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In-situ dissolution testing monitoring of pharmaceutical solid dosage forms by near-infrared spectroscopy and chemometrics

Dissertação do 2º ciclo de Estudos Conducente ao Grau de Mestre em Controlo da Qualidade, Área de Especialização: Fármacos e Plantas Medicinais

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Abstract

This thesis explores for the first time the application of near-infrared spectroscopy and multivariate data analysis to monitor *in-situ* and in real-time dissolution tests of pharmaceutical solid dosage forms.

For this goal a pharmaceutical immediate release formulation containing folic acid as the unique active pharmaceutical ingredient was selected. Dissolution testing is one of the most important experiments conducted by the pharmaceutical industry in the final quality control of solid dosage forms produced batches. Increase knowledge in this area and more efficient monitoring methods are fundamental for manufacturing processes improvement and control, following the quality-by-design concept defined by the International Conference on Harmonization Q8 (R2) guideline for industry.

Recommended analytical methods for the quantification of active pharmaceutical ingredient in immediate release formulations are often based on high performance liquid chromatography. However, due to the specificities of this method, e.g., the need of a high amount of reagents and the time spent in each analysis, it was found necessary to develop alternative methods for the active pharmaceutical ingredient quantification.

Identifying alternative methods for this task is therefore the motivation of this thesis.

The first part describes the development of a simple, accurate, precise, economic and sensitive ultraviolet spectrophotometric method for the determination of folic acid in commercial tablets and *in vitro* dissolution studies. This method revealed a good linearity in the studied concentration range, a good determination coefficient and an excellent recovery. The statistical comparison with the high performance liquid chromatography reference method showed excellent agreement and indicated no significant differences in accuracy and precision.

In the second part, the application of near-infrared spectroscopy and multivariate analysis to monitor *in-situ* dissolution tests was evaluated. Evaluation was performed with laboratory designed and commercial tablets of an immediate release formulation containing folic acid and four excipients. Near-infrared spectra were acquired *in-situ* with a transflectance probe immersed in the dissolution medium connected to a Fourier-transform near-infrared analyser. Partial least squares regression with leave-one-out cross-validation was used to correlate near-infrared spectra with the drug concentration.

Results demonstrate that it is possible to use *in-situ* near-infrared spectroscopy to monitor dissolution tests and that this method is a potential analytical technique candidate for the study of drug dissolution methods in a rapid way without any sampling process. Using this approach, it is possible to expand surrogate methods in quality control in the

pharmaceutical industry and to develop a better understanding of product critical quality attributes.

Keywords: Dissolution tests, folic acid, ultraviolet spectroscopy, near-infrared spectroscopy, multivariate analysis.

Resumo

Esta dissertação explora, pela primeira vez, a aplicação da espetroscopia no infravermelho próximo, juntamente com análise multivariada de dados, para monitorizar ensaios de dissolução de formas farmacêuticas sólidas *in situ* e em tempo real. Para tal, foi selecionada uma formulação farmacêutica de libertação imediata contendo ácido fólico como único princípio ativo. Os ensaios de dissolução são dos mais importantes testes efetuados pela indústria farmacêutica em formas farmacêuticas orais, no controlo da qualidade final dos lotes produzidos. O aumento do conhecimento nesta área e métodos de monitorização mais eficientes são fundamentais para a melhoria do processo de fabrico e do seu controlo, de acordo com a abordagem quality-by-design, definida pela guideline: International Conference on Harmonization Q8 (R2). Os métodos analíticos recomendados para a quantificação do princípio ativo em formulações de libertação imediata são frequentemente baseados em cromatografia líquida de alta eficiência. Contudo, devido às particularidades deste método (requerer elevadas quantidades de reagentes e análise demorosa) revelou-se necessário desenvolver um método alternativo para a quantificação do princípio ativo. Identificar métodos alternativos para esta tarefa é, portanto, a motivação de todo este trabalho. Assim, na primeira parte do trabalho, é descrito o desenvolvimento de um método simples, exato, preciso, económico e sensível por espetrofotometria no ultravioleta, para a quantificação de ácido fólico em comprimidos comerciais e em ensaios de dissolução. Este método demonstrou ter uma boa linearidade na gama de concentrações estudada, bom coeficiente de determinação e uma excelente taxa de recuperação. A comparação estatística dos resultados com o método referência (cromatografia líquida de alta eficiência) demonstrou não só uma ótima concordância entre os dois métodos, mas também não haver diferenças significativas na exatidão e precisão. Na segunda parte do trabalho, avaliou-se a aplicação da espetroscopia no infravermelho próximo juntamente com análise multivariada de dados, para monitorizar ensaios de dissolução *in-situ*. Esta avaliação foi efectuada em comprimidos comerciais e comprimidos concebidos no laboratório, de uma formulação farmacêutica de libertação imediata contendo ácido fólico como único princípio activo e quatro excipientes. Os espetros no infravermelho próximo foram adquiridos *in-situ* com uma sonda de transfletância submergida no meio de dissolução ligada a um espetrofotómetro de infravermelho próximo por transformada de Fourier. Através da regressão por mínimos quadrados parciais com validação cruzada estabeleceu-se uma correlação entre os espetros no infravermelho próximo obtidos e o teor de fármaco. Os resultados obtidos demonstram que é possível utilizar a espetroscopia no infravermelho próximo *in situ* para

monitorizar ensaios de dissolução e que este método consiste numa potencial técnica analítica para o estudo de métodos de dissolução de fármacos de forma rápida e sem efetuar amostragem. Através desta abordagem, é possível expandir os métodos substitutos no controlo da qualidade na indústria farmacêutica e desenvolver uma melhor compreensão dos atributos críticos de qualidade.

Palavras-chave: Ensaios de dissolução, ácido fólico, espetroscopia no ultravioleta, espetroscopia no infravermelho próximo, análise multivariada.

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List of abbreviations

AOTF – Acousto-optic tunable filter

API – Active pharmaceutical ingredient

BCS – Biopharmaceuticals classification system

CCDs – Charged coupled devices

CI – Confidence interval

CPP – Critical process parameters

CQA – Critical quality attribute

DoE – Design of experiments

EMA – European Medicines Agency

EMR – Electromagnetic radiation

FA – Folic acid

FDA – Food and Drug Administration

FIP – International Pharmaceutical Federation

FT – Fourier transform

FT-IR – Fourier transform infrared

FT-NIR – Fourier transform near-infrared

GI – Gastrointestinal

HPLC – High performance liquid chromatography

ICH – International Conference on Harmonization

InGaAs – Indium gallium arsenide

IVIVC – *In vitro-in vivo* correlation

LED – Light-emitting diodes

LOD – Limit of detection

LOQ – Limit of quantification

LV – Latent variables

MLR – Multiple linear regression

MNCN – Mean centering

MSC – Multiplicative scatter correction

NIR – Near-infrared

NIRS – Near-infrared spectroscopy

PAT – Process analytical technology

PbS – Lead sulfide

PC – Principal component

PC 1 – First principal component

PC 2 – Second principal component

PCA – Principal component analysis

PCR – Principal component regression

PDAs – Photodiode array spectrographs

Ph. Eur – European Pharmacopoeia

PLS – Partial least squares

QbD – Quality-by-design

QbT – Quality-by-testing

QC – Quality control

RMSEC – Root mean square error of calibration

RMSECV – Root mean square error of cross-validation

RMSEP – Root mean square error of prediction

RSD – Relative standard deviation

RSE – Relative standard error

SD – Standard deviation

SG – Savitzky-Golay

SGF – Simulated gastric fluid

SIF – Simulated intestinal fluid

SNR – Signal-to-noise ratio

SNV – Standard normal variate

USP – United States Pharmacopoeia

UV – Ultraviolet

UV-vis – Ultraviolet-visible

1. Introduction

1.1 Quality-by-design in the pharmaceutical industry

Under the current quality-by-testing (QbT) regulatory framework, product quality is ensured by raw material, in-process material and end product testing and fixed (inflexible) drug product manufacturing process with very strict specifications on variables that are monitored in a univariate manner. Finished drug products are tested for quality by assessing whether they meet the manufacturer's proposed and regulatory approved specifications. If the specifications are not met, they are rejected. Many times, root causes for failure are usually not well understood (1, 2). This framework causes significant burden on industry and consequently lead to manufacturing process that remain fixed and suboptimal (3).

The International Conference on Harmonization (ICH) describes pharmaceutical quality-by-design (QbD) as a systematic, scientific, risk-based, holistic and proactive approach to pharmaceutical development that begins with predefined objectives and emphases product and processes understanding and process control (4). QbD goal is to design and develop formulations and manufacturing pharmaceutical processes to guarantee a predefined product quality and performance objectives, as well as to provide robust manufacturing process through knowledge acquired from the manipulation of the process parameters in a constant way (1, 4-6). With the QbD paradigm, it is possible to use knowledge and data from product development studies to continuous improvement of the manufacturing process (5). QbD allows a more flexible regulatory approach based on optimisation and understanding of how design of a product and its manufacturing process may affect product quality (3).

The quality cannot be tested into products; it should be built-in or should be by design. This sentence describes the entire concept of QbD, where the quality built-in products is an emerging necessity and overcomes the pharmaceutical end-product quality control (QC) approach (7, 8). In a few words, the overall aim of QbD is to make more effective use of the latest pharmaceutical science, engineering principles and knowledge throughout the lifecycle of a product (3).

QbD identifies characteristics that are critical to quality and translates them into the attributes (critical quality attributes – CQAs) that the drug product should possess, and establishes how the critical process parameters (CPPs) can be varied to consistently produce a drug product with the desired characteristics (1).

The main contrasts between the QbT approach and an enhanced QbD approach concerning different aspects of pharmaceutical development are referenced in Table 1.

Table 1: Comparison between the QbT and QbD approaches for pharmaceutical development (adapted from references (5, 8)).

Aspect	QbT Approach	QbD Approach
Overall Pharmaceutical Development	-Empirical -Development with one variable at a time	-Systematic -Influence of material attributes, process parameters to drug CQAs -Multivariate experiments -Establishment of design space
Manufacturing Process	-Fixed -Validation with full-scale batches -Focus on optimization and Reproducibility	-Adjustable within design space -Continuous verification -Focus on control strategy and robustness -Use of statistical control methods
Process Control	-Go/no go decisions -Off-line analysis	-Process analytical technology tools use (real-time release testing)
Control Strategy	-Drug product quality controlled by end-product testing	-Drug product quality ensured by risk-based control strategy -Real-time release testing
Lifecycle Management	-Reactive, problem solving -Correction actions	-Preventive actions -Continual improvement

A key concept that supports QbD understanding and implementation is the design space concept (4). The ICH Q8 guidance describes the creation of a design space for pharmaceutical products and defines it as an established multidimensional combination and interaction of material attributes and/or process parameters demonstrated to provide assurance of quality. A design space can be described in terms of ranges of material attributes and process parameters, or through more complex mathematical relationships. It is possible to describe a design space as a time dependent function (e.g., temperature and pressure cycle of a lyophilisation cycle), or as a combination of variables such as components of a multivariate model (8). The design of space of an analytical method is established using a set of statistically designed experiments (design of experiments or DoE) which is a structured and organized method for determining the relationship

between factors affecting a process (inputs) and the output of that process (5). The knowledge and information from the product development studies and the manufacturing process experience are the base of the design space and support specifications and manufacturing controls. The changes in formulation and manufacturing processes during development and lifecycle management should be faced as opportunities to enrich additional knowledge and further support to the establishment of the design space. Design space is proposed and is subjected to regulatory assessment and approval (8). Changes within the design space of the method are not considered to be a change to the method (4). Otherwise, movement out of the design space is considered to be a change and would lead to a regulatory post approval change process. It is expected that an operation within the design space will result in a product meeting the defined quality (8).

The QbD approach plays an important role in facilitation a better process knowledge as well as creating opportunities for root-of-cause investigation and developing control strategies in formulations and processes development (9). This better product process and control knowledge can be gained by the application of the process analytical technology (PAT) paradigm (8).

1.2 Process analytical technology in the pharmaceutical industry

The production of pharmaceutical dosage forms is a multistage operation consisting of several validated processes managed by standard operating procedures (10). After an operation, it is carried out a laboratory assessment to evaluate product quality, which is actually based on off-line (removing the sample and analysing far from the process chain) testing of randomly collected samples of batches (11-14). Although, this traditional approach has been successful in providing quality pharmaceutical products to the population, is time consuming, labour intensive and often inefficient, since it does not assure zero defect product quality, since risk assessment and risk management are not included (10-13). Additionally, after the drug approval, a small change to how a drug is made requires another regulatory assessment and authorization requiring time and paperwork (15). This process highly discourage the updating by the pharmaceutical companies making manufacturing processes frozen in time (11, 15).

Today, there are opportunities to improve pharmaceutical development, manufacturing and quality assurance with technology innovation (11, 15). Food and Drug Administration (FDA) edited in 2004 a guideline on PAT: *Guidance for Industry PAT — A Framework for*

Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance. This guidance describes a regulatory framework on PAT that will encourage the development and implementation of innovative and efficient pharmaceutical development, manufacturing, and quality assurance. This PAT initiative includes chemical, physical, microbiological, mathematical and risk analysis conducted in an integrated manner (15).

The PAT initiative is encouraged by the most important pharmaceutical regulatory authorities (health agencies), European Medicines Agency (EMA), Japanese Ministry of Health, Labour, and Welfare and ICH guidelines Q8 and Q9 (1, 13, 14).

Figure 1 represents in a schematic way the PAT paradigm in comparison with the conventional approach.

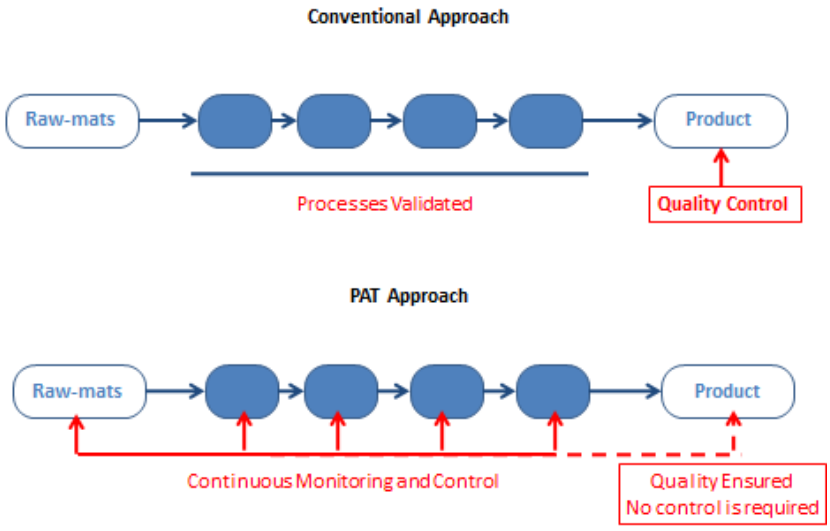


Figure 1: PAT paradigm.

PAT is defined as a system aimed for designing, analysing, and controlling manufacturing processes through timely and continuously measurements, during processing, of critical quality and performance attributes of raw and in-process materials with the purpose of guaranteeing final product quality (1, 11, 13, 15, 16). To implement PAT, a three-step process can be followed as illustrated in Figure 2 (14).

