial\ 40}$ (g)	$m_{final\ 40}$ (g)		$m_{initial\ 10}$ (g)	$m_{final\ 10}$ (g)			
C3	1	0.1228	23.7173	0.5793	24.6642	a	1.091	9.2779	0.836	0.836	0.001
									0.837		
									0.836		
						b	1.072	9.651	0.737	0.738	0.001
									0.738		
									0.739		
	2	0.1208	23.2158	0.5342	24.0565	c	1.2104	9.6055	0.754	0.755	0.001
									0.755		
									0.755		
						a	1.0712	8.9025	0.824	0.825	0.001
									0.825		
									0.825		
						b	1.1214	9.6072	0.777	0.776	0.001
									0.776		
									0.776		
						c	1.1366	9.6719	0.830	0.830	0.000
									0.830		
									0.830		

Table 16. Data corresponds to the pharmaceutical preparation called *D4*, where the weights of the respective solutions and the absorbance values measured at 244 nm are shown

Product name	number	Starting solution		Dilution (40x)		id	Dilution (10x)		A	$\bar{A}$	s
		m_{added} (g)	m_{final} (g)	$m_{initial}$ 40 (g)	m_{final} 40 (g)		$m_{initial}$ 10 (g)	m_{final} 10 (g)			
<i>D4</i>	1	0.1478	23.4568	0.3465	23.5700	a	1.0953	9.5436	0.750	0.749	0.001
									0.749		
									0.748		
						b	1.107	9.5457	0.763	0.763	0.001
									0.764		
									0.763		
	2	0.1501	24.1451	0.3698	24.1872	c	1.1054	9.5838	0.735	0.735	0.000
									0.735		
									0.735		
						a	1.1041	9.6958	0.722	0.722	0.001
									0.722		
									0.723		
	2	0.1501	24.1451	0.3698	24.1872	b	1.12	10.0969	0.680	0.679	0.001
									0.679		
									0.679		
						c	1.1618	10.2477	0.683	0.683	1.35E-16
									0.683		
									0.683		

Table 17. Data corresponds to the pharmaceutical preparation called E5, where the weights of the respective solutions and the absorbance values measured at 244 nm are shown

Product name	number	Starting solution		Dilution (40x)		Dilution (10x)			A	$\bar{A}$	s
		m_{added} (g)	m_{final} (g)	$m_{initial\ 40}$ (g)	$m_{final\ 40}$ (g)	id	$m_{initial\ 10}$ (g)	$m_{final\ 10}$ (g)			
E5	1	0.1386	24.7021	0.5119	24.3763	a	1.0719	9.7056	0.767	0.767	0.001
									0.767		
									0.768		
						b	1.052	9.6753	0.762	0.762	0.000
									0.762		
									0.762		
						c	1.0849	10.1754	0.747	0.746	0.001
									0.747		
									0.746		
						a	1.4532	10.0169	1.009	1.009	0.000
									1.009		
									1.009		
	2	0.1372	24.8416	0.8082	25.0228	b	1.2337	10.0337	0.869	0.868	0.001
									0.868		
									0.868		
						c	1.1163	9.736	0.822	0.822	0.000
									0.822		
									0.822		

Table 18. Data corresponds to the pharmaceutical preparation called F6, where the weights of the respective solutions and the absorbance values measured at 244 nm are shown

Product name	number	Starting solution		Dilution (40x)		Dilution (10x)			A	$\bar{A}$	s
		m_{added} (g)	m_{final} (g)	$m_{initial\ 40}$ (g)	$m_{final\ 40}$ (g)	id	$m_{initial\ 10}$ (g)	$m_{final\ 10}$ (g)			
F6	1	0.2155	24.5331	0.5324	24.2232	a	1.2548	9.6216	0.869	0.869	0.001
									0.868		
									0.869		
						b	1.2023	9.9999	0.831	0.830	0.001
									0.830		
									0.830		
	2	0.2161	24.6254	0.5099	23.7369	c	1.2224	10.2029	0.796	0.796	0.000
									0.796		
									0.796		
						a	1.1478	9.563	0.875	0.874	0.001
									0.874		
									0.874		
						b	1.2029	9.9171	0.873	0.873	0.001
									0.873		
									0.874		
						c	1.3601	9.7174	0.943	0.943	0.000
									0.943		
									0.943		

Table 19. Data corresponds to the pharmaceuticals preparations called G7, Griplex and I9, where the weights of the respective solutions and the absorbance values measured at 244 nm are shown

Product name	number	Starting solution		Dilution (40x)		id	Dilution (10x)		A	$\bar{A}$	s
		m_{added} (g)	m_{final} (g)	$m_{initial\ 40}$ (g)	$m_{final\ 40}$ (g)		$m_{initial\ 10}$ (g)	$m_{final\ 10}$ (g)			
G7	1	2.633	25.5818	0.646	23.8346	a	1.0515	9.9688	1.001	1.000	0.001
									1.000		
									1.000		
						b	1.107	9.9483	1.021	1.021	0.001
									1.020		
									1.021		
H8	1	0.4974	24.8492	0.4937	23.8319	c	1.1149	9.7569	1.048	1.048	0.001
									1.049		
									1.048		
						a	1.0622	9.722	0.869	0.870	0.001
									0.870		
									0.870		
						b	1.2309	9.8317	0.961	0.961	0.000
									0.961		
									0.961		
						c	1.1008	9.8754	0.896	0.895	0.001
I9	1	0.8686	24.99	0.5188	24.3036				0.896		
									0.896		
									0.894		
						a	1.1824	9.9277	0.782	0.782	0.000
									0.782		
									0.782		
						b	1.2405	9.5047	0.751	0.751	0.000
									0.751		
									0.751		
						c	1.0631	9.4264	0.791	0.791	0.000
									0.791		
									0.791		

Table 20. Calculated values for the quantification of acetaminophen in the rest of the pharmaceutical preparations in study

Product name	number	id	Curve of calibration		c (ppm)	$\bar{c}$ (ppm)	s (ppm)	$m_{tab\ calc.}$ (mg)
			b	R^2				
B2	1	a	0.08303	0.999	3299	3564	274	507
		b			3847			
		c			3545			
	2	a			3348	2978	380	442
		b			2590			
		c			2995			
C3	1	a			3583	3312	248	485
		b			3339			
		c			3011			
	2	a			3651	3651	97	532
		b			3539			
		c			3763			
D4	1	a	0.0799	0.999	5410	3925	1110	576
		b			5459			
		c			5280			
	2	a			5051	3479	1072	518
		b			4868			
		c			4790			
E5	1	a			4034	3370	515	518
		b			4069			
		c			4063			
	2	a			2642	3293	541	515
		b			2674			
		c			2711			
F6	1	a			3706	3464	238	349
		b			3839			
		c			3689			
	2	a			4148	3736	262	377
		b			4100			
		c			3842			
G7	1	a			4292	4135	94	658
		b			4153			
		c			4156			
H8	1	a			4699	3979	532	1248
		b			4541			
		c			4745			

I9	1	a			3752	3289	353	518
		b			3285			
		c			4009			

Similarly to Table 13, Table 20 shows the values obtained for the other pharmaceutical preparations after applying the formulae mentioned.

5.4.3. Acetaminophen content

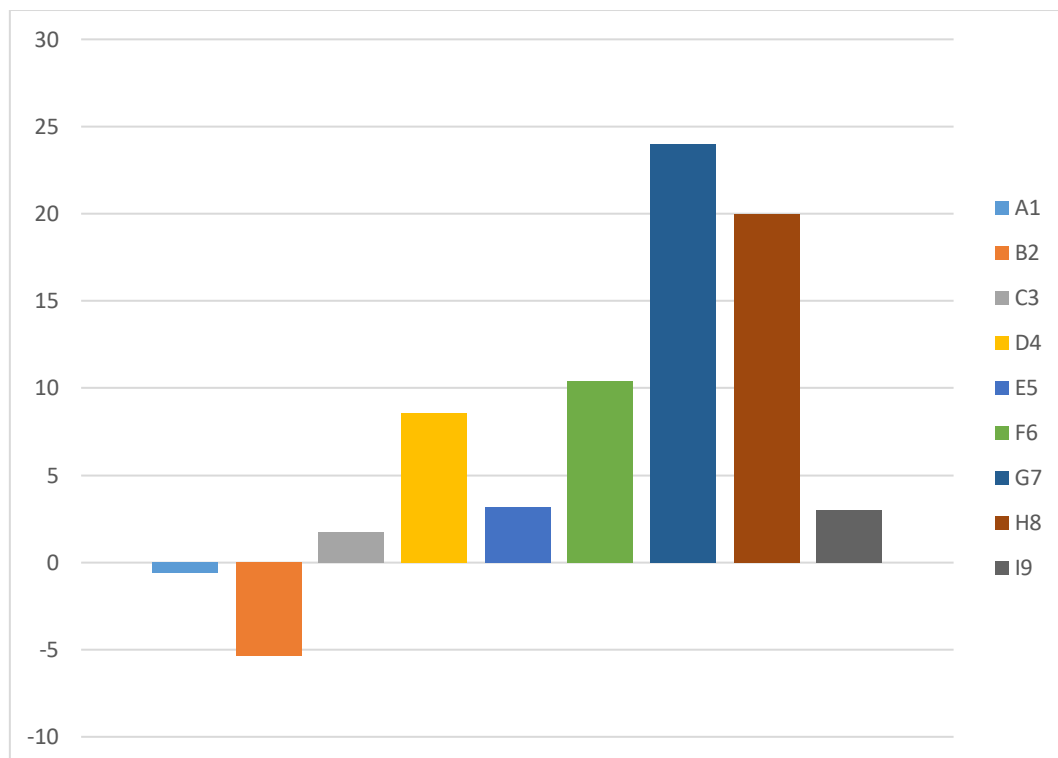
Following on from Table 20, the average acetaminophen content of the tablets/soluble powders submitted to the study in question was calculated for the various pharmaceutical preparations, enabling a comparison to be made between the value declared by the manufacturer and the value obtained analytically in the form of a percentage difference. These values are shown in Table 21 The difference in content was calculated using the following formula:

$$\Delta_m(\%) = \frac{\bar{m}_{tab} - m_{prod}}{\bar{m}_{tab}} \times 100$$

Table 21. The acetaminophen content of the analysed pharmaceutical preparations compared with the values declared by manufacturers

Product name	m_{prod} (mg)	$\bar{m}_{tab}$ (mg)	s (mg)	RSD (%)	Δm (%)
A1	1000	994	1	0.11	-0.60
B2	500	475	46	9.73	-5.37
C3	500	509	33	6.53	1.72
D4	500	547	41	7.56	8.60
E5	500	517	3	0.53	3.20
F6	325	363	20	5.42	10.38
G7	500	658	-	-	24.00
H8	1000	1248	-	-	20.00
I9	500	518	-	-	3.00

The following column chart (Graph 4) was drawn up based on the data presented in Table 21, in order to make it easier to compare the percentages obtained for the various pharmaceutical preparations under study.



Graph 4. Graphical representation of the values obtained for the percentage differential calculated for the various pharmaceutical preparations.

Upon scrutinizing the percentage differences between declared and analytically obtained values, a nuanced analysis unveils discernible patterns warranting thorough examination. Notably, sachet-format pharmaceutical preparations, *G7* and *H8*, emerge as outliers, exhibiting pronounced disparities. Specifically, *G7* demonstrates the most significant deviation, with a staggering 24 % variance above the manufacturer's indicated value. Similarly, disconcerting is the content observed in *H8*, surpassing the manufacturer's declaration by 20 %.

One possible explanation for the noticeable difference between the tabulated and experimental values obtained for the pharmaceuticals *G7* and *H8* could be related to the use of dry mass in the experimental analyses. Although unlikely, it is possible that the entity that produced the pharmaceuticals did not work with the dry mass of the excipient, which could certainly account for the observed differences.

Conversely, pharmaceutical formulations including *A1*, *B2*, and *C3* portray commendable adherence to stipulated compositions, highlighting percentage differences below 2 %. These findings underscore robust quality control practices and underscore the importance of stringent regulatory oversight in ensuring product integrity and safety.

Although *E5* and *I9* exhibit slightly elevated deviations, hovering around the 3 %, their values remain within acceptable. In contrast, tablet formulations such as *D4* and *F6* reveal conspicuous discrepancies, with percentages of 8.60 % and 10.38 %, respectively, exceeding anticipated values.

In essence, the observed discrepancies highlight the complexity inherent in pharmaceutical manufacturing and underscore the imperative for continual vigilance and adherence to stringent quality control measures. Addressing these disparities is paramount to ensuring product safety and efficacy, thereby safeguarding public health, and fostering consumer trust in pharmaceutical products.

5.5. Method validation

Throughout Chapter 2. of this work, numerous descriptions of validation parameters were made. These concepts were applied experimentally, namely parameters such as the determination of limits of detection and quantification , linearity, precision, and accuracy parameters.

To enable the calculation of these parameters, fifteen readings were taken of standards 1 and 20, which represent the lower and upper limits of the working range.

5.5.1. Calculation of limits of detection and quantification

To calculate the limits of quantification and detection, the values shown in Tables 7 and 8, as well as the equations presented in the respective section in the introduction were taken into account.

Thus, the values obtained for the detection and quantification limits were $LOD = 0.59 \text{ ppm}$ and $LOD = 1.70 \text{ ppm}$, respectively.

5.5.3. Linearity study

The first parameter to be assessed in this part of the study was whether or not there was homogeneity of variances. As previously mentioned, this checks whether there is a significant difference between the variances of two groups, in this case between the group of absorbance readings obtained for the first and last standards in the calibration curve.

For each group of 15 readings, the respective standard deviation and variance were calculated, and for standard 1 $s = 0.00140$ and $s^2 = 1.971 \times 10^{-6}$, and for standard 20 $s = 0.00184$ and $s^2 = 3.381 \times 10^{-6}$. Based on these values, for a 99 % degree of confidence, using the F (Fisher) test and defining that the degrees of freedom for both groups are fourteen, it was possible to calculate whether this is a calibration curve with homogeneity of variances, and the following results were obtained:

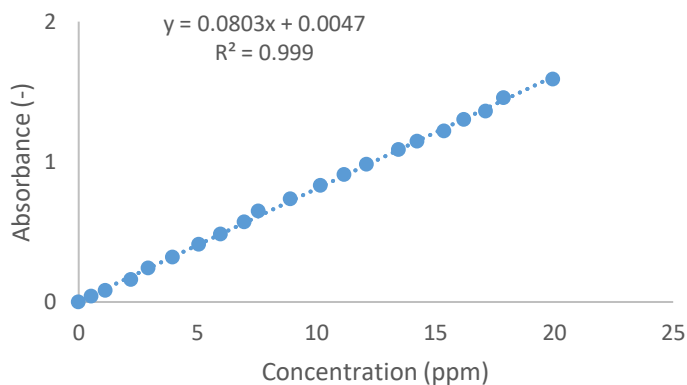
Table 22. Values for PG and F tabulated from Fisher's statistical test for 99 % confidence

PG	1.715
F	3.698

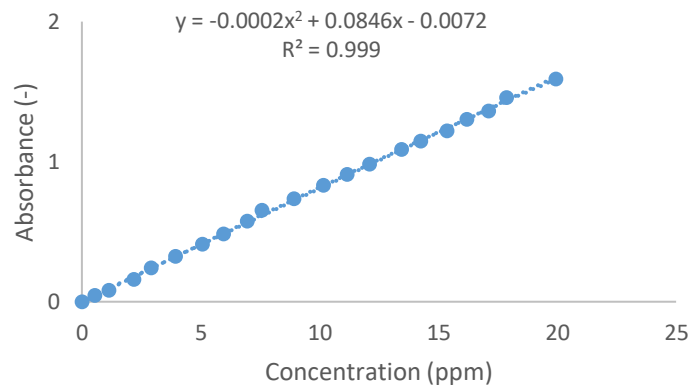
Based on data shown in Table 22, it can be concluded that $PG < F$, which at a significance level of 99 % means that there is homogeneity of variances, i.e. the difference between the variances is not considered significant and the working range is well adjusted.

Although the working range has been checked and is well adjusted, it is also necessary to carry out statistical tests to check the linearity of the calibration curve. The tests applied in this study were the Mandel test, the Rikilt test and the normalised areas test, all of which were introduced and described in the introduction of this thesis.