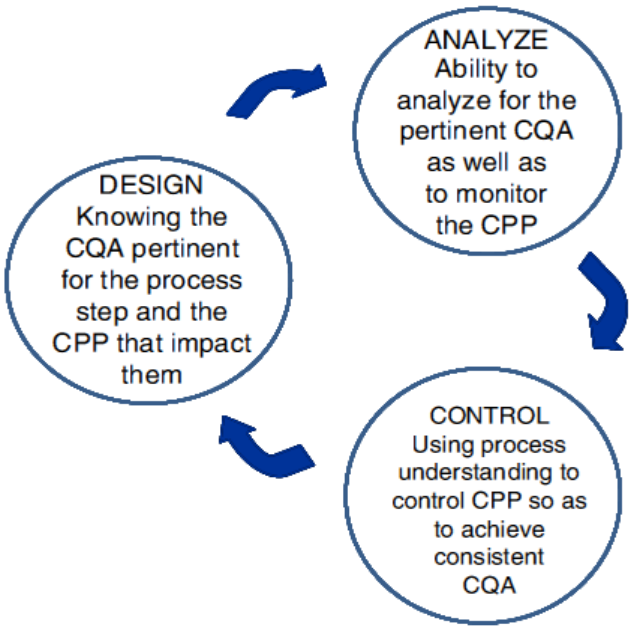


Figure 2: Three steps for PAT implementation and its main objectives (adapted from reference (14)).

The *design* phase starts early in the process development, in the beginning of a unit and later again to be optimized and characterized. Here, the CQAs that are affected by the process are identified along with the CPPs that have determined to affect the CQAs. The *design* phase allows an essential understanding which is crucial for the next two phases. In the *analyse* phase is selected a suitable analyser to monitor the CQA and to control the CPP, obtaining during the production, real-time information of all critical quality process aspects (14). The *control* phase includes planning a control scheme of the CPP based on process understanding such that the data from the analyser can be utilized for making real-time process decisions and adjustments and this way obtaining a consistent product quality within limits, avoiding batches loss (13-15). With this procedure the quality of the product is ensured and the efficiency of the manufacturing process is increased (5).

One of the PAT main goals is by continuously monitoring the process and thus being able to better understand it, contribute to an improvement of the process knowledge (17). A process is considered well understood when:

- all critical sources of variability are identified and explained;
- variability is managed by the process and
- product quality attributes can be predicted over the design space established for materials used, process conditions, manufacturing, and other (14).

The advantages of PAT, referring to quality, safety and efficiency will differ depending on the process and the product however, in a general way it can be summarized as:

- better process understanding;
- reducing production cycle times by using on-, in- and/or at-line measurements and controls;
- reducing costs, preventing rejects, waste and re-processing;
- increasing automation to improve operator safety and reduce human errors;
- decrease in energy consumption, increasing capacity and
- facilitating continuous processing to improve efficiency and manage variability (11, 14).

Some of the possible opportunities for PAT implementation are characterized by:

- low efficiency, long processing times and for high amount of waste processes;
- new products under development stages and
- improvement of pharmaceutical products which patent is expiring, and this way can avoid the generic drug manufacture competition (15).

Available tools in the PAT context enable process understanding for scientific, risk-managed pharmaceutical development and quality assurance. These can be categorised according to the following items.

1. Multivariate tools for design, data acquisition and analysis

These tools include multivariate mathematical approaches such as statistical techniques as DoE and multilinear regression analysis, which allows a quantitative understanding of the effects of different inputs upon the output of a system, this is, the effects of the interaction of product and process variables (11).

2. Process analysers

For real-time process monitoring and control there are available tools that take univariate process measurements such as pH, temperature and pressure but also there are tools like near-infrared spectroscopy (NIRS), that provide multivariate information related to physical and chemical attributes of the materials that are being processed (11, 13). These multivariate process analysers constitute the essential PAT tools that provide data from each relevant process contributing to process and product understanding as well as continuous improvement (7). Those PAT analysers can be done on timely in-line, on-line and at-line measurements. The definitions are:

- at-line: measurements where the sample is removed and analysed close to the process chain;

- on-line: measurements where the sample is diverted from the manufacturing process, and may be returned to the process chain;
- in-line: measurements that can be invasive or non-invasive, where the sample is not removed from the process chain (11, 13, 15, 18).

Spectroscopic techniques such as near-infrared (NIR) and Raman spectroscopy are the tools for reaching and ensuring QC at every step of manufacturing process (7). Due to its low cost per analysis, the availability of compact and robust process sensors and its high sensitivity to moisture and several chemical properties, NIRS was established as a fundamental PAT tool for pharmaceutical applications. Raman spectroscopy, thanks to the development of cheaper and more rugged laser technology, has recently emerged as an alternative PAT in-line monitoring and non-invasive tool for pharmaceutical processes analysis (19).

3. Process control tools

Process monitoring and control strategies are intended to monitor the state of a process and actively manipulate it to maintain a desired state. These tools provide a means for measuring process parameters and acting on those measurements.

Most pharmaceutical processes are based on time-defined end-points, but they not consider the effects of physical differences in raw materials that are not detected in the actual QC of raw materials. Concerning the PAT framework, a process end-point is the achievement of the desired material attribute (11).

4. Continuous improvement and knowledge management tools

Continuous learning through data collection and analysis over the life cycle of a product is essential and contributes to justifying proposals for post-approval changes. Information technology systems that support knowledge acquisition from such databases are valuable for the manufacturers.

A knowledge base can be of most benefit when it consists of scientific understanding of the relevant multifactorial relationships (e.g., between formulation, process, and quality attributes) as well as a means to evaluate the applicability of this knowledge in different situations (11).

By performing a real-time testing approach with PAT, the QC testing of the end-product is no longer required once it already provides a guarantee that the product is within specifications (4, 5).

In order to achieve a thorough understanding and control over the solid dosage form manufacturing, the QbD approach using PAT tools need to be incorporated into the field. In summary, using the PAT framework for solid dosage form manufacturing will lead to fewer batch rejections, less laboratory tests and waste, deeper understanding of the process, which is the ultimate prerequisite for process control (2).

1.3 Dissolution testing

The dissolution testing is one of the most important experiments conducted by the pharmaceutical industry for testing new drug candidates, new drug delivery systems, new formulations and also for generic drugs approval to ensure drug products compliance with the quality standards (20-22).

The drug dissolution testing has been applied since 1950s and was included in the United States Pharmacopoeia (USP) in 1970 (5, 23). Until this date, there is no other test that allows the prediction of a drug product dissolution in the human body (5). Dissolution test is a valuable *in vitro* test which allows the determination of the drug release rate and extent in function of time at determined conditions defined by the official monographs, which has an enormous predictive power of the gastrointestinal (GI) absorption process of the drug (24-26).

In a dissolution test of a tablet or capsule two main steps occur, that are represented in Figure 3 (27):

1. Disintegration (release of the drug from the formulation matrix).
2. Dissolution (solubilization of the drug in the liquid medium).

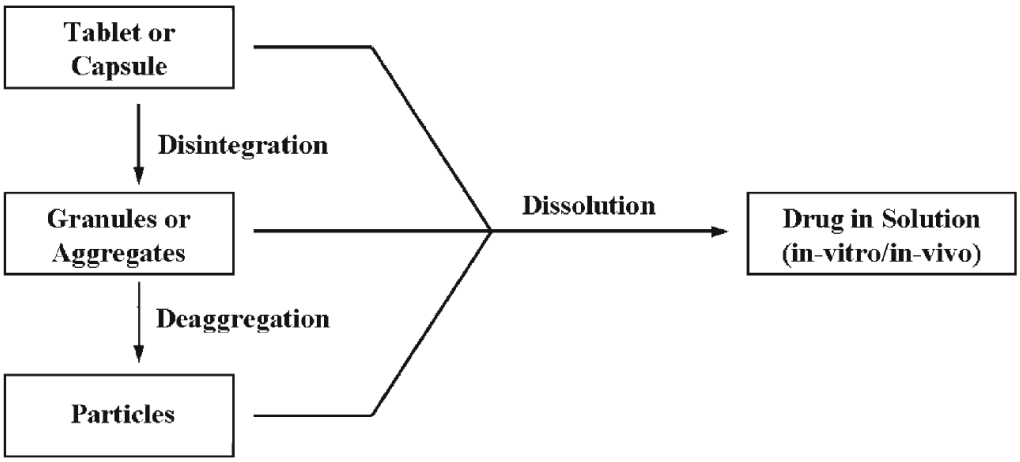


Figure 3: Steps of a drug dissolution process (adapted from reference (28)).

Disintegration test is also a standardized test intended to determine the capacity of tablets and capsules to disintegrate at a certain time in certain conditions determined in the pharmacopoeias. Disintegration is different from dissolution, and disintegration test does not implies the last one (29). A capsule or tablet may disintegrate into smaller particles, but if the active pharmaceutical ingredient (API) do not dissolve, it won´t be available to be absorbed in the small intestine (5).

In the final product QC routine in the pharmaceutical industry, every batch of a certain drug product has to be tested before its release, and its approval or rejection depends if the dissolution test is similar or has deviations relatively to the reference values (25). This test enables the characterization of the drug and tablet pharmacotechnical performance (25, 27). In case of drugs under development, it predicts *in vivo* performance of the drug and its correlation with drug *in vitro* behaviour (establish *in vitro-in vivo* correlation (IVIVC)) and also it can help the selection of a suitable formulation (excipients and API) (21-23). To controlled release drugs, this pharmacotechnical performance test is particularly important for evaluate release and further dissolution of the API, which is considered to be a determining step in the *in vivo* absorption (30).

Dissolution tests have proven to be a relevant tool for indicating alterations in crystallinity, pore structure of polymeric excipients, polymorphisms, gelatine capsule cross-linking and moisture content (5).

Bioequivalence between drugs are evaluated by dissolution tests in case of post-approval and scale-up changes (modifications of some critical parameters in the manufacturing process or in the formulation) (21, 27). In this context, it is necessary to take into account the entire drug dissolution profile instead of a single point, when examining batch-to-batch variability. Exemplifying, despite tablets from two different batches may dissolve at a certain percentage at a specific time, the entire dissolution profiles between these two batches may differ significantly (9).

There is a continuous need for the improvement of *in vitro* dissolution testing, aiming the approaching to the *in vivo* dissolution processes in the human body (31).

Under the QbD ideals, the dissolution tests should be developed to reflect *in vivo* performance as much as possible (1). That is why it is important to perform these tests with many variables as possible, to achieve a complete picture of the dissolution process (26).

Drug absorption from a solid dosage form after oral administration depends on the release of the drug substance from the drug product, the dissolution or solubilization of the drug under physiological conditions, and the permeability across the GI tract (31).

Dissolution of an active ingredient consists of a dynamic process dependent of the medium and hydrodynamics, and fundamentally, this process is controlled by the affinity

between the solvent and the solid substance and the way by which the pharmaceutical system releases the drug (32).

There are many factors that influence the drug dissolution rate, like the drug physicochemical properties (e.g. particle size or solubility, molecular structure, polymorph forms), formulation characteristics (e.g. excipients, presentation type - capsule, pill), release form (immediate or extended) and dissolution method and conditions (e.g. apparatus type, medium pH, paddle speed, temperature and surfactant type if present) (32, 33). Additionally, the dissolution rate shows dependence on the presence of manufacturing variables such as compression force, hardness, packaging type, storage conditions and changes in the surface area (5). Therefore, this test allows the detection of the different excipients (binders, disintegrators), mixing effects, granulation procedure and coating influence, providing a better control of the manufacturing process (22). Dissolution tests must be reliable, precise, simple and reproducible and must detect all the influences referenced above once they may also affect *in vivo* performance of the drug (27).

1.3.1 Guidelines

The first guidelines for dissolution testing of solid oral drugs were published in 1981 and were elaborated by the International Pharmaceutical Federation (FIP). These guidelines were intended as suggestions primarily directed to compendial committees, working on the introduction of dissolution / release tests for the respective Pharmacopoeias (34). In 1997 was edited the final version of the FIP guidelines and the FDA *Guidance for industry for dissolution testing of immediate release solid oral dosage forms* which provides several information such as general recommendations for dissolution testing and approaches for setting dissolution specifications (18). Also in 1997 was edited the *guidance for industry of development, evaluation, and application of IVIVC for extended release oral dosage forms* providing recommendations to pharmaceutical sponsors who intend to develop documentation in support of an IVIVC for an oral extended release drug product (35).

In 2000, FDA published the *Guidance for Industry - Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System* where recommendations are provided for the sponsors of investigational new drug applications, new drug applications and abbreviated new drug applications, and supplements to these applications who wish to request a waiver of *in vivo* bioavailability and/or bioequivalence studies for immediate release solid oral dosage forms (36). In 2010 was published the *Guidance for Industry: The Use of*

Mechanical Calibration of Dissolution Apparatus 1 and 2 – Current Good Manufacturing Practice (CGMP). This guidance is intended to support drug manufacturers in calibrating USP pharmacopoeia dissolution apparatus 1 and 2 guaranteeing that critical parameters associated with the dissolution apparatus meet certain mechanical calibration tolerances (37).

1.3.1.1 Instrumentation

There are several standardized dissolution apparatus described in the different pharmacopoeia. The most used are the USP type 1 (basket method) and 2 (paddle method) apparatus (38). These devices are simple and robust, and are recommended in several guidelines as well in different pharmacopoeias as a first choice for the *in vitro* dissolution testing of immediate as well as controlled/modified release drugs (34). These methods are the official methods for the *in vitro* dissolution testing and should be used except in case of showing to be unsatisfactory. They are flexible once they allow the dissolution testing of a variety of drug products (18). The paddle or the basket rotation promotes agitation in the medium in order to remove the drug saturated layer of dissolution from around the dosage and replace it with new medium. The rotation speed of the paddle and the quantity and composition of the dissolution medium can be varied to fit the *in vivo* condition, while the shape and position of the paddle and the vessels are regulated by the relevant pharmacopoeia guidelines (5).

Other systems are the USP apparatus 3 which describes the reciprocating cylinder and the flow-through cell, USP apparatus 4. The last one is also monographed in the USP, Japanese pharmacopoeia and European Pharmacopoeia (Ph. Eur) (34). This flow-through cell emerged as a solution for low solubility drugs that saturated in 900 mL of medium. In this apparatus 4, the tablet is placed in a cell where there is a continuous flow of fluid which provides simultaneously the dissolution medium and the stirring (39).

If an individual drug product cannot be accommodated by one of the apparatuses, described above, alternative models or appropriate modifications (automation, auto-sampling) have to be developed and approved (18, 34). In case of superiority of the alternative or the modification, they have to be proven in comparison to the well-established and standardised apparatuses and demonstrate to have the ability to differ between batches with acceptable and non-acceptable performance (18, 34, 40).

The dissolution procedures have been harmonized for the entire pharmacopoeia internationally, although there are some sections that remain unique to each pharmacopoeia (34). Thus, the official standard methods of the Ph. Eur should be

considered in case of a dissolution test methodology development for a product aimed to the European Union market (5). Generally a drug tablet or capsule is immersed in a known volume of water or an aqueous solution (500 to 1000 mL), where it dissolves under heating and stirring (34, 41, 42). The percentage of the API dissolved in this solution is then calculated at a specific time (41, 42).