With regard to the Mandel test, performed at a confidence level of 99 % and also using Fisher's F test, this compares two models, the linear and the non-linear (polynomial), and which of them best fits the values shown in Table 6. The following graphs refer to both models:



Graph 5. Linear regression.

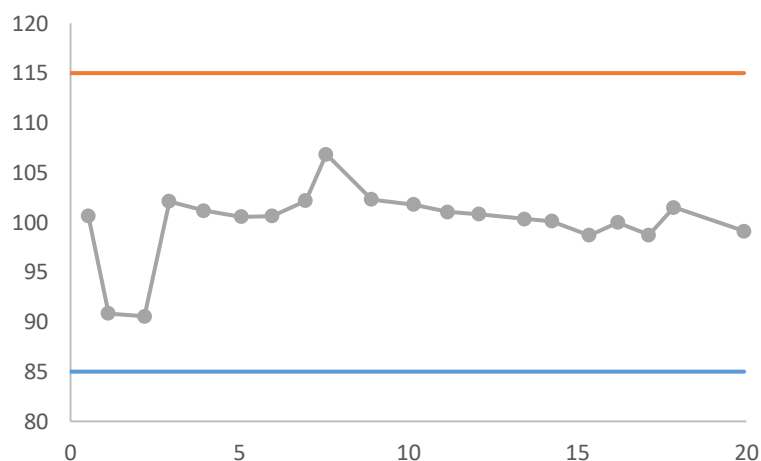


Graph 6. Non-linear regression (second degree polynomial).

As with Table 8, the respective parameters for Graphs 5 and 6 were also calculated in order to be able to carry out the Mandel test. This resulted in $DS^2 = 0.00110$, $PG = 6.979$ and $F = 8.285$. Thus, with $PG < F$, at 99 %, it can be concluded that the non-linear calibration function does not lead to a significant fit, so the calibration function is the one represented by the linear model.

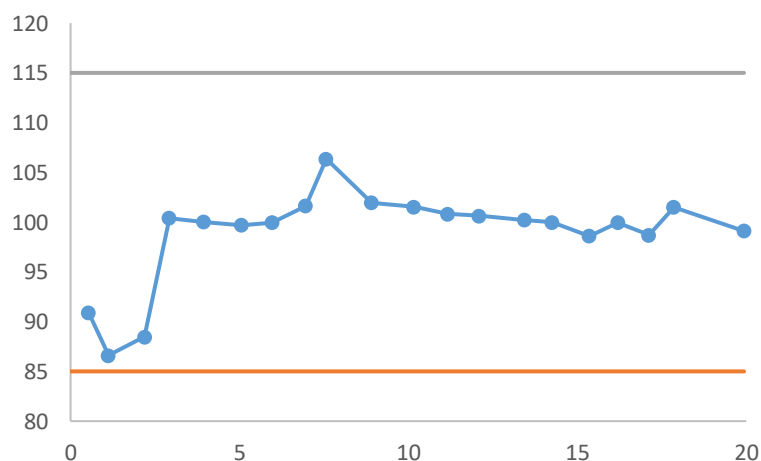
Moving on to the Rikilt test, as in the previous test, it is carried out with the aim of identifying whether the linear model is the best fit for the working range applied. After performing the calculations as described above, Graph 5 was obtained, showing the upper and lower limits of 115 % (orange) and 85 % (blue), respectively, and the relationship between the concentrations of the standards and the percentage ratio between the reference value and the signal/concentration ratios.

Graph 7 shows that all the points are within the established limits, which leads to the conclusion that the linear model is a good fit for all the points in the calibration curve in question. In this way, this test corroborates the Mandel test, which both consider that the linear calibration presents an acceptable fit.



Graph 7. Rikilt test to assess linearity.

Along the same line of thought, the normalised areas test was also carried out to assess the linearity of the calibration in question. As with the previous tests, this also resulted in the conclusion that the linear model was a good fit for the working range applied. Using Graph 8, which relates the calculated normalised areas to the concentrations of the standards and checks whether these are within the limits considered acceptable, it is possible to visualise this same conclusion.



Graph 8. Graph of the study of normalised areas.

5.5.4. Precision study

Two parameters were used to study precision in the work carried out: repeatability and intermediate precision.

With regard to the repeatability parameter, in a comparable way to the homogeneity of variances, the fifteen readings taken for the last and first standard on the calibration curve were also taken into account. Based on these, it was possible to apply the equations presented in section 2.2.1., taking into account the variance values calculated for each group. This made it possible to obtain the values shown in Table 23 for the repeatability study.

Table 23. Values for the repeatability parameter calculations

$s_{r\text{pooled}}$	0.0016
r	0.0046
$\%CV_r$	0.20

Moving on to the calculation of intermediate precision, in this case, given that these are solutions that are not stable over time, and that each test required a calibration curve to be run for each day, values from different standards run over time that have similar analyte concentrations in their composition should be used for the calculation. Having stipulated this condition, the standard selected was the one identified as 11, which in its theoretical constitution should have a concentration close to 10 ppm. Thus, all the ten standards carried out between October and January were selected and based on the absorbance values corresponding to 3 readings of each of the solutions, the calculation was carried out using the formula presented in the respective section, and a value of $S_I = 0.00082$.

5.5.5. Accuracy

In the section 2.1. of the introduction to this dissertation the procedure to be adopted when verifying the accuracy of a method is described. This same procedure, called the recovery test, was applied in the context in question. Two samples were prepared, one simple (unfortified) and the other fortified.

Using the Portuguese pharmaceutical preparation *A1*, the solution labelled as simple corresponds to the one without any addition of the standard material. As for the fortified solution, as its name indicates, in addition to containing *A1* in its composition, it will have an addition of 50 % of the reference material acetaminophen.

The samples described were initially prepared in containers with a final volume of 25 mL, the primary solutions, similar to the procedure described in section 5.4.1., were then diluted 40 times (intermediate solution), removing 0.625 mL of the respective solutions into a 30 mL container, and were in turn diluted 10 times using a syringe with a filter and adding 0.05 mL of hydrochloric acid to a container with a final volume of 10 mL. The only change to this procedure was the addition of the standard material, in a known quantity as shown in the following table, to the fortified primary solution. All the contents were duly weighed on an analytical balance so that the true concentrations could be calculated.

Again, using a UV-Vis spectrophotometer, absorbance readings were taken at the wavelength of 244 nm, and the values obtained are shown in Table 24. The same table shows the masses of *A1*, and standard material added to the respective solutions as well as the initial and final weights of each solution under study.

As in previous tests, a calibration curve was performed on the same day that the solutions in question were prepared, and this is shown in Table 25.

Table 24. Values corresponding to the different weighing's carried out during the preparation of the various solutions, and the respective absorbance values measured at 244 nm

Solutions	Starting solution			Dilution (40x)		Dilution (10x)			$\bar{A}$	σ
	m_{added} (g)	$m_{standard}$ (g)	m_{final} (g)	$m_{initial\ 40}$ (g)	$m_{final\ 40}$ (g)	id	$m_{initial\ 10}$ (g)	$m_{final\ 10}$ (g)		
Simple	0.1220	0.0000	24.5167	0.5925	24.5665	a	1.0055	9.4757	0.716	0.001
						b	0.9886	9.5655	0.726	0.000
						c	1.1176	10.2197	0.761	0.001
Fortified	0.1211	0.0503	39.5097	0.9025	24.7775	a	0.9136	9.2424	1.126	0.001
						b	1.0005	9.6925	1.197	0.000
						c	0.8749	9.1162	1.216	0.001

Table 25. Results obtained through the calculations carried out, together with the respective concentration values obtained and the recovery test carried out

Solutions	id	Curve of calibration		c (ppm)	$\bar{c}$ (ppm)	s (ppm)	$c_{standard}$ (ppm)	Recovery (%)
		b	R^2					
Simple	a	0.08303	0.9986	3299	3381	73	0	-
	b			3437				
	c			3406				
Fortified	a	0.08303	0.9986	3716	3881	227	1008	85
	b			3787				
	c			4140				

Using the values shown in Table 24 and the following equation, it was possible to calculate the concentrations of acetaminophen present in each solution, simple and fortified:

$$C_{standard} = \frac{m_{standard} \times \rho}{m_{final (fortified)}} \times 10^6$$

Based on the calculated acetaminophen concentrations in the samples and the added concentration, the percentage recovery shown in Table 25 was calculated using the following equation:

$$Recovery (\%) = \frac{\overline{C_{sol.fortified}} - \overline{C_{sol.simple}}}{C_{standard}}$$

In this last equation, the $\overline{C_{sol.fortified}}$ corresponds to the calculation of the average of the values obtained for the concentrations corresponding to the various readings taken for the fortified solution, the constitution of which takes into account the addition of the reference material, and $\overline{C_{sol.simple}}$, in turn, represents the average of the calculated values of the various concentrations corresponding to the unfortified solution.