Because the dissolution experiments are sensitive to mechanical and physical/chemical factors (stirring, position of aliquotation, vibrations, paddle or vessel shape) the dissolution apparatus should be appropriately calibrated to ensure compliance with regional good manufacturing practice requirements (43, 44). To standardise the conditions, USP recommends two different dissolution calibrator tablets: disintegrating (prednisone tablets) and non-disintegrating/eroding (salicylic acid tablets). To increase the method repeatability the prednisone calibrator tablets are recommended for apparatus 2 (44).

1.3.1.2 Dissolution medium

As already mentioned above *in vitro* dissolution data should allow some interpretation regarding the *in vivo* performance. According to this most dissolution tests conditions are based on the human body: they are conducted at 37^{o}C and a range pH value 1.2 to 6.8, mimicking the pH of the GI tract (5, 34).

To simulated intestinal fluid (SIF), a dissolution medium of pH 6.8 should be employed. A higher pH should be justified if necessary, but in general, it should not exceed pH 8.0. To simulated gastric fluid (SGF), a dissolution medium of pH 1.2 should be employed without enzymes. The need for enzymes in SGF and SIF should be evaluated on a case-by-case basis and should be justified. It is possible the need for enzymes (pepsin with SGF and pancreatin with SIF) to dissolve pellicles, if formed, to permit the dissolution of the drug. For water insoluble or sparingly water soluble drug products, the use of a surfactant such as sodium lauryl sulfate is recommended. The need for and the amount of the surfactant should be also justified (18). A pH gradient may be appropriate for gastroresistent formulations and products for which dissolution testing at one pH-level or at different pH-levels in parallel does not give biopharmaceutically relevant results (34). The use of apparatus 3 allows an easy change of the medium. Apparatus 4 can also be adopted for a change in dissolution medium during the dissolution run (18).

Agitation typically should be obtained in the basket/paddle apparatus by stirring at 50 to 100 rpm and in general should not exceed 150 rpm. Regarding media temperature, 37 ± 0.5° C should generally be used for oral dosage forms. Slightly increased test

temperatures (e.g. 38 ± 0.5° C) are under consideration for special applications e.g. for rectal dosage forms, lower temperatures (e.g. 32 ± 0.5 °C) for transdermal systems (34).

1.3.1.3 Specifications

In vitro dissolution specifications are established to ensure batch-to-batch consistency and to indicate potential problems with *in vivo* bioavailability.

Three categories of dissolution test specifications are described in the FDA guidance.

1. **Single-point specifications:** for immediate release formulations where the drug is highly soluble and rapidly dissolving; it is used as a routine quality control test.
2. **Two-point specifications:** for characterizing the quality of the drug product and as a routine QC test for certain types of drug products (e.g. slow dissolving or poorly water soluble drug product).
3. **Dissolution profile comparison:** (a) for accepting product sameness under scale-up and post-approval changes related changes, (b) to waive bioequivalence requirements for lower strengths of a dosage form, (c) to support waivers for other bioequivalence requirements (18).

According to the Biopharmaceuticals Classification System (BCS), the rate and extent of drug absorption are controlled by the dissolution rate, aqueous solubility and GI permeability (5). This classification can be used as a basis for setting *in vitro* dissolution specifications and can also provide a basis for predicting the likelihood of achieving a successful IVIVC (18).

The drug substances are divided in four classes according to the dissolution, solubility and permeability:

- BCS class I (high-solubility and high-permeability drugs);
- BCS class II (low solubility - high permeability drugs);
- BCS class III (high solubility - low permeability drugs) and
- BCS class IV (low solubility - low permeability drugs) (21).

The solubility and permeability parameters are standardized in the FDA *Guidance for Industry Dissolution Testing of Immediate Release Solid Oral Dosage Forms* (18).

In the USP and FDA guidances, dissolution testing is mandatory for all drugs when the API belongs to BCS class II, class III or IV (45).

According to the BCS guidance, biowaivers may be accepted for BCS class I products if the drug product is rapidly dissolving (21).

1.3.2 Analytical methods for dissolution tests analysis: emerging methods

The analytical methods for API quantification should be sensitive, accurate, and precise (32). The majority of the methods for monitoring dissolution use ultraviolet-visible (UV-vis) spectroscopy or high performance liquid chromatography (HPLC) with UV-vis detection, based on manually or automatically removed aliquots from the dissolution vessel (46). The current reference methods have a significant number of limitations such as requiring large amounts of drug and long sampling times (30s to 60s) (20). This sampling process is disruptive to the dissolution profile since the removal of aliquots from the dissolution vessel disturbs the solution (46). These standard methods provide no information of the process that takes place within the tablet like water sorption, swelling, polymer matrix erosion and drug diffusion as well as limited or no information on chemical processes that takes place within a dissolution vessel (13). Moreover, the sampling will decrease the total volume of the vessel (42, 46). HPLC method is time consuming and labour intensive, demand the use of high quantities of organic reagents and require sample preparation which can be a source of potential errors (20, 26). The UV-vis measurements present some limitations: not every drug is suitable for this technique, because some result in a large amount of undissolved particles (15). The presence of excipients, air bubbles and undissolved particles in the window of the detector or in the solution may interfere with the measures (leading to scattering of UV-vis radiation) and it explains the inferior accuracy of this method (1, 20).

The lack of simultaneous real-time information concerning the solution concentration and the solid-state composition makes the detection of solid-state changes during dissolution difficult since these often initiate almost instantaneously (47). This lack of real-time information can compromise the understanding of the formulation behaviour and the dissolution mechanisms, which represent one of great challenges to the pharmaceutical industry (46, 47).

The health authorities have challenged the pharmaceutical industry to understand dissolution and make the dissolution test more biologically relevant (46). The PAT approach brought the necessity for the development of new analytical tools that promote the increase of the understanding of complex dissolution behaviour this is, techniques that provide an insight of what is happening in real-time (46, 47). Imaging techniques such as Fourier transform infrared (FT-IR), NIRS and magnetic resonance imaging spectroscopy have been applied for the study of dissolution processes (47). Also Raman spectroscopy

have been applied in the dissolution monitoring, contributing to the process knowledge (13).

These new real-time monitoring methods brought by PAT approach require validation against the official analytical methods employed in dissolution testing. This validation has to conform with *Validation of Analytical Procedures* defined in the ICHQ2(R1) guideline and *Validation of Compendial Methods* in <1225> USP (48, 49). The validation aspects to be considered in the new method are accuracy, precision (repeatability, reproducibility, intermediate precision), specificity, linearity, range, detection limit, quantification limit, and robustness (49).

1.4 Near-infrared spectroscopy

The NIRS technique uses the electromagnetic radiation (EMR) in the region 12821 cm^{-1} to 4000 cm^{-1} (780 to 2500 nm) (50).

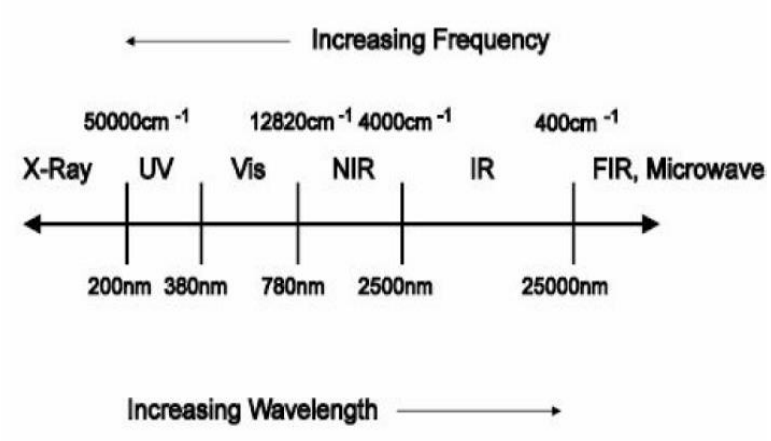


Figure 4: Electromagnetic radiation regions between X-rays and microwaves (adapted from reference (51)).

The NIRS technique is a vibrational spectroscopic technique that studies vibrational transitions in molecules and provides important information of the compounds structure (13, 51). In NIRS samples irradiated with NIR light, absorb it, causing molecular overtones (electron excitations to higher energy levels) and combination vibrations of C-H, O-H, N-H and S-H (51, 52). Combinations come from interaction of two or more vibrations taking place simultaneously in polyatomic molecules and arise in absorption bands called combination bands, the frequencies of which are the sums of multiples of each interacting frequency (51, 53). The combination bands region is located at higher NIR wavelengths (1900-2500 nm) (54). Overtones are absorption bands caused by transitions between

non-contiguous vibrational states at approximately, multiples of the fundamental vibrational frequency (53). Transition from the ground state to the first excited state absorbs light strongly in the NIR region and give rise to the intense bands called the fundamental bands. Transition from the ground state to the second excited state with the absorption of NIR radiation give rise to weak bands called first overtone in NIR. Transition from the ground state to the third excited state with the absorption of NIR give rise to weak bands called second overtone in NIR. Equally, third and fourth overtone bands will occur based on the transition to the fourth and fifth excited state with the absorption of NIR (51). Figure 5 represents these transitions between the ground state and the different excited states.

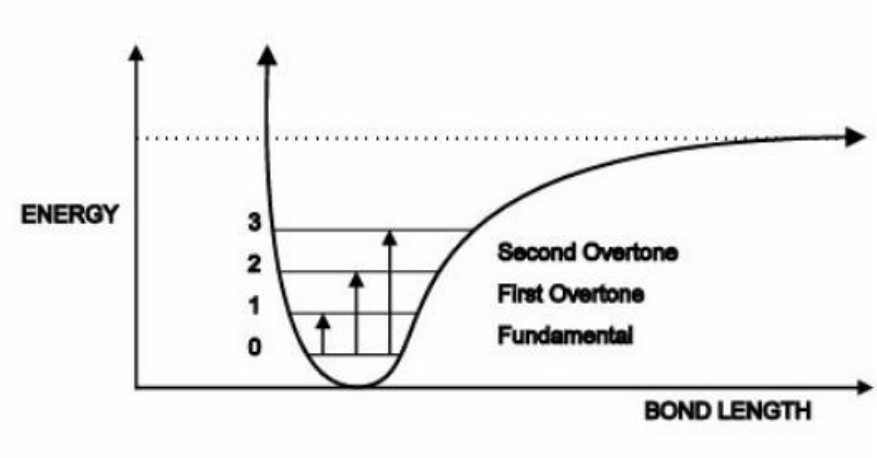


Figure 5: Transitions between the ground state and different excited states (adapted from reference (51)).

These overtones are much less likely than the fundamental transitions, so the bands are much weaker (the band for the first overtone is 10–100 times weaker than that for the fundamental frequency, depending on the particular bond). These bands appear between 780 nm and 2000 nm, depending on the overtone order and the bond nature and strength (53).

A molecule to be analysed by NIR spectroscopy should possess the change of dipole moment (52). This is, only vibrations that result in change of dipole moment of a molecule, can absorb NIR radiation. Therefore, the majority of organic and some inorganic compounds shows good reflectance or transmission properties in this region of the EMR spectrum (51).

Interactions between atoms in different molecules (for example hydrogen bonding and dipole interactions) alter vibrational energy states, thereby shifting existing absorption bands and giving rise to new ones, through differences in crystal structure. This allows crystal forms to be distinguished and physical properties (such as density, viscosity, and

particle size in pulverulent solids) to be determined. It can be affirm that the NIR spectrum contains not only chemical information of use to determine compositions, but also physical information that can be employed to determine physical properties of samples (53).
NIR measurements for analytical purposes can be done in three ways, depending on the nature of the sample.

- **Transmittance (700-1800nm):** the radiation passes through the sample and what is measured is the decrease in radiation intensity due to the radiation absorbed and scattered by the sample (10); it is used for transparent materials (solutions) and for lower concentration samples (51); transmittance measurements are more accurate, best taken at lower wavelengths because they are more energetic and have more penetration power (54).
- **Diffuse reflectance (1000-2500nm):** it is used for solids, turbid liquids and semi-solid solutions; it has lower sensitivity than transmittance; here, most of the radiation is reflected; physical characteristics affect reflectance measurements especially at higher wavelengths (combination bands region), hence, any sample changes will create an additional source of variability and noise in the measurements (54) and
- **Transflectance:** hybrid mode of transmittance and reflectance; here, the radiation is transmitted through the sample, reflected from a ceramic tile or other reflector surface and then transmitted back through the sample before finally reaching the detector, therefore the pathlength is doubled (51); it is used for solids, semi-solids and turbid liquids (10).

The ability of great penetration depth of the sample by NIR radiation is explained by the low absorption coefficient, which is an analytical advantage, since it allows direct analysis of strongly absorbing and even highly scattering samples, such as turbid liquids or solids in either transmittance or reflectance mode without further pre-treatments (10).

1.4.1 Instrumentation

NIR spectroscopy instrumentation has evolved dramatically in response to the need for speed in analyses and flexibility in adapting to different sample states. Spectrophotometers used to record NIR spectra are essentially identical with those employed in other regions of the electromagnetic spectrum. But NIR equipment can incorporate a variety of devices, depending on the characteristics of the sample and the particular analytical conditions and needs (such as speed, sample complexity and environmental conditions), so the technique is very flexible (53).

Any commercial NIR spectrophotometer has five basic sections: (1) sample compartment, (2) light source, (3) monochromator, (4) detector/s, and (5) signal processor or computer (54).

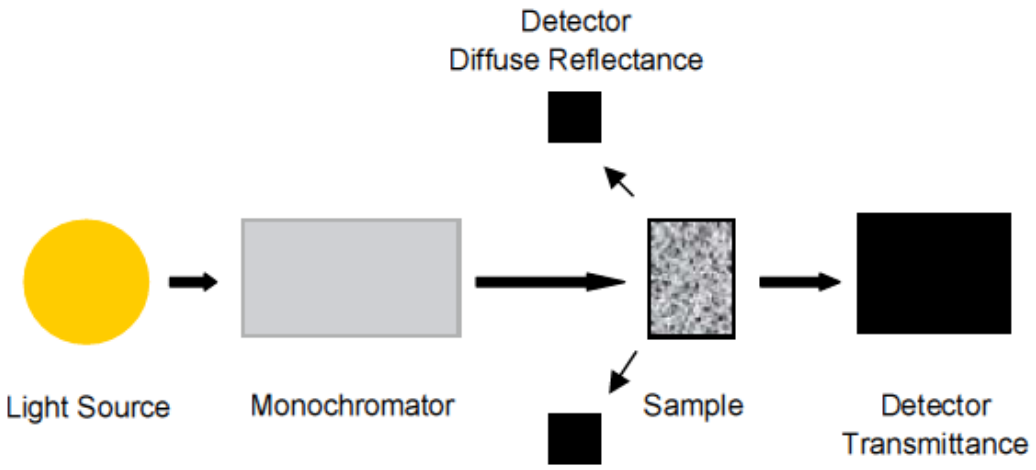


Figure 6: Basic NIR spectrometer configuration (adapted from reference (10)).

1. Light source

One of NIR light source most used is the tungsten halogen lamp, which has wavelength emission ranges from 320 to 2500 nm; it is small and rugged (10, 54). Light-emitting diodes (LED) is the most important light source of NIR (51). The low power consumption and price, small size, and long lifetime (around 25 years) of LEDs still make them the most suitable light sources for miniaturized instruments and specific screening applications outside the laboratory environment. Conventional LEDs emit in short wavelength ranges (30–50 nm) around their centre point (54).

2. Monochromator

A number of optical configurations exist that can be used to separate the polychromatic NIR spectral region into “monochromatic” frequencies (10).
NIR spectrophotometers can be of two types concerning to wavelength selection, namely discrete wavelength and whole spectrum (53). In discrete-wavelength spectrophotometers, wavelengths can be selected by using as light sources filters that allow the passage of variably broad wavelength bands or LEDs covering only a narrow spectral range of 50–100 nm (10, 53).

Whole-spectrum coverage NIR instruments usually include a diffraction grating, diode array or acousto-optic tunable filter (AOTF). They are much more flexible than discrete wavelength instruments, so they can be used in a wider variety of situations (53). AOTF allows faster tuning for wavelength selection (numerous readings per second without being sensitive to vibrations), and provide better reproducibility without the need for mechanical devices because one filter can create several wavelengths. NIR equipments called Fourier transform (FT) analysers (encompassing an interferometer often the Michelson interferometer) show some advantages. Essentially, an FT analyser has three major advantages over a dispersive instrument: the multiplex or Fellgett's advantage (information from all wavelengths collected simultaneously yielding a higher signal-to-noise ratio (SNR) for a given scan-time or a shorter scan-time for a given resolution), the throughput or Jacquinot's advantage (no slit restrictions) and the "Connes' advantage" (better wavelength accuracy). Inside an FT spectrometer, the light beam is split in two: one beam is reflected to a fixed mirror, and the other is reflected to a mirror that moves forward and backward at carefully controlled speed. The reflected beams are recombined back in the beam splitter to generate the interferogram signal, which is a result of light interferences. The processed signal or output looks like the spectra obtained by any traditional spectrometer, but with the expectation of higher throughput and frequency accuracy. One of the drawbacks is the fact that Fourier transform near-infrared (FT-NIR) instruments are complex and expensive, and mainly suitable for controlled environments (such as laboratories) due to their sensitivity to external factors such as temperature and vibrations (50, 54).

3. Sample compartment

This compartment encompasses a sample holder or a sample presentation interface. To measure good NIR spectra, the proper sample presentation is essential especially when measuring solid samples, since scatter effects and stray light induced by variations in packing density of powders or sample positioning of tablets or capsules may cause large sources of error in the spectra (10). Instruments working by diffuse reflectance it is common to use open sample cups or sample cells confined by silica or quartz (materials transparent to NIR light) and adjusted sample holders for tablets and capsules (10, 54). Transmission instruments may also work with confined sample cells, but with specific pre-set pathlenghts ranging from 0.1 to 10 cm, depending on the product to be analysed (54).
One important reason for the increasing acceptance of NIR spectroscopy is the possibility of using it directly on the production line allowing continuous real-time measurements (54). The probes can be directly inserted into the process line or connected to a flow-cell,

through which the sample can be diverted from the production line. Reflectance measurements in the production line can be made through a window. Transmission measurements can be made by inserting two optic probes facing each other. Transflectance measurements can be made by sending the light first through the probe, then through the sample, and subsequently to the detector back through the probe following reflection. The use of fibre optic probes either for transmission, diffuse reflectance or for transflectance measurements allows sampling by immersion in liquids for controlling liquid processes (54).

4. Detector

Detectors transform the incident light energy to electric analogue signal. The electrical signal is then amplified and transformed to digital, which may later be further processed by the computer. Detectors and amplifiers are considered the most common sources of non-systematic noise in instruments (random noise). Random noise is reduced in most commercial instrumentation by averaging several spectra from a same sample, improving the SNR (53).

Photo-sensitive detector materials are chosen according to the NIR region to be covered. From 400 to 1100 nm, silicon detectors are common. Silicon detectors are stable, low noise, fast, not too expensive, and sensitive to low light intensity to achieve good performance. Lead sulfide (PbS) or indium gallium arsenide (InGaAs) detectors can cover higher wavelength regions than silicon detectors, being usual having both types combined in a same instrument. PbS detectors are slower, but very popular since they are sensitive from 1100 to 2500 nm and provide good signal-to-noise properties. The most expensive, InGaAs detector, combines the speed and size characteristics of the silicon detector with the wavelength range of the PbS detector (10). Photodiode array spectrographs (PDAs) have a set of InGaAs detectors or charged coupled devices (CCDs) in array. While InGaAs PDAs offer high signal precision, high SNR, and less sensitivity to high light intensities when compared to CCDs, CCDs have higher signal sensitivity and resolution. PDAs take faster measurements (all wavelengths measured at once) (54).

Selection of the appropriate analyser depends upon the required analyte sensitivity, reliability, ease of use, calibration transferability and implementation needs. For those reasons, laboratory and process analysers have to be differentiated. Laboratory analysers normally are used in off-line or at-line measurements in QC or in research. They must be able to reach analyte sensitivity, have optimum sample presentation and high SNR. Diffraction grating and interferometer-based instruments are the most recommended.

Process analysers are intended for in-line or on-line measurements to provide real-time data while operating in industrial, many times harsh, conditions. Robust and fast analysers are necessary in those conditions (10).

1.4.2 Advantages and limitations

The use of NIRS in qualitative and quantitative analysis offers many advantages to pharmaceutical industries by providing not only chemical but also physical information rapidly with little or no sample preparation, unlike most traditional methods (48).

The NIR spectra can be obtained *in-situ* without any sample pre-treatment, and can be performed in solids, powders, pastes, gases and liquids (55). The fact that there is no need for sample preparation, makes NIR a convenient technique to monitor the synthesis of toxic compounds once the intensive physical contact with the compounds is reduced as well as the possibility to reuse samples after measurement (56).

Once the laborious task of preparing samples is eliminated, the productivity increases which compensates the decrease in the precision by using NIR (57). Also, in this way avoids important steps responsible for error sources (56).

The NIR spectrum has low intensity and wide bands as well as several overlaps, which means low sensitivity. For this reason it becomes difficult to use NIR to measure samples with low concentrations (58). The NIR signal is a complex function of physical and chemical parameters and result in a great amount of spectral data that need to be resolved with chemometric models so that useful information can be extracted (13, 57). Moreover, physical conditions of samples and measuring environment also influence the spectra, making it even more complex to interpret the data (56). For these reasons, NIR is not usually used as a direct analysis technique (10). NIR requires using multivariate calibration models that are constructed with samples of all variability of sources, in order to ensure adequate accuracy, precision, and robustness (12).

Table 2 summarizes the advantages and disadvantages of the NIR technique.

Table 2: Main advantages and disadvantages of NIRS (adapted from references (52, 53, 56)).

Advantages	Disadvantages
Non-destructive and non-invasive method	Low sensitivity of the signal when determining substances with concentration below 0.1% (w/w) (not suitable for trace analysis, only for major components)
Fast spectral measurements (less than 1 minute)	High financial investment for the instrumentation in the beginning
Low cost analysis: no need of chemical reagents and one operator can measure a big amount of samples (automation)	High trained personnel for the development of calibration models
Several spectra can be obtained on the same object leading to more representative sample composition and more accurate results	Requires an accurate and robust calibration with a large data set and variation, which can be difficult to obtain
Minimal or no sample preparation	Use chemometric tools to extract useful information; scarcely selective
Several components of the sample can be measured at the same time and also its physical characteristics (density, particle size)	Difficulty on transferring calibration between instruments of the same manufacture or between different manufactures
Easy application in different environments (industry, laboratory)	It requires an accurate chemical and physical analysis of reference samples
Measurements can be on-, in- and at-line	
Analysis *in-situ* thanks to robust optical probes	
Availability of portable instruments that permit the measurements in the field	Interference of water in the spectrum of the substances that are being measured due to the water high absorbance
Penetrate glass containers	
Can be used for qualitative and quantitative analysis	

1.4.3 Pharmaceutical industry applications

NIR spectroscopy is considered to be one of the most implemented PAT tools (59).

Although being a well-known technique for many years, only recently NIR spectroscopy started to be applied in the pharmaceutical industry (26). Pharmaceutical companies have gradually adopted NIR spectroscopy as their technique of choice for the manufacturing process control (53).

NIR spectroscopy is considered a reliable technique to:

1. Process monitoring and control, being part of the product QC (in process and final product) (51, 52).
2. Predict product characteristics at all stages of a solid dosage form manufacturing process (51, 52).
3. Raw material analysis through quantification and identification of excipients and API, assessment of a spectral signature of raw materials, intermediates and final dosage forms (10, 56).
4. Monitoring and control of unit operations such as blending, granulation, compression, film coating process evaluation, drying, tabletting and capsule-filling (10, 56).
5. Prediction in real-time the drug release from coated tablets as well as pre-determined dissolution times (60).
6. Study of the particle size, content uniformity and hardness testing of solid formulations (10, 57).
7. Perform analysis of intact dosage forms: tablets, capsules, lyophilized products, polymeric implants and microspheres (10).
8. Analysis of polymorphs, as they exhibit spectral differences in the NIR region as well as optical isomers (61).
9. Determining the water content of a sample, although water absorbs very strongly in the NIR region, specially between 1400 nm and 1450 nm and between 1900 nm and 1940 nm (56).

An internet search on web pages of the main manufactures of NIR instrumentation evidenced a remarkable concern of these manufactures with the PAT approach. They provide platforms and the necessary skills required to implement PAT on a single or multiple unit process as well as diverse instrumentation and technologies to many types of industry, including pharmaceutical industry (62-64).

Concerning the application of PAT into the pharmaceutical industry, the following tables (Table 3 and Table 4) present examples of FT-NIR equipments available in the market. They are divided into two groups: laboratory analysers and process analysers.

Table 3: Examples of FT-NIR laboratory analysers available in the market.

Laboratory Analyser Equipment	Instrument Picture	Applications/Features
ABB® MB3600-PH		-Laboratory QC analysis; -Raw material identification and qualification; -Research and development; -At-line PAT measurements (62).
ABB® MB-Rx		-Provides real-time insight into reaction monitoring for various processes in chemical and biotechnology applications; -Its optics are non-hygroscopic, and do not require desiccant cartridges; -Intended for laboratories and pilot plants; -Rugged insertion probe (62).
Antaris ® II		-Analyse any sample type rapidly and accurately (solids, powders, tablets, paste, gel, films, and liquids); -Contains all the tools necessary to make regulatory compliance easy; -Industry-leading method transfer performance; -Rugged design ready for fast, precise and accurate measurements in the lab or in the plant (64).

Table 4: Examples of FT-NIR process analysers available in the market.

Process Analyser Equipment	Instrument Picture	Applications/Features
ABB ® FTPA2000-260PH		-Real-time monitoring of continuous and batch processes in pharmaceutical industries; -Method development for monitoring of product CQA; -PAT implementation, as part of QbD initiatives (62).
Bruker® Tandem		-Automatic on-line PAT tool; -Allows the collection of process data and control the tablet compression process; -Provide both physical (weight, thickness, diameter, hardness) and chemical (content uniformity analysis; simultaneous quantification of multiple components, such as APIs and moisture content) characteristics of pharmaceutical tablets (65).
Büchi® NIRFlex N-500		-Polarization interferometer insensitive to mechanical disturbances; -Possibility to change the measurement options within a matter of seconds; -Autosampler; -Measurements can be performed directly in bags and containers using fibre optic probes (63).

1.4.4 Guidelines

The major Pharmacopoeias such as USP and Ph. Eur. have generally adopted NIR techniques. Both contain a general chapter on NIRS where is referred the suitability of NIR instrumentation for use in pharmaceutical analysis focussing on operational qualification and performance verification related to wavelength scale and repeatability, response repeatability, photometric linearity, and photometric noise. Only some limited guidance is provided in terms of developing and validating an application (10).

The Pharmaceutical Analytical Science Group – PASG - edited in 2001 *Guidelines for the development and validation of near-infrared spectroscopy methods* where is presented a discussion of the characteristics for consideration during the design, development and validation of NIR methods included as part of registration applications (48).

In 2012, the EMEA published a draft guideline on the use of NIRS by the pharmaceutical industry: *Guideline on the use of Near-infrared Spectroscopy (NIRS) by the pharmaceutical industry and the data requirements for new submissions and variation*. This guideline describes the general regulatory requirements and its procedures on developing a NIR method. According to this guideline, the development and implementation of an NIRS procedure is iterative and the stages are interdependent (49). The main stages in developing and establishing NIRS procedures are summarised in Figure 7.

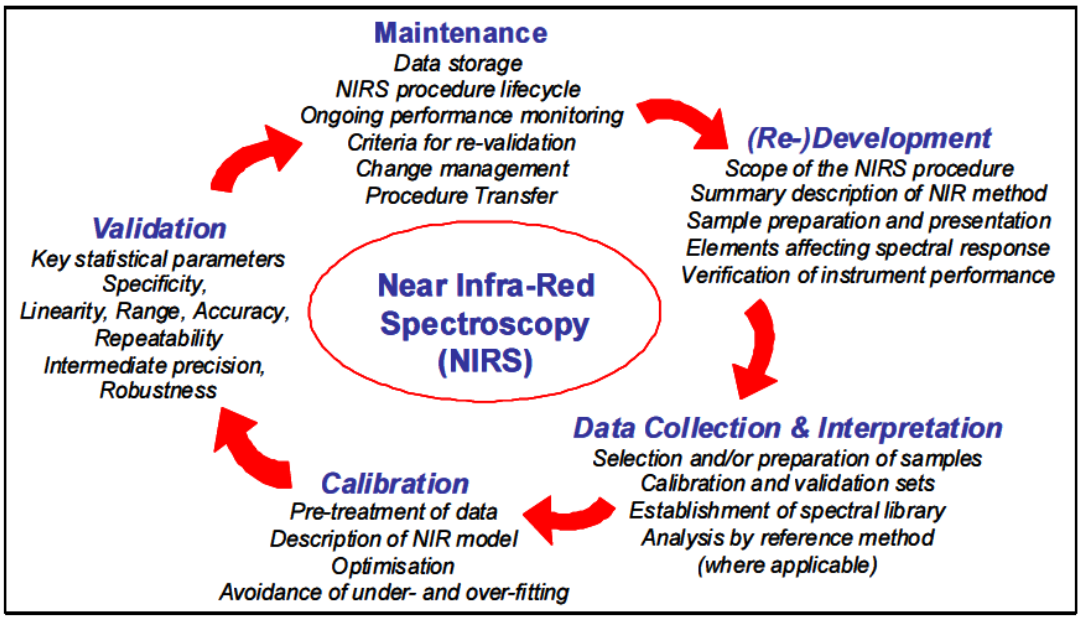


Figure 7: Main stages in developing and establishing NIRS procedures (adapted from reference (49)).

In general, NIRS procedures used for the release testing of drug substances or drug products need to be developed and validated in conjunction with the reference methods. For PAT, NIRS procedures, e.g., dynamic process monitoring of a powder blend, may not be possible to refer to a conventional reference method (49).

1.5 Data processing

PAT tools such as NIRS generate a great amount of data which need organization and extraction of the relevant information and consequently, reduce irrelevant information, in order to make QC decisions. Moreover, the analytical data of several PAT tools is typically not suitable for direct interpretation, therefore it is indispensable to use chemometric analysis (7, 14). As an example, NIR signal results in highly overlapped and broad peaks influenced by a number of physical, chemical and structural variables, and chemometrics made the extraction of that information possible (54). Since vibrational spectroscopy methods are non-selective, the information retrieved is multivariate in nature (16). Thus, multivariate analysis can be used to handle large data sets, simplifying the analysis, gaining essential knowledge of processes under investigation and retrieving qualitative and quantitative information from the spectral data (10). This amount of data generates greater confidence in conclusions that come from the statistical analysis and allows the construction of more robust multivariate models (5).