After performing all the calculations, the recovery value obtained was 85 %, which is within the acceptable range of 80 to 120 % as set out in the introduction. In this way, the statistical study carried out leads to the conclusion that the method applied can be classified as an exact method, which along with its vast application advantages, demonstrates that it is a viable method leading to acceptable results.

5.6. Chemical profile of different matrices corresponding to different pharmaceuticals under study using the FTIR-ATR method

Several techniques were mentioned in Chapter 1.5, that, in addition to qualitatively identifying the active substance, also lead to the crucial identification of excipients, which play a fundamental role in the constituent matrix of pharmaceuticals due to their key functions. Among all the techniques outlined, FTIR-ATR emerged as the most appealing and suitable for this and was thus applied in the subsequent research.

5.6.1. Experimental procedure

With access to the same samples used in the previous acetaminophen quantification studies and their respective medical leaflets, they were then studied using PerkinElmer FTIR equipment, model Spectrum Two (ATR module), where they were subjected to 4 scans per spectrum, in a wave number range between $4000\text{--}400\text{ cm}^{-1}$, with the aid of the Spectrum software (Figure 10). Access to this equipment was provided by the laboratory **FCUP|DQB - Lab&Services**.

One of the main advantages of the equipment in question is that there is no need for sample preparation and that it can be recovered after analysis, i.e. it is non-destructive. Thus, without sample preparation, small quantities of the nine pharmaceutical preparations under study, previously ground, were placed precisely on top of the equipment's crystal with the aid of a spatula.

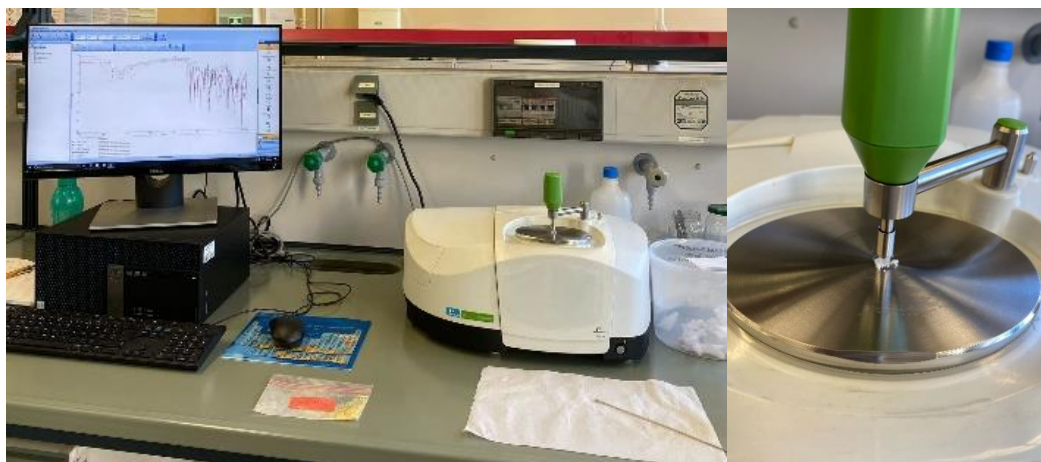


Figure 10. PerkinElmer FTIR equipment, model Spectrum Two (ATR module), with approximate image of its press system under the crystal, with sample in place.

Prior to any reading of the samples, a blank assay is carried out, which consists of a reading of the equipment in the surrounding air/environment, i.e. where the equipment is clean and devoid of sample, in order to eliminate any interference that could affect the sample reading, since solid samples do not have the capacity to completely cover the surface of the crystal, and there are spaces that will be filled by the air circulating in the environment where the equipment is located.

Based on the described procedure, infrared spectra were obtained for each pharmaceutical preparation, where clear similarities and distinctions between the various samples under study can be observed, as well as the possibility of identifying some excipients in their constitutions.

5.6.2. Analysis of the FTIR-ATR spectra of pharmaceutical preparations

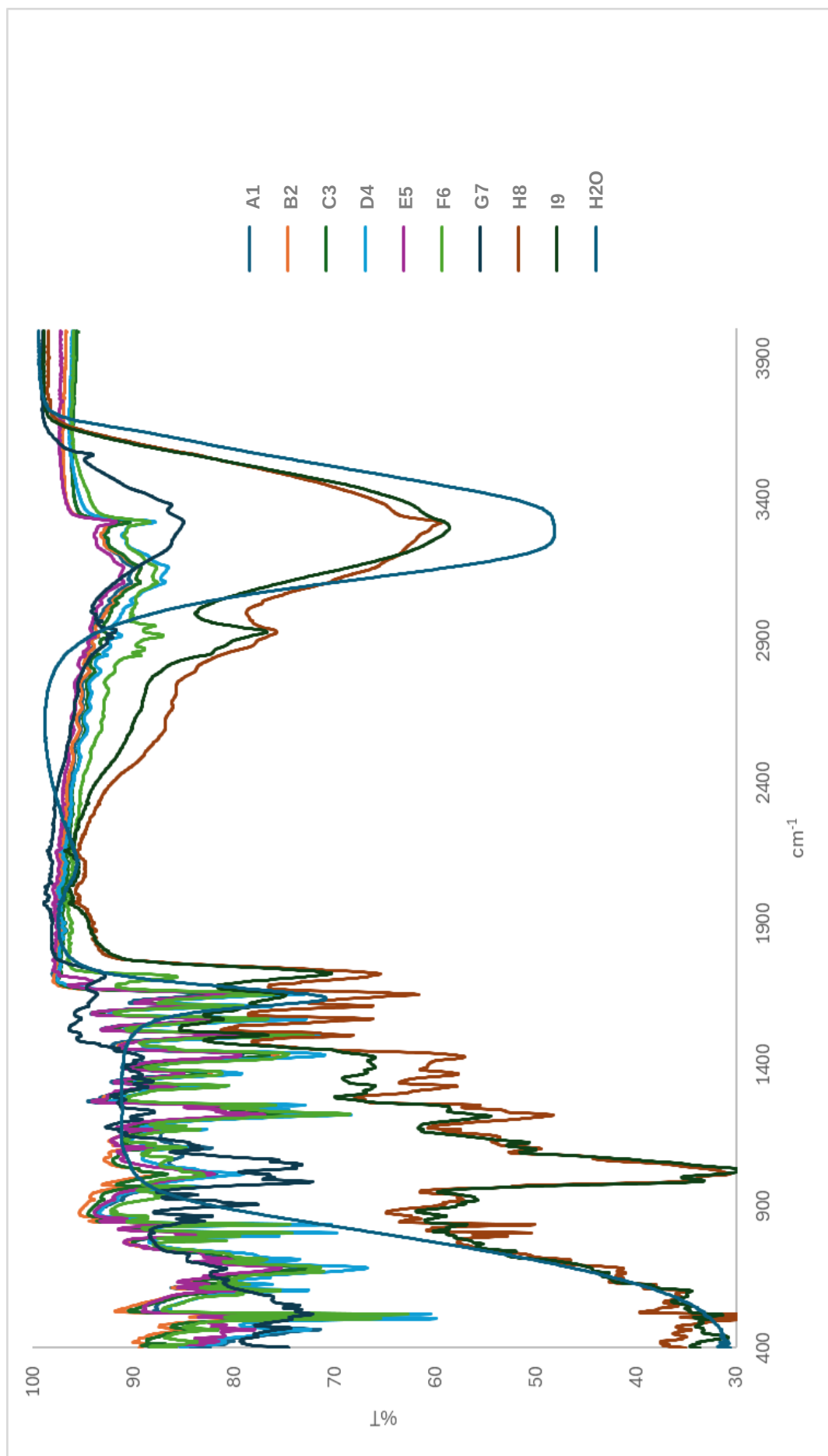
The main factor in common among all the pharmaceuticals under study is the active substance present in the constitution of all the preparations. Acetaminophen, whose chemical structure is shown in Figure 3, can be presented in the form of five different polymorphs; however, the one that arouses most interest in this study is the monoclinic crystalline form, also known as the commercial one [80].