1.5.1 Chemometrics

Chemometrics is the science of extracting relevant information from chemical processes through mathematics, statistics and computational devices in chemical analysis, being essential for effective data analysis and to obtain real-time information from data (14, 54).
In a summarized way, chemometric methods help in the development of an empirical model based on the collected data that can be used for the prediction of the properties of a chemical process and hence help in process analysis, optimization, and control using mathematical and statistical methods for the data treatment of chemical analyses (14, 67).
There are several advantages of using multivariate methods over univariate techniques such as robust modelling, noise removal, handling of interacting variables or overlapping spectral profiles, outlier or fault detection, variable-reduction and understanding the causal relationships (2). The fast, precise, accurate, and non-destructive PAT analysers in

combination with chemometrics are suitable for process analysis and optimization leading to improved product quality, productivity, and efficiency (14).

1.5.2 Experimental design

Experimental design is a powerful technique in the beginning of the experiments, to support decisions such as regarding the number of experiments and the conditions in which these can be conducted. The goal of the DoE is the determination of a relation between factors acting on a system and the response or properties of the same system. This relation is, in many cases, in the form of a mathematical model. An experimental design can help to optimise, costs, time, equipment, materials, manpower among others. A number of different experimental designs can be used, the choice is depending on the final goal of the experiments (61).

1.5.3 Spectral processing

Interfering spectral factors, such as light scattering, pathlength variations, noise and also the different physical properties (particle size) of the samples demands for mathematical corrections, called data pre-treatments (10, 53). In these situations, spectral pre-treatment minimize those contributions, which incorporate irrelevant information into spectra, in order to be able to develop simpler and robust calibration models (53). The main objective is to decrease background and increase the signal conveying chemical information.
Basically, pre-processing methods can be classified as baseline correction-normalization, signal enhancement, and statistical filtering of signal noise (54).

1.5.3.1 Mean centering

Mean centering (MNCN) the spectra is a basic pre-treatment that involves the subtraction of each variable´s response from the mean response of that variable over all of the samples in the data set, enhancing the absorbance from each individual wavelength. Centering the data to the mean value reduces the final model complexity, often reducing the number of variables to be employed by one (54).

1.5.3.2 Derivatives

One way to remove baseline differences is to apply derivatives to the spectra (61). The first derivate spectrum is the slope at each point of the original spectrum. The second derivate is the slope of the first derivate (68). Derivatives can be applied to improve the resolution of overlapping bands, enhancing the signal, and removing an additive baseline -1st derivate and linear baseline – 2nd derivate (54). Since spectral noise is also amplified by derivation, derivatives are usually combined with smoothing algorithms, being the most used the Savitzky-Golay (SG). The idea behind the SG algorithm is to use the first and second derivative of the fitted curve at a certain point to estimate the first and second derivative of the original spectrum. The use of derivatives shows some disadvantages, since they change the shape of the original spectrum. However, the second derivative has the advantage that the peaks appear in the same location as in the original spectrum but with opposite sign (68). In most cases the second derivative presents more features than the original spectrum (61). Concerning the spectra interpretation, this fact can be an advantage if the original spectrum is very simple but can also be a disadvantage if the original spectrum is already a very complex one (68).

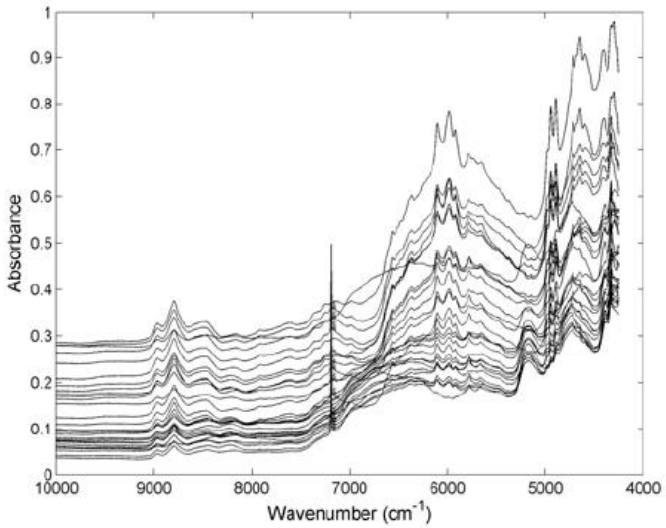
1.5.3.3 Normalization

Some of the more frequent pre-treatments for NIR spectra include the normalization methods: the multiplicative scatter correction (MSC) and the standard normal variate (SNV). Both methods process reflectance and transmittance spectra. Baseline shifts and intensity differences resulting from variable positioning or pathlength variations may be reduced or eliminated by normalization algorithms (10).

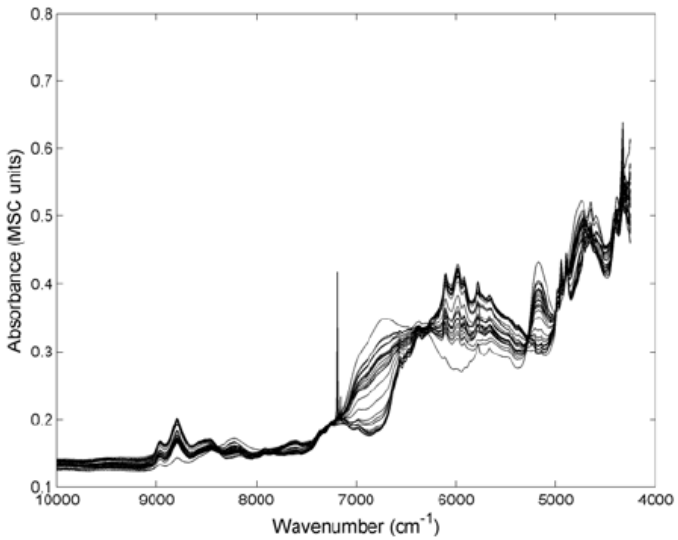
MSC removes the additive and multiplicative scatter effects in the spectra, eliminating most of the variations among the spectra (see Figure 8). The MSC works very well in cases where the scatter effects are the dominating source of variability, for example in many applications of diffuse reflectance NIR spectroscopy. This transformation should however be used very carefully when scatter is not the dominant effect. Two important consequences of MSC application have been observed, from a calibration point of view: simplification of the calibration model by reduction of the components required and possible improvement of linearity besides being used in many spectroscopic applications with good results (68).

The SNV method centres and scales individual spectra, having a very similar effect to MSC. The main difference between SNV and MSC, besides the vertical scale adapted

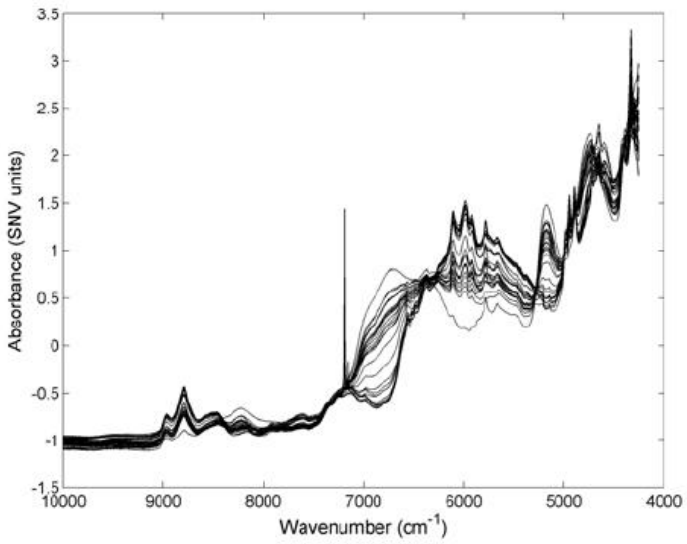
from SNV, is that SNV standardises each spectrum using only data from that same spectrum (68). Figure 8 shows a NIR spectroscopy raw spectrum, pre-processed with MSC and pre-processed with SNV from a powder paracetamol formulation.



(a)



(b)



(c)

Figure 8: NIR spectroscopy spectra from a powder paracetamol formulation (a) raw data, (b) pre-processed with MSC and (c) pre-processed with SNV (adapted from reference (61)).

1.5.4 Principal component analysis

There is a need for variable-reduction methods because of the vast amount of spectral information provided by NIR spectrophotometers, the substantial number of samples required to construct classification and calibration models, and the high correlation in spectra (14). Variable-reduction techniques allow the dimensions of the original data to be reduced to a few uncorrelated variables containing only relevant information from the samples. The best known and most widely used is principal component analysis (PCA) (56).

PCA is a mathematical procedure that converts the original variables into new, called principal component (PC) (56). These PC´s are linear combinations of the original ones and can be interpreted like spectra (56, 67). In this way, the relevant information for the system is contained in a reduced number of variables (53).

They represent the direction of the largest variation in the data: the first principal component (PC 1) explains the largest variation in the data and contains the most information. Each data point can be projected on this PC and a “score” can be attributed to each of these points, this is, each spectrum is represented by a score on each PC. Once PC 1 is constructed, a second (PC 2) can be drawn according to the same principle. This PC 2 is by definition orthogonal to PC 1 and represents the largest variation around

PC 1. The main advantage of this procedure is that the original information, represented by a high number of original variables (wavelengths) can be represented by only a few latent variables (LV). Feature reduction allows visualizing the data structure by plotting the scores of the samples on the first few PC's (56). The PCA data obtained can be used as new variables, instead of the original data, in subsequent calculations (53). PCA is the basis for several other chemometric techniques, like principal component regression (PCR), Partial Least Squares (PLS) and soft independent modelling of class analogy - SIMCA (56).

Figure 9 represents the transformation procedure on the basis of three original variables, this is, three wavelengths per spectrum. For real spectra with x wavelengths the transformation leads to a x dimensional space (10).

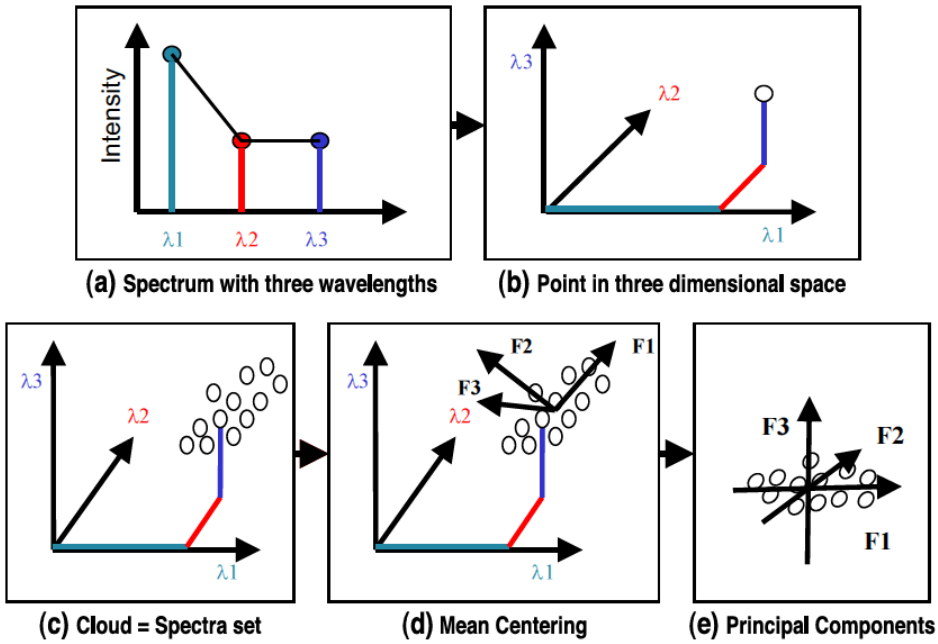


Figure 9: Transformation of a spectrum with three variables, or wavelengths (a) to a new coordinate system with one axis for each wavelength, converting the spectrum to a single point in a three-dimensional space (b), cloud formation of several spectra (c), MNCN (d), and determination of principal components F1, F2 and F3 (e) (adapted from reference (10)).

In pharmaceutical NIR analysis, it is often possible to compress most of the spectral variability to only a few principal components, with only a rather small loss of information (10).

1.5.5 Multivariate calibration for quantitative analysis

To perform a qualitative analysis using NIR data it is necessary to calibrate using multivariate methods (10). The purpose of multivariate analysis methods is to construct models capable of accurately predicting the characteristics and properties of unknown samples by correlating raw or pre-processed products spectra with one or more chemical-physical properties of a set of samples (53, 54). The process involves the steps described in Table 5.

Table 5: Steps in the multivariate model construction process (adapted from reference (53)).

Step	Purpose
1. Calibration samples selection	Select a set of samples representative of the whole population.
2. Determination of the target values by using the reference methods	Determine the value of the measured property in an accurate and precise manner. The quality of the value dictates that of the calibration model.
3. NIR spectra record	Obtain physicochemical information in a reproducible manner.
4. Pre-treatment of the spectra	Reduce unwanted contributions (such as shifts and scatter) to the spectra.
5. Model construction	Establish the spectrum-property relationship using multivariate methods.
6. Model validation	Ensure that the model accurately predicts the property of interest in samples not subjected to the calibration process.
7. Unknown samples prediction	Predict rapidly the property of interest in new, unknown samples.

A number of multivariate analysis methods can be classified according to their purpose and the algorithms or computational procedures that they use. The method of choice will depend on the purpose of the analysis, the characteristics of the samples and the complexity of the system concerned (for example its non-linearity). The simplest and oldest, however, less used, quantitative multivariate analysis method is multiple linear regression (MLR), which usually uses fewer than five spectral wavelengths. MLR

assumes concentration to be a function of absorbance (according to Beer´s Law), which implies the knowledge of the concentrations of not only the target analytes, but also all other components contributing to the overall signal (53).

The multivariate-regression methods most frequently used in NIR spectroscopy are PCR and particularly PLS regression (10).

PCR uses the PCs provided by PCA to perform regression on the sample property to be predicted (68).

PLS finds the directions of greatest variability by considering both spectral and target-property information, with the new axes called ‘‘PLS components’’ or ‘‘PLS factors’’(10). The main goal of the PLS is to establish a linear link between two matrices, the spectral data X and the reference values Y. This technique consists of modelling both X and Y in order to find out the variables in X matrix that will best describe them Y matrix. This can be explained by the representation of the spectra in the space of wavelengths in order to show directions that will be linear combinations of wavelengths called factors which describe best the studied property (67).

The main difference between the two methods is that the PC 1 or factor in PCR represents the largest variations in the spectrum, whereas in PLS it represents the most relevant variations showing the best correlation with the target property values. In both cases, the optimum number of factors used to build the calibration model depends on the sample properties and the analytical target (10).

For regression modelling, PLS is the main tool employed having advantages such as of handling collinear variables (e.g. spectral data) and handling of modest amounts of missing data. This is an appealing property, for instance if one needs to analyse process data where some probes may be malfunctioning or data from a certain day is missing. The method also assumes that there is noise present both in X and Y measurements, which is lacking in an ordinary regression, such as MLR (2).