According to the literature [61,81,82], this agent has a unique spectral fingerprint in the infrared, exhibiting several characteristic bands that are crucial for its identification. Some expected bands are presented in the following ranges: 3300-3500 cm^{-1} , which corresponds to a broad band due to the stretching vibration of the O-H bond of the hydroxyl group; 3037-3130 cm^{-1} , a band due to the vibration of the CH_3 (methyl) group; 3100-3000 cm^{-1} , related to the stretching vibrations of the N-H bond of the secondary amine group; 1665-1650 cm^{-1} , a prominent band due to the stretching vibration of the C=O (carbonyl) bond of the amide group; and 1610-1580 cm^{-1} , a band associated with the stretching vibration of the C=C bond of the aromatic ring.

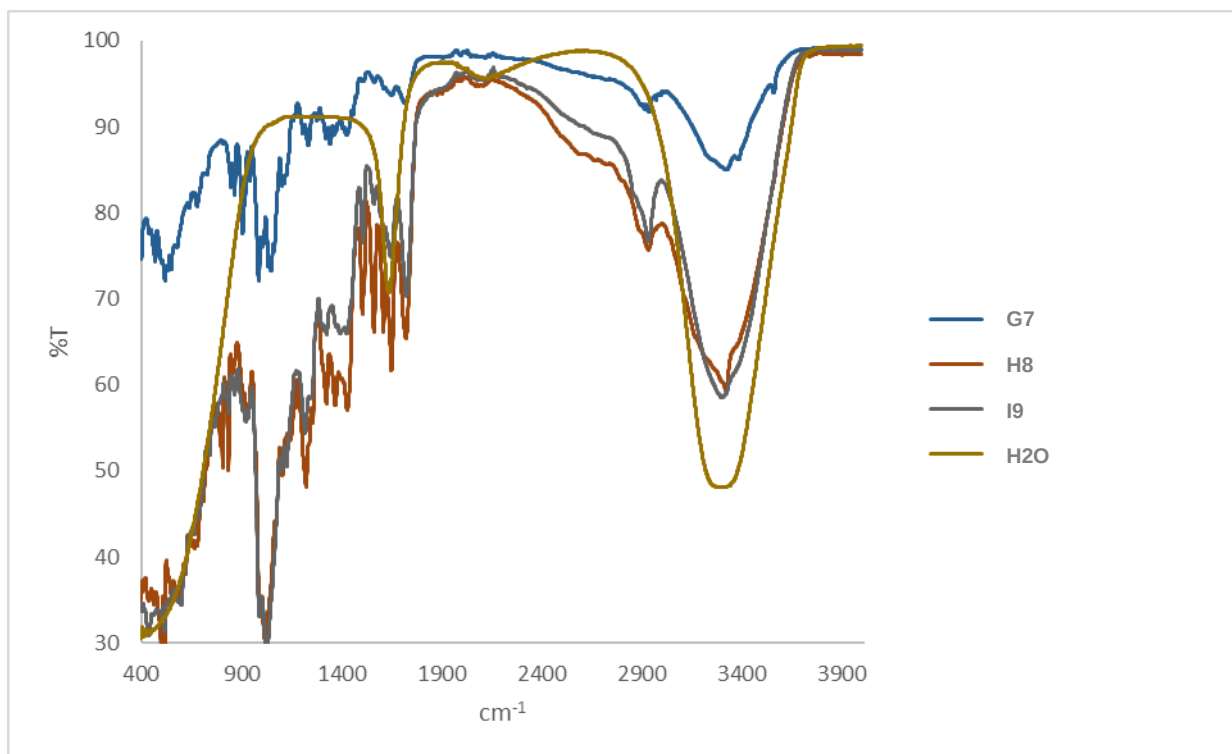
In a more comprehensive approach, Graph 9 illustrates all overlaid spectra to visually represent the differences and similarities among the nine pharmaceutical preparations under study, with the spectrum for H_2O included for comparison. It can be clearly observed that two samples, corresponding to the pharmaceuticals *I9* and *H8*, are markedly distinct from the others; however, they exhibit a high degree of similarity to each other, suggesting a comparable composition or formulation. The pharmaceutical *G7*, while showing significant differences similar to those of *I9* and *H8*, does not present

these differences as intensely. This indicates that although *G7* has some unique characteristics, it is somewhat closer in spectral profile to the other samples than *I9* and *H8* are. These spectra exhibit a marked resemblance to that of water, attributable to the fact that they are soluble powder compounds with a greater tendency and ease to react with water. This likely interaction with water is probably due to the ambient humidity to which the samples were exposed during storage in Poland prior to their analysis using the FTIR-ATR equipment at FCUP, a couple of months later.

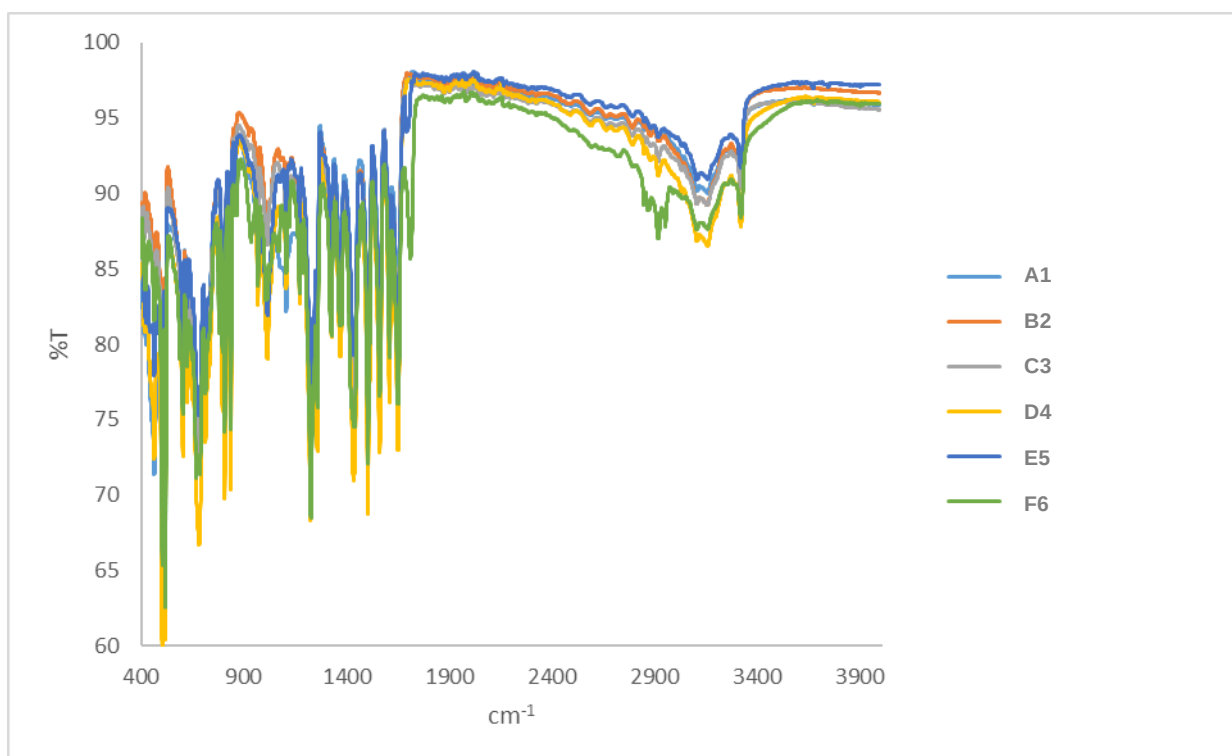
The remaining studied samples, when viewed overall, exhibit very similar spectral features in terms of band positions and their intensities. This similarity between the other preparations suggests a common point in their chemical composition or manufacturing processes. Consequently, this analysis leads to an initial classification, effectively dividing the pharmaceutical preparations into two main groups: soluble powder preparations (Graphic 10) and tablet (Graphic 11) forms.