1.5.5.1 Calibration model validation

A proper validation of the calibration equation is a crucial step to determine its suitability to predict new samples, which is the whole purpose of developing NIR calibrations (54).

Calibration error

The root mean square error of calibration (RMSEC) is a measuring of the fit of the model to the calibration data. It is defined as:

$$RMSEC = \sqrt{\frac{\sum_{i=1}^{N}(\hat{y}i - yi)^2}{N - A - 1}}$$

(Equation 1)

In Equation 1, $\hat{y}i$ are the values obtained by testing the calibration equation directly on the calibration data, N is the number of samples and A is the number of principal components. This estimate is an estimate of model error and not prediction error (68).

Internal validation: cross-validation

Validation should rather be performed with distributed samples which were not previously used for calibrating, but independent validation may not always be possible. In this way, cross-validation can provide a basic assessment of the calibration performance with calibration data only (54). This technique is done by successively deleting samples from the calibration itself. First, a sample (or set of samples) is deleted from the calibration set. A calibration is performed and that sample (or set of samples) is projected. In the next step the first removed sample (or set of samples) returns to the calibration set and the next sample (or set of samples) is removed. The procedure continues until all samples have been deleted once. The root mean square error of cross-validation (RMSECV) is defined by the following equation (68):

$$RMSECV = \sqrt{\frac{\sum_{i=1}^{N}(\hat{y}cv, i - yi)^2}{N}}$$

(Equation 2)

In Equation 2, $\hat{y}cv, i$ is the estimate for $\hat{y}i$ based on the calibration equation with the sample i deleted and N is the number of samples (68).

The cross-validation may be performed using the following methods to split the data:

- leave-one-out cross-validation: when only one sample is deleted at a time; this method is also called full-cross-validation;
- segmented cross-validation: contiguous blocks, in which a set of samples can be deleted in each iteration; this should be done when replicates exist in the calibration set; the replicates should be removed together, if not, over-optimistic results may be obtained;

- venetian blinds, in which every i^{th} sample is deleted, and
- random subsets, in which set of samples are randomly chosen to be removed (68).

The RMSECV it is not an error that directly estimates the actual predictor, but an estimate of the average prediction error of calibration models based on N-1 samples (68). Any statistic reported from cross-validation cannot be directly compared or interpreted the same way as statistics from a real validation of the final model with new samples (54).

External validation

One way to validate the calibration model is to split the data into two sets: one for calibration and one for validation/testing. In this case, the root mean square error of prediction (RMSEP) is calculated according to:

$$RMSEP = \sqrt{\frac{\sum_{i=1}^{N}(\hat{y}i - yi)^2}{Np}}$$

(Equation 3)

in which $\hat{y}i$ and yi are the predicted and measured reference values, respectively, for the test samples and the Np is the number of samples in the validation/test set. The RMSEP is the simplest test that can be made to validate a model (68).
When the RMSEP or RMSECV are used to predict the number of model components a plot of RMSEP(CV) against the number of components can be used (Figure 10).

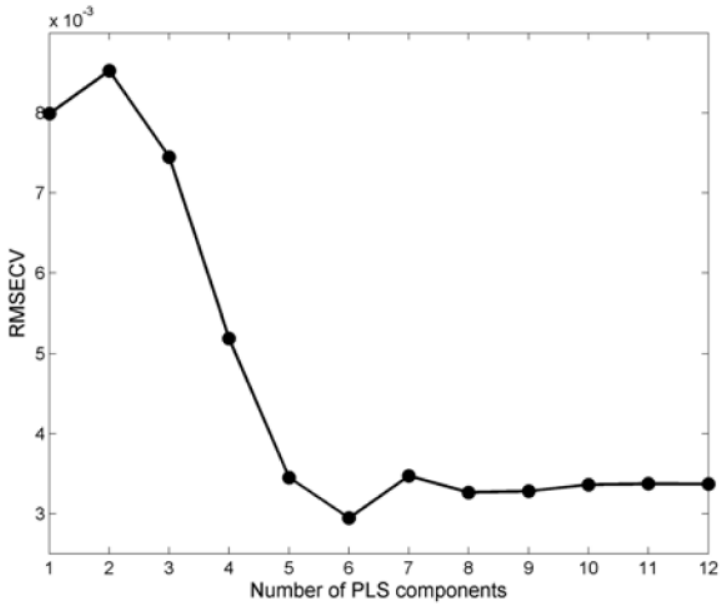


Figure 10: RMSECV in function of the number of PLS components (adapted from reference (61)).

Typically, in this plot the value of the error for a small number of components is high and decreases as the number of components increases. If the number of components is very high the error tends to increase again. A normal procedure when using this plot is searching for the number of components that gives the smallest error. If, however a smaller number of components give approximately the same error value, it would be preferably to choose the smaller number of components, leading to a more robust model. It should be emphasised that if the RMSECV is used to predict model architecture, RMSEP should be used to test the final performance of the model.

The plot of $\hat{y}_i$ versus y can also be useful to see how the model is behaving in terms of predictions abilities. An example can be visualized in Figure 11. Good predictions should be in a 45° line (and having a high correlation coefficient). However, some care must be taken when using the correlation coefficient in a model validation. Correlation only measures the degree of linear relationship between the measurements. A calibration equation can be clearly biased and still have high correlation between measured and predicted values. Also, extreme points may distort the correlation coefficient. So, a comparison between RMSEP(CV) is always preferred (68).

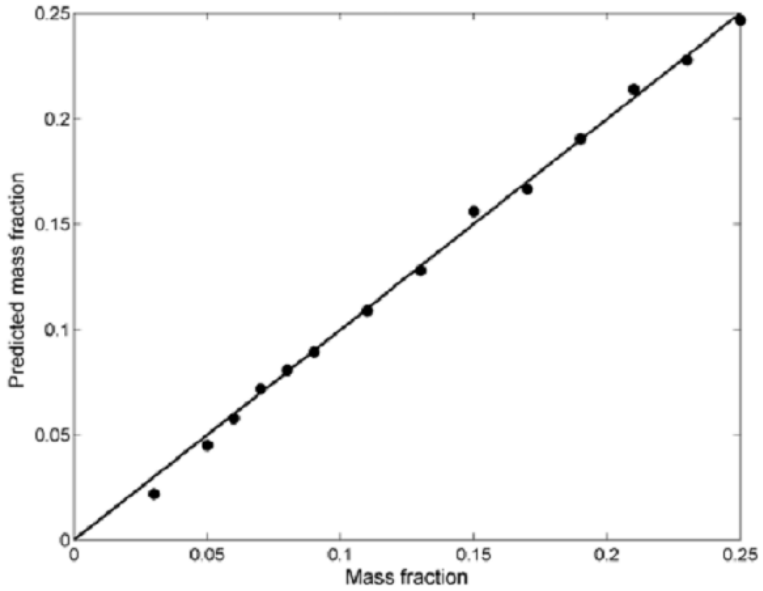


Figure 11: Mass fraction of an API (neomycin sulphate) versus the predicted mass fraction (adapted from reference (61)).

1.5.6 Outliers

There are always some observations that for some reason are different from the rest of the data set (68). Outliers from either reference values or spectral data exist and most calibration methods are highly sensitive to them (54). There are several reasons for an observation to be an outlier:

- when a sample belongs to another population than the “normal” samples;
- when an instrument is not functioning properly and therefore gives an erroneous or misleading signal, affecting one or all *x*-variables, and
- when there are errors in *y* caused by reference method failure or transcription error, resulting in a sample that will not fit into the regression model obtained from the rest of the data (68).

It is important to refer, that an outlier is not necessarily an erroneous observation, but merely an observation different from the rest of the population. Such observation can represent new and valuable information for the researcher.

Calibration and prediction outliers have to be considered separately. The calibration outliers are very important because they will affect the equation prediction with consequences for all future samples. A prediction outlier does not affect the calibration model however the prediction of *y* for such samples will be erroneous.

It is also useful to distinguish between *x*- and *y*-outliers. The *x*-outliers are those *x*-vectors that are in some way abnormally positioned relatively to the majority of *x*-data (Figure 12 (c)). The *y*-outliers are defined as those observations that have a different relationship between *y* and *x* (Figure 12(a)) (68).

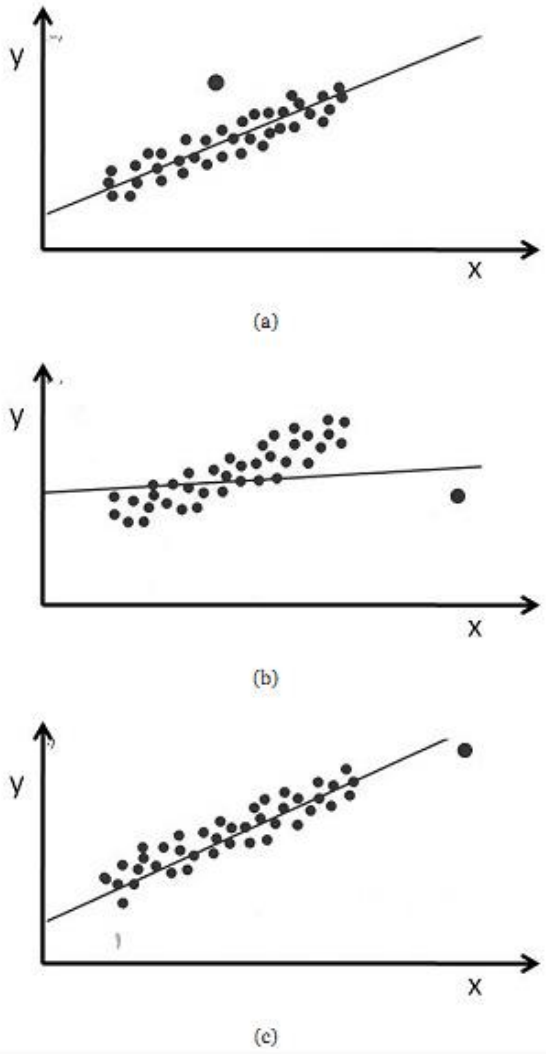


Figure 12: Outlier detection in linear models. The outlier in (a) is an *y*-outlier, the outlier in (c) is a *x* -outlier and the outlier in (b) is both *x*- and *y*-outlier (adapted from reference (68)).

In one dimension, as in figure above, it is easy to define a x-outlier, however with more than one dimension it becomes more difficult to detect this kind of outliers. The plots of principal components scores and PLS scores are very important to detect *x*-outliers. If outliers are present this will show up as points in the scores plots lying outside the normal range of variability (68).

1.6 Application of NIRS to dissolution tests

The studies concerning NIRS application in dissolution tests published until now were all performed off-line in diffuse reflectance mode. No study using NIRS *in-situ* to monitor dissolution studies was found after an exhaustive bibliography search.

The studies on NIR application in dissolution tests that were performed until this date are presented in Table 6. All of them show satisfactory results regarding the use of NIRS and chemometrics.

Table 6: Compilation of NIRS applications in dissolution tests with chemometric tools.

Author	Aim of the study	Main conclusions
Zannikos et al, 1991	-Predict the dissolution rate of carbamazepine tablets using NIR in diffuse reflectance mode and chemometric methods (PCR), validating with the reference method. -Study the influence of moisture in the dissolution rate profile of the drug.	-The dissolution rate was affected by the degree of hydration in high humidity, and could be non-destructively predicted by NIR.
Donoso et al, 2004	-Use NIR in diffuse reflectance mode as an alternative method in measuring the dissolution rate from theophylline tablets compacted at different compressional forces. -Obtain model equations by using different mathematical techniques for regression. -Test the model equations predictive ability, and statistically compare the predictive values to laboratory results to check for the agreement of the model with the reference methods.	-NIR in diffuse reflectance mode showed to be an alternative and non-destructive method for measurement of drug dissolution from tablets. -Models were developed for prediction of percentage of drug dissolved from tablets and were successfully validated and applied for prediction. -The predicted values were very similar to laboratory data.
Freitas et al, 2005	-Application of PLS regression method to correlate dissolution profiles obtained by using a dissolution apparatus (conventional method) and the NIR diffuse reflectance spectra of a series of clonazepam tablets. -Ten different formulations with fixed amount of clonazepam and varying proportions of excipients were analysed.	-Good correlation results were obtained of predicted dissolution percentage by NIR versus dissolution percentage by the reference method. - NIR diffuse reflectance spectroscopy method is an alternative, non-destructive tool for measurement of drug dissolution from tablets. - PLS showed to be an adequate regression method in building the calibration models.

Author	Aim of the study	Main conclusions
Blanco et al, 2006	- Develop PLS calibration models to determine the percentage of API dissolved at a given time by using tablets pressed at variable pressures and spanning a wide range of dissolution profiles using NIR in reflectance mode, validating against the reference method dissolution procedure and analysis.	-It was possible to accurately determine the API content and percentage of dissolution at a time of pharmaceutical tablets by using NIRS (in diffuse reflectance) in combination with multivariate calibration. -The pressure used to compact the tablets have a pronounced effect on their NIR spectra. -PLS model was used to determine compliance with the dissolution specifications, the results were comparable to those obtained with the reference method.
Otsuka et al, 2007	-Develop multivariate regression models that predicted the change in dissolution properties for indomethacin tablets pressed under varying compression pressures used both transmittance and diffused reflectance NIRS with chemometrics.	-The dissolution of the tablets was delayed by an increase in maximum compression pressure. -It is possible to predict the dissolution properties of pharmaceutical preparations using both diffuse reflectance and transmittance with chemometric methods. - The transmittance mode was more accurate for predictions of the dissolution behaviour of tablets than the diffuse reflectance mode.

Author	Aim of the study	Main conclusions
Tabasi et al, 2009	-Study the dissolution behaviour of sustained release theophylline tablets using NIR diffuse reflectance spectroscopy and multivariate calibration models.	-The results of this study showed that NIRS with multivariate modelling (PLS) was able to successfully differentiate variations on an excipient for drug coating in tablet formulation and correlate dissolution profiles of each batch to its corresponding tablet composition. -Using NIRS, dissolution results could be accurately predicted without having to actually analyse the product. -The results of this study expand the application of NIRS in sustained release pharmaceutical products.
Neves et al, 2012	-Develop a quantitative methodology to simultaneously determine the dissolution testing of four API (isoniazid, rifampicin, pyrazinamide and ethambutol) in tablets using NIR in diffuse reflectance, validating the results with the reference method.	-NIRS by diffuse reflectance, coupled with chemometric methods showed to be an efficient analytical technique, when used in the study of the dissolution testing of tablets, when compared to HPLC. -PLS is presented as a good regression method to be used together with pre-treatment steps that must be performed initially on the sample spectra.

1.7 Folic Acid

The study presented in this thesis is based on an immediate release tablet formulation containing folic acid as the API.

Chemically, the folic acid is constituted of three components: (1) a bicyclic pterin linked by a methylene bridge (C9-N10) to (2) para-aminobenzoic acid which is joined by peptide linkage to a single molecule of (3) L-glutamic acid (69, 70).

The folic acid molecule belongs to the vitamin B group and is present in many foods as free folic acid but mainly conjugated with glutamic acid residues (69). The human metabolism is not able to produce this molecule, so they have to obtain it from diet (70). Moreover, the folic acid obtained from pharmaceutical preparations is more bioavailable than the ones obtained from diet, once most of it is lost in the cooking process (71).