Graph 9. Overlapping of the FTIR-ATR spectra of all the pharmaceuticals under study together with the H₂O spectrum.



Graph 10. Overlapping of the FTIR-ATR spectra of all the soluble powder preparations under study.



Graph 11. Overlapping of the FTIR-ATR spectra of all the tablet preparations under study.

Initiating the analysis of the FTIR spectra for the tablet-form pharmaceuticals, Figures 11 through 16 reveal the presence of the characteristic bands of the active substance known as acetaminophen, as previously indicated. From analysing these spectra, it can also be concluded that they show a high degree of similarity to each other, suggesting a consistent formulation in these samples. Furthermore, the spectra imply that the matrix complexity of these tablet formulations is comparable, indicating uniformity in their chemical composition and manufacturing processes. This uniformity is evident in the consistent presence and intensity of the acetaminophen-specific bands across the different samples, reflecting similar levels of matrix complexity and potentially similar excipients and manufacturing conditions.

The pharmaceutical under study, originating from Portugal and named A1, is represented in the FTIR-ATR spectrum in Figure 11. In addition to exhibiting the characteristic bands of the active ingredient, which constitutes 83 % of its composition, the spectrum also displays peaks arising from the vibrational modes of the excipients present in its formulation. Notably, three primary excipients in its composition are highlighted: corn starch, polyvinylpyrrolidone, commonly known as povidone, and hydrated colloidal silica [83].

The presence of corn starch is confirmed by multiple characteristic bands. The O-H stretching is represented by the broad band 3324 cm^{-1} , indicative of the hydroxyl groups present in starch. Additionally, the C-H stretching band 2929 cm^{-1} is associated with the aliphatic hydrogen atoms in the starch structure [84].

Regarding the excipient povidone, its presence is evidenced by the distinct C=O stretching (carbonyl group of the lactam) band represented in the range of $1660\text{--}1600\text{ cm}^{-1}$, 1653 cm^{-1} , which also refers to the N-C elongation [85]. These bands are characteristic of the povidone structure, confirming its presence in the formulation. This excipient is common to all pharmaceuticals in tablet form.

In the range of $1100\text{--}1000\text{ cm}^{-1}$, there is a prominent Si-O-Si stretching vibration, which corresponds to the chemical structure of hydrated colloidal silica. The bands 1109 and 1011 cm^{-1} in the spectrum, highlight the presence of silica within the pharmaceutical formulation [86,87]. Hydrated colloidal silica is commonly used as a glidant and thickening agent in tablet formulations.

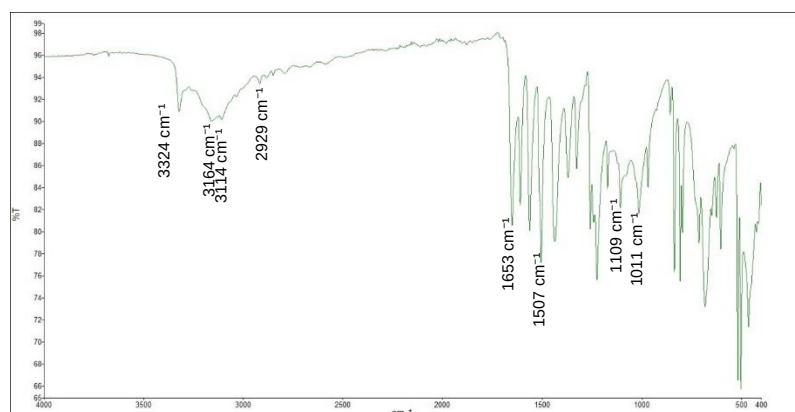


Figure 11. FTIR-ATR spectrum of the pharmaceutical A1.

These observations indicate the contributions of these excipients to the overall spectral profile of A1. The detailed analysis of these characteristic bands not only confirms the presence of the active ingredient and the excipients but also provides insights into the formulation's complexity. The specific functional groups associated with the active ingredient and excipients are reflected in their respective FTIR-ATR bands, validating the composition, and ensuring the quality and consistency of the pharmaceutical product.

Figures 12 and 13 correspond to the pharmaceutical preparations B2 and C3, respectively, and are very similar in terms of bands, intensity, and complexity of the constituent matrix. A distinct difference can be seen between these, and the spectrum of A1 shown in Figure 11, where the presence of the excipient hydrated colloidal silica, which is not included in the composition of the pharmaceuticals B2 and C3, stands out in the 1000 cm^{-1} zone. This is the biggest visual difference between these spectra.

Although they appear very similar in terms of spectra, these pharmaceuticals are made up of only two common excipients, povidone, and stearic acid. Both are fabricated of starch, but in the case of B2 it is pregelatinised starch and in the case of C3, like A1, it is constituted of maize starch.

Proceeding with the analysis of the FTIR-ATR spectrum of the common Polish medication known as B2, beyond the potential identification of the active agent, the presence of the excipient pregelatinized starch is attributed to the bands at 3325 cm^{-1} and 2929 cm^{-1} , as illustrated in Figure 12. These bands correspond, respectively, to the hydroxyl groups (O-H) present in the starch structure and the C-H bonds.

Regarding the excipient povidone, it is indicated by the presence of a band at 1654 cm^{-1} , which represents the C=O bond of the carbonyl group in the excipient's structure and the stretching of the N-C.

The last excipient identified in this spectrum is stearic acid, represented by the carboxyl and alkyl groups that are part of its structure. The carboxyl group is reflected in the bands at 3318 and 1647 cm^{-1} , due to the presence of O-H and C=O bonds, respectively. The alkyl group is indicated by the C-H stretching visible in the region $2850\text{--}2960\text{ cm}^{-1}$.

Similarly, the FTIR-ATR spectrum shown in Figure 13, related to the pharmaceutical preparation C3, reveals the presence of the same excipients that are part of its composition. Therefore, it is possible to attribute the bands represented in the figure, in the same manner as previously described, to the excipients corn starch, povidone, and stearic acid.

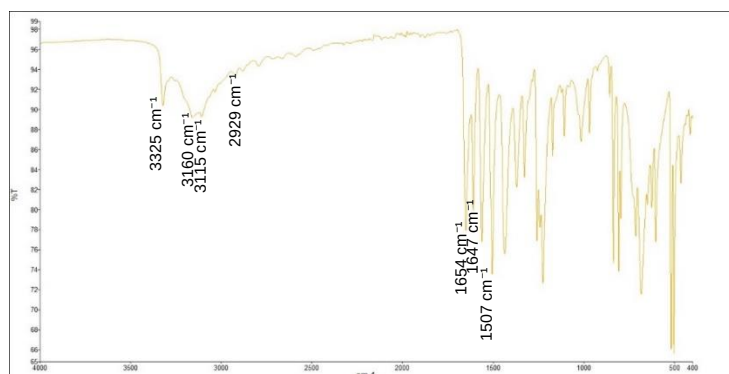


Figure 12. FTIR-ATR spectrum of the pharmaceutical B2.

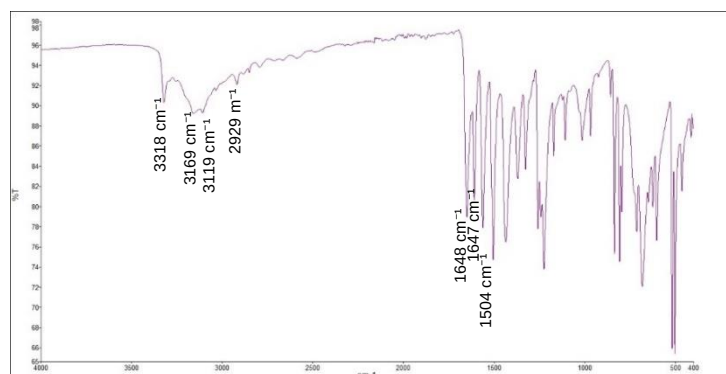


Figure 13. FTIR-ATR spectrum of the pharmaceutical C3.

In the pharmaceutical preparations *D4*, *E5*, and *F6*, given in Figures 14, 15, and 16 respectively, notable differences are observed in their FTIR-ATR spectra. Beyond the characteristic bands of the common active ingredients present in all three medications, the shared excipients identified in these pharmaceutical formulations include starch and povidone.

A more detailed examination of Figure 14 reveals similarities to previously presented spectra. The starch from potato, as indicated in the Table 2, is distinctly identified by the stretching vibrations of O-H and C-H bonds, represented by bands at 3327 and 2924 cm^{-1} .

¹, respectively. Furthermore, the presence of the excipient povidone is indicated by the band at 1653 cm^{-1} .

E5 exhibits a spectrum similar to the previous one, with identifiable excipients such as povidone, corn starch, and pregelatinized starch (Figure 15). A notable difference is the appearance of the band at 1704 cm^{-1} , attributable to the asymmetric stretching of the COOH group within the sodium croscarmellose structure [88,89]. This distinctive feature is also evident in the spectrum of the pharmaceutical *F6* (Figure 16).

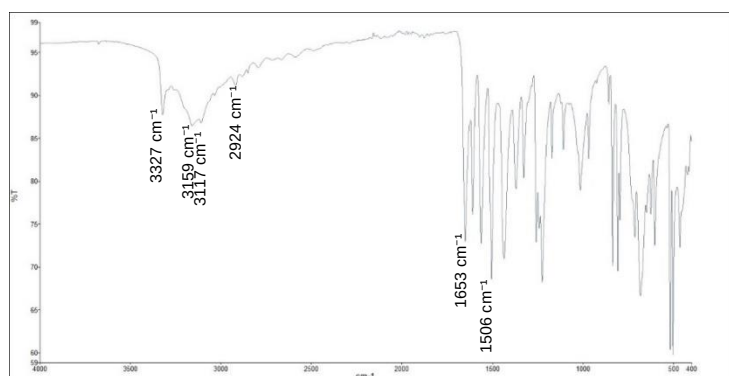


Figure 14. FTIR-ATR spectrum of the pharmaceutical *D4*.

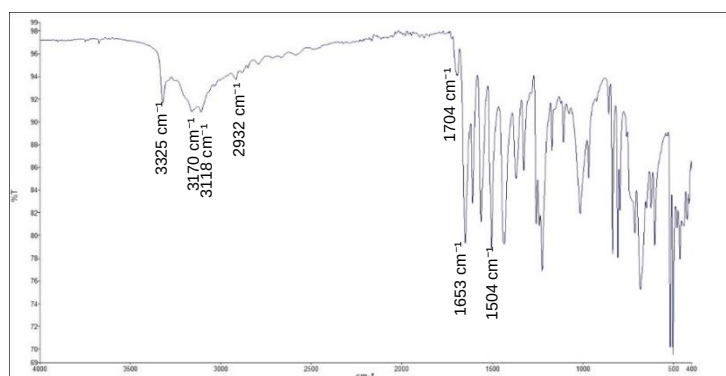


Figure 15. FTIR-ATR spectrum of the pharmaceutical *E5*.

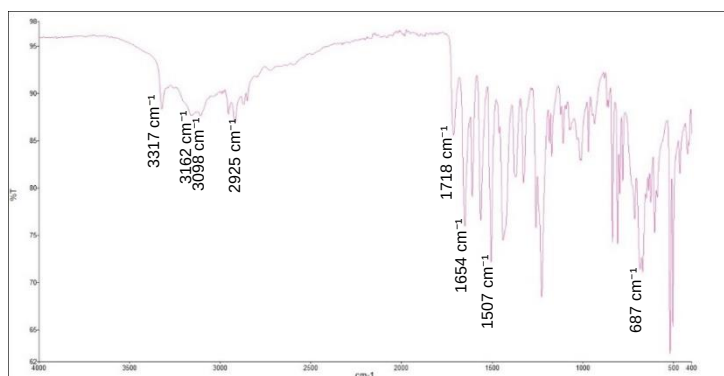


Figure 16. FTIR-ATR spectrum of the pharmaceutical *F6*.

F6, in addition to its active ingredient acetaminophen, exhibits therapeutic properties associated with ibuprofen, represented by the deformation outside the aromatic plane at 687 cm^{-1} . Another distinct characteristic of its FTIR-ATR spectrum is the elongation observed at 2925 cm^{-1} , which can be attributed to the presence of magnesium stearate in its formulation. Despite magnesium stearate being an excipient found in previously mentioned pharmaceuticals; its presence was uniquely identified in this specific pharmaceutical preparation.

The next set of pharmaceuticals, represented in Figures 17, 18, and 19, belong to a distinct category from those previously discussed as they are soluble powder medications. This characteristic signifies their enhanced ability to react with water, despite their original solid (powder) form intended for ingestion in liquid form. This feature grants them a heightened affinity for water, facilitating easy formation of bonds or interactions with water molecules essentially, as they are hydrophilic substances.

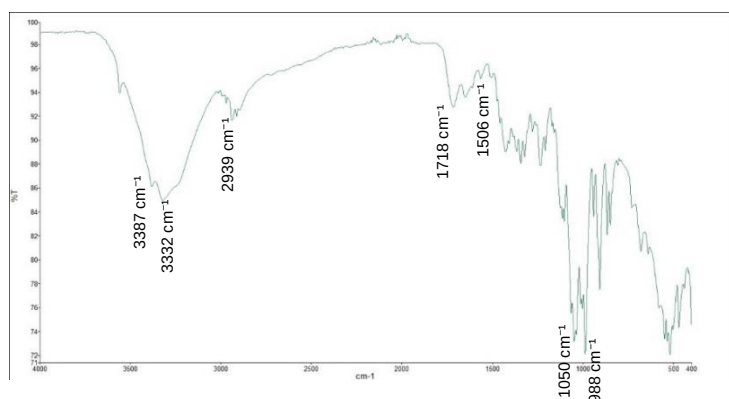


Figure 17. FTIR-ATR spectrum of the pharmaceutical G7.

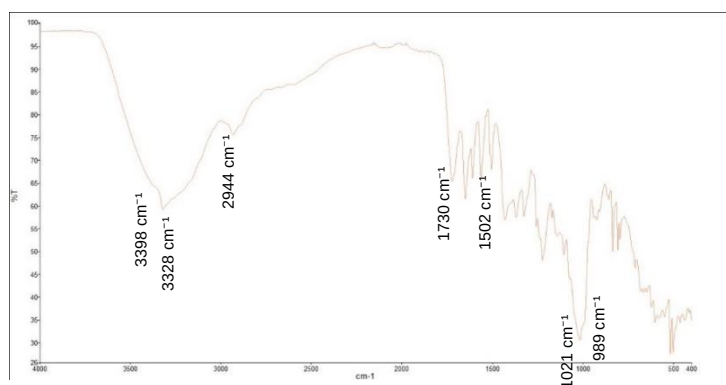


Figure 18. FTIR-ATR spectrum of the pharmaceutical H8.

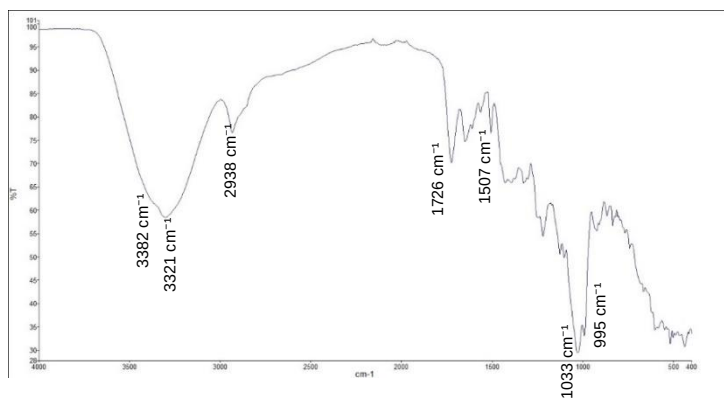


Figure 19. FTIR-ATR spectrum of the pharmaceutical I9.

Due to their extended storage periods outside their original packaging, these pharma exhibit in their FTIR-ATR spectra a pronounced and broad band indicative of water. This suggests the possibility that the pharmaceuticals have been exposed to humidity over a prolonged period, resulting in partial dissolution and leaving traces of this interaction observable in their spectra. Concurrently interpretable are the major bands corresponding to the active ingredient acetaminophen, as well as the bands at 3332, 3328, and 3321 cm⁻¹ in Figures 17, 18, and 19, respectively, which represent a prominent

region in the spectra attributed to symmetric stretching vibrations of the O-H bonds in water.