This vitamin has received considerable attention because of its role in the prevention of diseases. Its function is vital to the biochemical process of DNA synthesis and repair. Pre-conception consumption of folic acid plays a major role in the prevention of neural tube defects, primarily anencephaly and spine bifida (69, 70).

This yellow crystal has limited solubility in water and organic solvents. However, it is soluble in alkaline solutions so the folic acid standards used for quantification are prepared in basic solution, where the molecule demonstrates more stability (69).

Several methods have been developed for the quantification of folic acid in pharmaceutical formulations. Chemiluminescence using a flow injection technique and with fluorescence detection was used to quantify folic acid in tablets (72, 73). Another flow injection method, multicommutation, with flourimetric detection was used to quantify folic acid in tablets after irradiation of the samples with UV (ultraviolet) radiation (74). Other methods such as, a sensor with electrocatalytic detection and square wave voltammetry were also used to quantify folic acid in pharmaceutical preparations (75, 76).

Several different chromatographic methods have been developed and applied in the determination of folic acid in individual tablets or in multivitamin tablets such as microemulsion electrokinetic chromatography, high performance thin layer chromatography - HPTLC - and ultra-high pressure liquid chromatography - UHPLC - (77-80). In addition hyphened methods as liquid chromatography/tandem mass spectrometry - LC/MS-MS - and high performance liquid chromatography/electrospray ionization-mass spectrometry - HPLC/ESI-MS - were also used to determine folic acid in multivitamin tablets (81, 82).

The British pharmacopoeia and USP methods for the quantification of folic acid recommend the use of HPLC (83). Even though HPLC is a sensitive and selective method

and is very well established in pharmaceutical applications, there are several disadvantages which already have been reference before: HPLC has a complicated operation and maintenance system that requires large solvent volumes, with high cost of consumable supplies and the generation of substantial quantities of hazardous organic solvents and the time required to perform a HPLC method is many times very high and not in accordance with the requirements of the QC of a pharmaceutical industry that entails a more expeditiously method (77).

2. Experimental

This work was made in collaboration with a pharmaceutical industry and with the pharmaceutical technology department of the faculty of pharmacy of the University of Porto.

The experimental section is divided in two parts, in the first one the development and validation of the UV spectrophotometric method for folic acid quantification is shown. In the second part, the application of the NIRS method to monitoring dissolution tests is explained.

2.1 Development and validation of a UV spectrophotometric method for the determination of folic acid

2.1.1 Instrumentation, samples, reagents and solutions

High performance liquid chromatography

In this work two different HPLC apparatus were used: one for the quantification of folic acid in commercial pharmaceutical formulations and a second for the quantification of folic acid in the dissolution tests. The reference method procedure was performed according to the USP folic acid monograph (83).

For the quantification of the folic acid content in commercial tablets, the method was performed on a HPLC system (Merck Hitachi LC system, Ltd. Tokyo, Japan) comprising two LC L-7100 pumps, an interface D-7000 and a Diode Array Detector. Chromatography Data Station Software was used for chromatographic control and data processing. The chromatographic conditions are defined in Table 7.

For the quantification of folic acid during dissolution tests, the HPLC method was performed in an instrument with an automatic injection system (autosampler) and four pumps from UltiMate 3000 LC Systems, (Dionex, Germany). Software Chromeleon 6.70 (Dionex, Germany) was used for data control and processing. The chromatographic conditions are defined in Table 8.

A reversed-phase Spherisorb ODS2 column (pore size 5.0 µm, 4.6 x 250 mm, Waters, Dublin, Ireland) with a security guard cartridge (4.0 x 3.0 mm, Phenomenex, USA) was used for separation in both HPLC apparatus. Each chromatographic run was performed in isocratic elution mode using a mobile phase consisting of a buffer solution with pH 7.20 ± 0.05 (mixture (1:1) of 0.60 mol L^{-1} sodium perchlorate with 0.02 mol L^{-1} potassium

monobasic phosphate) and methanol (90:10). The pH was adjusted with potassium hydroxide 1.00 mol L^{-1} or phosphoric acid 1.00 mol L^{-1}. All solvents used in the chromatographic system were filtered through a 0.45 µm nylon membrane filter (HNWP, Millipore) using a vacuum pump (Büchi, Switzerland) and degassed for 15 minutes in an ultrasonic bath (JP Selecta, Barcelona, Spain).

Table 7: Chromatographic conditions for the quantification of folic acid in commercial pharmaceutical formulations.

Column	C18, 4.6 x 250 mm, 5.0 µm
Mobile Phase	Isocratic buffer solution pH 7.20 / methanol (90:10) (v/v)
Injection volume	20 µl
Column temperature	Ambient room temperature (25 ± 1ºC)
Flow rate	1.0 mL min^{-1}
Run time	13 minutes
Detector	280 nm

Table 8: Chromatographic conditions for the quantification of folic acid in dissolution tests.

Column	C18, 4.6 x 250 mm, 5.0 µm
Mobile Phase	Isocratic buffer solution pH 7.20 / methanol (90:10) (v/v)
Injection volume	20 µl
Column temperature	Ambient room temperature (25 ± 1ºC)
Flow rate	1.0 mL min^{-1}
Run time	13 minutes
Detector	280 nm

Ultraviolet spectrophotometry

All UV measurements were made on a Jasco V-660 double beam UV–vis spectrophotometer (Jasco, USA) using 1 cm optical path quartz cells. The UV measurements were performed with a spectral resolution of 1 nm, a scan speed of 400nm min^{-1}, on a spectral range between 200 nm and 450 nm.

Figure 13: The UV-vis spectrophotometer Jasco V-660 (adapted from (84)).

pH measurements

A Crison GLP22 pH-meter (Crison, Spain) calibrated with standard buffer solutions was used for pH measurements.

Dissolution tests

Tablets dissolution testing was performed in accordance to the USP Pharmacopoeia general method and folic acid tablets monograph (38, 83). Table 9 summarizes the dissolution testing apparatus and specifications used in this work.

Table 9: Dissolution testing apparatus and specifications.

Equipment	Erweka model ZT3-1 automatic dissolutor
Dissolution medium	500 ± 1% mL distilled water
Rotating apparatus	Apparatus 2 (paddle)
Temperature	37 ± 0.5ºC
Stirrer speed	50 rpm

The dissolution medium temperature was controlled at 37 ± 0.5°C using a Crison TM65 thermometer.

Samples, reagents and solutions

MilliQ ultra-pure water and grade glassware were used for the preparation of all solutions and samples. All chemicals and reagents were of analytical or HPLC grade.

Phosphate buffer (0.10 mol L^{-1} solution of NaH_2PO_4/Na_2HPO_4) was used to prepare solutions of different pH's in a pH range between 7.0 and 9.5 with a pH increment of 0.5.

Folic acid reference standard was purchased from Sigma (St. Louis, MO, USA) with a purity of 89.9%.

A folic acid stock solution (500.00 µg mL^{-1}) was prepared by dissolving an equivalent of 50.00 mg of folic acid reference standard in 100 mL of 0.10 mol L^{-1} phosphate buffer at pH 9.0. Working standard solutions were obtained by appropriate dilution of this stock solution with phosphate buffer.

All pharmaceutical excipients used in the interference studies were of pharmaceutical grade and were purchased from Sigma (St. Louis, MO, USA).

In this study three IR folic acid commercial brands were selected, hereby designated by A, F and G. These commercial folic acid products were purchased from local drugstores. All folic acid products were labelled to contain 5.00mg of folic acid per tablet.

2.1.2 Determination of folic acid in pharmaceutical formulations

To verify the pH influence on the UV spectra of folic acid, six folic acid solutions with different pH's were tested. Therefore, 0.875 mL of a folic acid stock solution (500.00µgmL^{-1}) was diluted to a volume of 25 mL with 0.10 mol L^{-1} of phosphate buffer (in the range pH 7.0 to pH 9.5 with pH increments of 0.5) to obtain folic acid working standard solutions with a concentration of 17.50 µg mL^{-1}. The folic acid working standard solutions with different pH's were then measured on the UV spectrophotometer at a temperature of 25ºC (controlled room temperature).

To confirm the maximum of absorbance (λ_{max}), a volume of 0.875 mL of folic acid stock solution was diluted to a final volume of 25 mL with 0.10 mol L^{-1} of phosphate buffer at pH 9.0 to obtain a folic acid reference solution of 17.50 µg mL^{-1}. Buffer was used as the blank on all UV measurements.

For the linearity study, seven solutions at different concentrations were prepared using seven different aliquots of folic acid stock solution at pH 9 according to Table 10. The solutions were measured by UV spectrophotometry using a buffer solution as blank. The absorbance was measured at 282.5 nm, and the respective values were used to determine the method's linearity by least squares. This procedure was made in three

different days. The limit of detection (LOD) and the limit of quantification (LOQ) for the assay were also calculated (85).

Table 10: Standard solutions used for the calibration curve assessment.

Standard solution	Concentration of folic acid (µg mL^{-1})	Volume of stock solution (µL)	Final volume (mL)
1	1.00	50	25
2	2.50	125	25
3	5.00	250	25
4	10.0	500	25
5	12.5	625	25
6	15.0	750	25
7	17.5	875	25

To verify the folic acid solutions stability, a folic acid working solution with a concentration of 17.50 µg mL^{-1} was prepared and preserved for 48h at room temperature (25 ºC) and analysed to test the folic acid stability in alkaline medium (pH 9).

Since the aim of this study was to determine folic acid in pharmaceutical formulations, the effects of the most commonly used excipients were examined. The analysed excipients were magnesium stearate and Ludipress®. Solutions containing folic acid (10.00 µg mL^{-1}) and the excipients in the proportion 1:1 and 1:10, were stirred with 0.10 mol L^{-1} of phosphate buffer at pH 9.0 in a magnetic stirrer for 20 minutes. Then the solutions were filtered with a filter pore of 0.45 µm, diluted, and analysed by UV spectrophotometry with the conditions already described.

After establishing the best experimental conditions, the three commercial tablets containing folic acid (brand A, F and G) were analysed by UV spectrophotometry as follows.

The average tablet weight was calculated from 20 tablets and then they were finely powdered in a porcelain mortar. A portion of this powder, equivalent to 50.00 mg of folic acid was accurately weighed and dissolved in 80 mL of 0.10 mol L^{-1} phosphate buffer at pH 9.0 and shaken for 20 minutes in a mechanical stirrer. The solution was filtered with a filter pore of 0.45 µm and transferred into 100 mL graduated flasks. The volume was completed with phosphate buffer at pH 9. Aliquots from this solution were transferred into 25 mL graduated flasks and were analysed by UV spectrophotometry. This procedure was

made in triplicate for each tablet brand. The amount of folic acid per tablet was calculated from the standards calibration curve.

To confirm the accuracy, precision of the proposed method and to check the interference of the excipients (matrix) present in commercial tablets, recovery experiments were carried out by the standard addition method. This study was performed by addition of known amounts (2.50, 5.00, and 7.50 μg mL^{-1}) of standard folic acid solution to a known concentration of the previously analysed commercial tablets. The resulting mixtures were analysed by UV spectrophotometry with the conditions already referenced. The drug recovery was calculated by comparing the concentration obtained from the spiked mixtures with that of the pure drug.

2.1.3 Determination of folic acid in dissolution tests

Dissolution tests, using the three commercial brands already mentioned (brand A, brand F and brand G) were performed to assess the possibility of quantify folic acid during dissolution by UV spectrophotometry.

The volume of the dissolution medium was placed in the vessel and was controlled at 37 ± 0.5°C. One tablet was placed in the vessel and the dissolution vessel was immediately operated at the specified agitation rate for 15 minutes. Thirteen sampling points were defined to monitor the dissolution. At each sampling time point, a 3.0 mL sample was collected using a syringe with cannula, between the surface of the dissolution medium and the top of the rotating paddle. The mixture temperature was periodically verified and the vessel kept covered over the entire duration of the dissolution test.

Samples were filtered with a membrane filter with a pore dimension of 0.45 μm prior to the analysis by HPLC and UV spectrophotometry. Table 11 presents the dissolution testing conditions used in this work.

Table 11: Dissolution testing conditions.

Rotating apparatus	Apparatus 2 (paddle)
Dissolution medium	500 ± 1% mL distilled water
Temperature	37.0 ± 0.5 ºC
Stirrer speed	50 rpm
Duration of the test	15 minutes
Number of vessels	1
Quantification method	Off-line UV spectrophotometric proposed method and by the reference method (off-line HPLC)
Sampling time-points	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 14 and 15 minutes

Figure 14: Erweka model automatic dissolutor.

2.2 Development of a NIRS method for dissolution testing monitoring

2.2.1 Samples

Two different types of samples were used to develop the NIRS method, commercial tablets and laboratory scale tablets.

The commercial tablets used were an immediate release commercial formulation (brand F) constituted by folic acid (2.55%) and two excipients, magnesium stearate (0.91%) and Ludipress® (96.54%). Ludipress® is a blend of lactose (93%), povidone (3.5%) and crospovidone (3.5%) (86).

The laboratory scale tablets were based on the IR commercial formulation (brand F).

To have different dissolution profiles, a full factorial experimental design (Table 12 batch A to G) was created varying the compression force in two levels and the proportion of binder (povidone) in three levels in a total of six batches. In these batches the excipients that constitute Ludipress® were added separately to be able to change their relative amounts. Besides these six batches, two additional ones were made in which Ludipress® was added in the concentration present in the commercial formulation (batch H and I from Table 12) and the compression force was varied in the two levels used in the experimental design. From each batch 20 tablets were fabricated.

Table 12: Composition and compression force of the laboratory scale tablets.

Batch	Folic Acid (%)	Lactose (%)	Povidone (%)	Crospovidone (%)	Magnesium stearate (%)	Ludipress (%)	Compression force (ton)
A	2.55	89.79	3.38	3.38	0.91	-	2
B	2.55	88.34	4.83	3.38	0.91	-	2
C	2.55	86.41	6.76	3.38	0.91	-	2
D	2.55	89.79	3.38	3.38	0.91	-	5
F	2.55	88.34	4.83	3.38	0.91	-	5
G	2.55	86.41	6.76	3.38	0.91	-	5
H	2.55				0.91	96.54	2
I	2.55				0.91	96.54	5

The laboratory scale tablets were prepared according to the following:

a) Powders were weighted in the proportion showed in Table 12 weighing a total mass of 50.00g.

b) The powder mixture were blended in a turbula (WAB T2F, Switzerland) (Figure 15) for 15 minutes in a plastic 500 mL recipient.

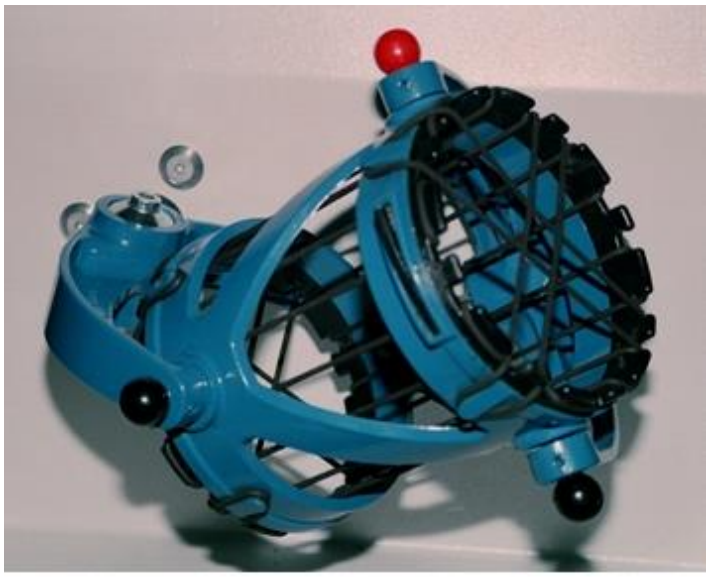


Figure 15: The turbula (WAB T2F, Switzerland) used for powder blending (adapted from (87)).

c) The blend was compressed with a compression force of 2 or 5 ton (see Table 12) in a hydraulic press (Specac, United Kingdom) for 5 seconds using a die and punch of 10 mm diameter.