Overall, in the spectra of the pharmaceutical preparations *G7*, *H8*, and *I9*, only one excipient common to all pharmaceuticals could be identified: sucrose. This shared excipient exhibits characteristic bands due to its structure, particularly vibrations of the C-O-C and C-O bonds, predominantly in the 950-1050 cm^{-1} region, as evident in the provided spectra [90,91]. The presence of this excipient can be related to the hydrophilic characteristics of the pharmaceuticals in question, since sucrose is a disaccharide made up of several molecules of hydroxyl groups that are polar, capable of forming hydrogen bridges with water molecules, making this excipient highly soluble and can also be classified as a hydrophilic substance [92].

In summary, the FTIR-ATR technique served its intended purpose by enabling comparison of the various matrices under study, revealing significant similarities at a superficial analysis level, thereby categorizing them into two distinct groups: tablets and soluble powder medications. Each spectrum in the specific study exhibited characteristic bands of the active substance, as seen in Figures 11 to 19. Despite their apparent similarities, these spectra displayed distinct features between the two formulations. Overall, common components across all formulations were identified, including povidone and various starches.

In the case of soluble powder medications, their FTIR-ATR spectra highlighted their hydrophilic nature, with sucrose predominantly identified as the excipient, showing distinct deformations of its functional groups.

The application of FTIR-ATR has proven to be an indispensable tool in the realm of pharmaceutical sciences. This sophisticated spectroscopic technique not only facilitates the differentiation of various pharmaceutical formulations by examining their unique chemical profiles but also highlights the critical importance of comprehensive molecular analysis in the fields of pharmaceutical research and quality assurance.

By delving into the intricate details of composition and the interactions among components, FTIR-ATR provides invaluable insights that are pivotal in ensuring the integrity, stability, and therapeutic efficacy of pharmaceutical products. The method's capacity to detect and analyse subtle variations and specific functional groups within the

molecular structure enhances our understanding, thereby enabling the optimization of drug formulations to achieve superior therapeutic outcomes.

Furthermore, FTIR-ATR's ability to offer precise and detailed molecular information is essential for the rigorous quality control processes required in the pharmaceutical industry. This ensures that each batch of medication meets stringent safety and effectiveness standards. As a result, FTIR-ATR plays a critical role not only in the development and improvement of new drugs but also in maintaining the high standards of existing pharmaceutical products.

6. Conclusion

The main goal of this dissertation was to quantitatively assess the acetaminophen content within a range of pharmaceutical preparations, employing UV-Vis spectrophotometry as the analytical technique. After acquiring the quantities of active substance present in the various pharmaceuticals, a comparative analysis was carried out with the values indicated by the manufacturer. In addition, the analytical method used undergoes meticulous validation tests to verify its reliability and suitability.

In the initial phase of the experiments, efforts were directed towards optimizing the procedural workflow. This involved systematic exploration of multiple calibration curves and sample preparation methodologies until identifying the most suitable approach aligned with the study's objectives and future experimental requirements. Subsequently, a determination was performed by utilizing twenty standards encompassing the working range of 0 to 20 ppm. Furthermore, an absorption maximum was established at wavelength equal to 244 nm, serving as the choice for all absorbance measurements.

The investigation into the stability of acetaminophen solutions unveiled a noteworthy photosensitivity-induced degradation phenomenon, posing a significant constraint throughout the experimental work. This inherent limitation required immediate preparation and analysis of solutions to preventively mitigate the adverse effects on resultant values.

Furthermore, the quantitative evaluation of the active substance across diverse pharmaceutical formulations yielded insightful observations. Particularly noteworthy was the adherence of five formulations to manufacturer-specified compositions, as evidenced by *A1*, *B2*, and *C3*, demonstrating percentage differentials below 2 %. Although *E5* and *I9* exhibited slightly elevated percentages around 3 %, their deviations remained acceptable.

These findings underscore the importance of meticulous formulation consistency and adherence to standardized manufacturing processes in ensuring product integrity and efficacy. They also highlight the significance of routine quality control measures to mitigate deviations and uphold consumer confidence in pharmaceutical products.

The examination of pharmaceutical preparations unveiled notable disparities in acetaminophen content, particularly evident in *D4* and *F6*, exhibiting elevated percentages of 8.60 % and 10.38 %, respectively. Such deviations from desired levels emphasise potential inconsistencies in manufacturing processes and underline the

imperative for stringent quality control measures. Of greater concern are formulations such as *G7* and *H8*, displaying alarmingly higher acetaminophen concentrations of 24 % and 20 %, respectively. This led to the hypothesis that the discrepancy may be linked to the use of dry mass in the experimental analyses, while unlikely, it is possible that the pharmaceutical manufactures did not account for the humidity content.

Such findings underscore the critical importance of rigorous quality assurance protocols and regulatory oversight in safeguarding consumer health and well-being. Furthermore, they accentuate the necessity for ongoing vigilance and refinement within pharmaceutical manufacturing practices to mitigate the risk of inadvertent consumer exposure to potentially harmful substances.

In the context of method validation, an exhaustive examination of various statistical parameters was conducted, encompassing limits of detection and quantification. Notably, values of 0.59 and 1.70 ppm were determined, respectively, underscoring the method's sensitivity and efficacy in quantifying acetaminophen content within pharmaceutical formulations. Moreover, the application of linear regression analysis affirmed the appropriateness of a linear model for the specified range, indicative of the method's robustness and suitability for analytical purposes. Additionally, comprehensive evaluation revealed the method to be characterized by sensitivity, selectivity, and precision within the calibration range, as evidenced by repeatability and intermediate precision values of 0.0016 and 0.00082, respectively. Of equal significance is the assessment of accuracy, with the method demonstrating a commendable accuracy level of 85 %, falling within the ideal range of 80 % to 120 %. This validation study corroborates the method's reliability and accuracy in quantifying acetaminophen content.

Upon the extensive experimental procedures undertaken and comprehensive consideration of the gathered data, it is evident that the proposed method stands as a valid approach for analysing acetaminophen content within pharmaceutical preparations. Remarkably, this method offers distinct advantages, notably its cost-effectiveness and operational efficiency, obviating the requirement for costly reagents and intricate procedural steps typically associated with alternative analytical techniques. However, it is imperative to acknowledge the inherent limitation necessitating immediate readings post-solution preparation and the iterative execution of calibration curves. While this requirement may impose logistical challenges and constrain analytical flexibility, it does not detract from the method's overall efficacy and utility. In essence, the spectrophotometric method emerges as the method of choice for studies akin to the

present investigation, offering a judicious balance between analytical rigor and practical feasibility.

The application of FTIR-ATR to the nine medications studied proved instrumental in not only identifying the active ingredient acetaminophen but also in pinpointing several excipients that comprise their formulations, while also revealing similarities across the matrices under investigation. This spectroscopic technique demonstrated its efficacy in discerning and delineating crucial functional groups present within the diverse chemical structures of these medications. Particularly noteworthy were the presence of excipients such as povidone and various starches in the tablet formulations, highlighting their roles and contributions. In contrast, for soluble powder formulations, sucrose emerged as the predominant excipient identifiable by FTIR-ATR. This method stands out as a spectroscopic tool, playing a critical role in the pharmaceutical industry by ensuring comprehensive characterization and qualitative assessment of pharma formulations. Its ability to analyse molecular interactions and composition provides essential insights into the formulation's stability, quality, and efficacy over time. By facilitating detailed chemical profiling, FTIR-ATR supports rigorous quality control measures, thereby safeguarding the integrity and therapeutic effectiveness of pharmaceutical products.

The studies carried out have provided a greater understanding of the constitution of pharmaceuticals, shedding light on the intricate relationships between active ingredients and excipients within various pharmaceutical formulations. Through meticulous qualitative and quantitative analyses, these studies have validated a reliable and robust method for assessing the acetaminophen content in different pharmaceutical products. The use of UV-Vis spectrophotometry and FTIR-ATR techniques has proven to be effective, efficient, and cost-effective, offering significant utility for the pharmaceutical industry.

Additionally, the findings underscore the importance of rigorous analytical validation in the pharmaceutical industry, emphasizing the need for continual methodological advancements to keep pace with the evolving complexities of pharma formulations.

7. Bibliographical References

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