To characterize the laboratory scale tablets, 10 tablets of each batch were subjected to a hardness test according to the Portuguese Pharmacopoeia IX in a Erweka TBH 28 (Germany) equipment (88).

Figure 16: Hydraulic press used to produce tablets with variable hardness (adapted from (89)).

2.2.2 Near-infrared spectroscopy

Near-infrared spectra were recorded on a FT-NIR analyser (FTLA 2000, ABB, Québec, Canada), with an InGaAs detector, controlled via the Bomem-GRAMS software (ABB, Québec, Canada) and equipped with two different accessories:

a) A powder sampling accessory (ACC101, ABB, Québec, Canada) for diffuse reflectance measurements with a 6 mm diameter illumination area (Figure 17). Each spectrum was acquired with an 8 cm^{-1} resolution, recorded as the average of 64 scans over a wavenumber range between 10000 cm^{-1} and 4000 cm^{-1}. In the beginning of the measurements, a background spectrum was taken by placing the reference material PTFE (Teflon) over the sampling window. Three measurements were made for each tablet on each side.

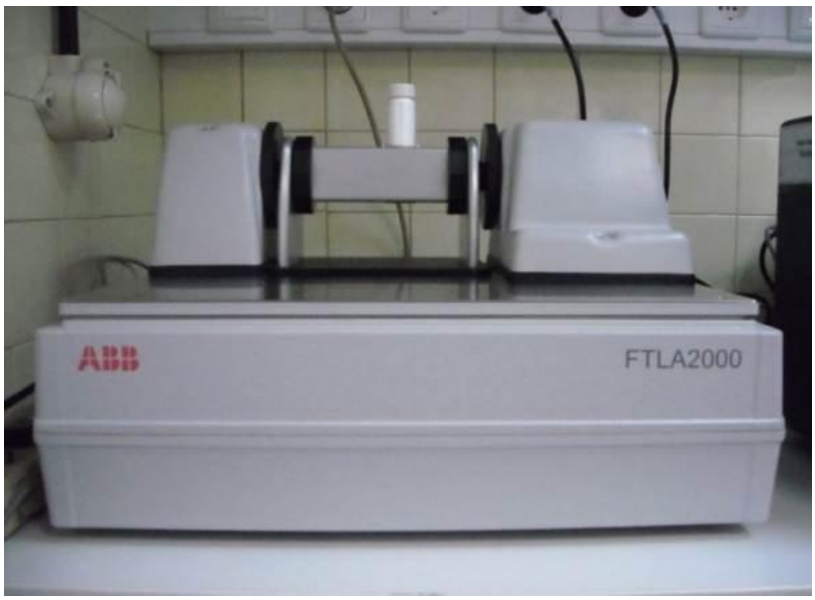


Figure 17: FT-NIR spectrometer FTLA2000 from ABB, equipped with a reflectance powder sampling accessory.

b) A transflectance probe (Flex, Solvias, Basel, Switzerland) with a mechanical pathlength of 1 mm (total optical pathlength of 2 mm) (Figure 18). Each spectrum was acquired with an 8 cm^{-1} resolution, recorded as the average of 16 scans over a wavenumber range between 10000 cm^{-1} and 4000 $cm^{-1.}$ The measurements were performed *in-situ* with the probe inside the dissolution vessel. In the beginning of each dissolution test, a background spectrum with air was taken with the probe clean and dry.

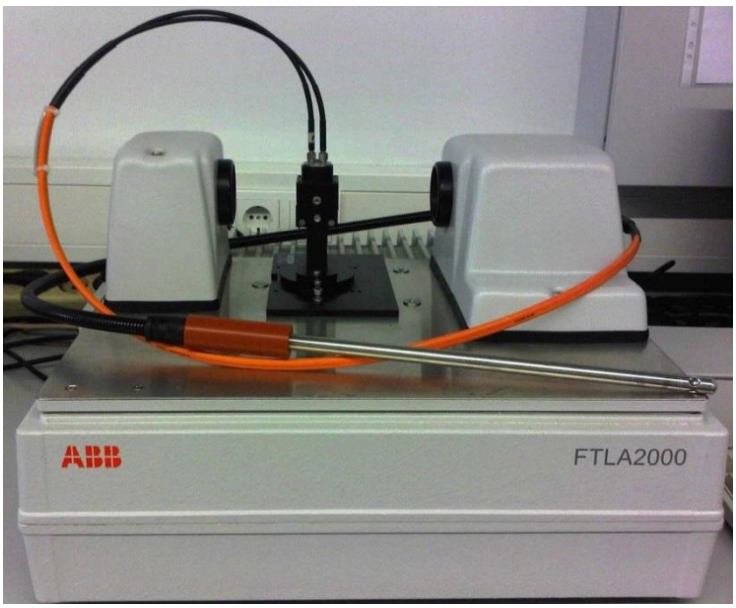


Figure 18: FT-NIR spectrometer FTLA2000 from ABB, equipped with a transflectance probe.

2.2.3 Tablet dissolution test

The dissolution tests were performed using the apparatus shown in Figure 14 using the conditions stated in Table 9.

The volume of the dissolution medium was placed in the vessel and was controlled at 37 ± 0.5°C. The transflectance probe was placed inside de dissolution medium always in the same position and depth. Once the tablet was inserted in the vessel, the apparatus was immediately operated at the specified agitation speed. At each sampling point, a sample of approximately 3 mL was collected, using a syringe with cannula, between the surface of the dissolution medium and the top of the rotating paddle, for UV spectrophotometry analysis. Samples for UV analysis were filtrated through a 0.45 µm syringe filters without dilution.

The mixture temperature was periodically verified and the vessel kept covered over the entire duration of the test.

Each sample for UV analysis was obtained at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 minutes of the dissolution testing. The NIRS measures were automatically made by the software at the specific times in order to coincide the UV sampling times.

2.2.4 Data processing

After spectral acquisition, all calculations were carried out using the Matlab version 7.9 (MathWorks, Natick, MA, USA) and the PLS Toolbox version 5.5.1 (Eigenvector Research, Inc., Seattle, WA, USA).

The way that the laboratory scale tablets were constructed renders them some difference in physical properties, predominantly in terms of particle size. To minimise these physical differences, different pre-processing methods were employed to the spectral data, e.g. SG filters, derivatives, SNV, MSC and normalization.

These pre-processing methods remove interferences such as, baseline drifts, light scattering effects and other instrumental variations. Additionally, the pre-processing methods remove interferences due to physical phenomena such as differences in the particle size distribution. However, it is known to be very difficult to remove entirely the effects of uncontrolled physical phenomena from the NIR spectra using simple pre-processing methods (10).

The multivariate technique used to relate the UV concentration of folic acid with the NIR spectra, was PLS. To assess the PLS model accuracy (bias), the RMSECV estimated

according to Equation 2 was used. The model robustness was evaluated in terms of the RMSEP according to Equation 3.

3. Results and discussion

As in the case of the experimental section, this section will be divided in two parts. In the first part it will be presented and discuss the development and validation of the UV spectrophotometric method for the determination of folic acid in pharmaceutical formulations and in dissolution tests. The validated method will be used in the second part as the reference method for the NIRS method. In this second part the results regarding the NIRS method to monitor dissolution methods will be presented and discussed.

3.1 Development and validation of a UV spectrophotometric method for the determination of folic acid

3.1.1 Determination of folic acid in pharmaceutical formulations

Studies at different pH's were carried out to verify its influence on folic acid absorption in the UV-vis region. The influence of pH was studied between 7.0 and 9.5 in intervals of 0.5 pH units (see Figure 19). Four broad absorption bands with absorption maxima at 217 nm, 257 nm, 282.5 nm and 361.5 nm could be observed in the UV-vis folic acid spectrum. All bands were dependent of the pH, and the band centered at 361.5 nm underwent a bathochromic shift moving between 346.5 nm at pH 7.0 and 363.5 nm at pH 9.0.

Since in the literature it is referenced that folic acid is soluble and more stable in alkaline solutions the remaining experiments were carried at pH 9.0 (69).

As can be seen in Figure 19, the UV-vis spectra of folic acid showed that the drug absorbed appreciably at 282.5 nm ($\lambda_{max.}$), so this wavelength was selected as the detection wavelength for determination of folic acid in pharmaceutical formulations.

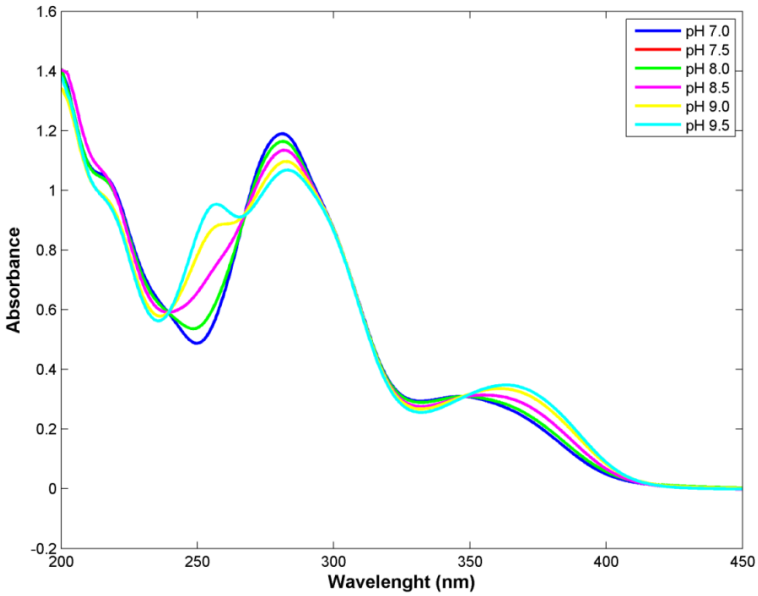


Figure 19: UV-vis spectra of a 17.50 µg mL^{-1} folic acid solution at different pH values. The buffer used was of 0.1 mol L^{-1} NaH_2PO_4/Na_2HPO_4.

To evaluate the linear range of the calibration curve seven standards with concentrations of 1.00 µg mL^{-1}, 2.50 µg mL^{-1}, 5.00 µg mL^{-1}, 10.00 µg mL^{-1}, 12.50 µg mL^{-1}, 15.00 µg mL^{-1} and 17.50 µg mL^{-1} were measured by UV spectrophotometry and the absorbance value at 282.5 nm taken for further calculations. The measurements were made in triplicate and the average value was determined. The calibration curve (Figure 20) was obtained by least squares, and validated by evaluating the linear dynamic range, precision, LOD and LOQ (85).

Under the described experimental conditions, Beer's law is obeyed in the concentration range from 1.00 to 17.50 µg mL^{-1} of folic acid with an excellent determination coefficient (R^2 = 0.9999). This range was chosen because the final objective of this part of the work is to assess the possibility of using UV spectroscopy to measure the concentration of folic acid during dissolution tests. During dissolution the range of concentration is from approximately 2 µg mL^{-1} in the first measuring point until 10 µg mL^{-1} in the end of the dissolution.

The absorbance values for this concentration range were adjusted by the Equation 4.

$$A = 0.00247 + 0.06184 \times [Folic\ Acid] \qquad \text{(Equation 4)}$$

In Equation 4, $[Folic\ Acid]$ is the concentration of folic acid in µg mL^{-1} and A is the UV absorbance at 282.5 nm.

The LOD ($3.3.SD_{blank}$/slope of curve) and LOQ ($10.SD_{blank}$/slope of curve) were 9.3×10^{-3} µg mL^{-1} and 28.2×10^{-3} µg mL^{-1} of folic acid, respectively (85).

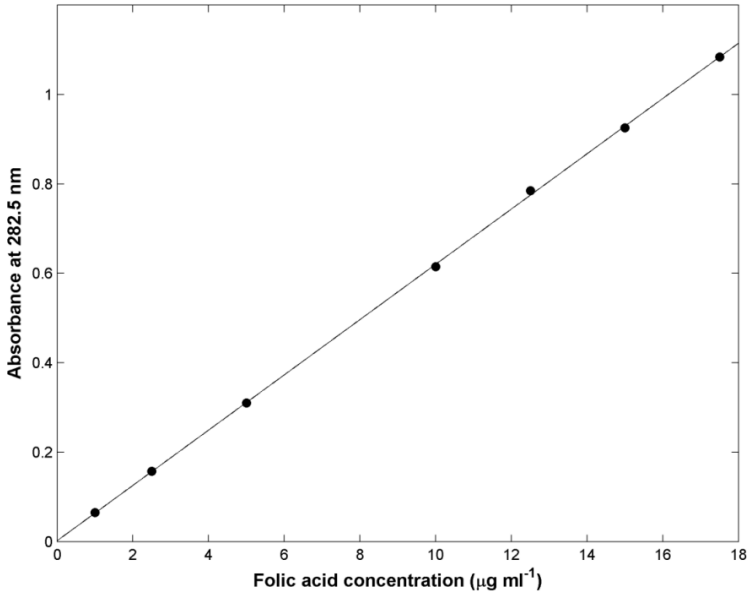


Figure 20: UV calibration curve for the determination of folic acid in pharmaceutical formulations.

Folic acid solutions are known to degrade with light and temperature during time, to check the stability of the solutions a folic acid working solution with a concentration of 17.50 µg mL^{-1} was investigated (69). The data given in Table 13 shows the values of absorbance at 282.5 nm of this solution which remain unchanged after standing for 48 hours at room temperature (approximately at 25ºC) protected from light.

Table 13: Folic acid working solution stability at room temperature (25 °C).

Time (h)	Absorbance (282.5nm)
0	1.08468
1	1.08509
2	1.08437
24	1.07699
48	1.07716

The interference of the excipients was studied to verify the specificity of the method to folic acid. The effect of each excipient was considered interference when the absorbance signal showed a variation coefficient equal or more than 5.3% or a mean recovery between 90-107% in the determination of the drug. The percentage of folic acid found in the test solutions ranged from 98.2 to 102.5 %, with variation coefficients values less than 1.1% for three replicates, indicating that no interferences of the excipients were observed under these conditions (90). In view of these results, the method is considered to be specific for the analyte, folic acid.

The proposed UV method was successfully applied on the determination of folic acid in three tablet formulations (brand A, F and G). The results, presented in Table 14, compare favourably with the reference method (HPLC) described in the USP, at a 95% confidence level using the t-student and Fisher test (91). The tests show no significant differences between the results of the proposed and the official methods, at the 95% confidence level. These results testify the applicability of the proposed UV method for the determination of folic acid in pharmaceutical formulations.

Table 14: Determination of folic acid in commercial pharmaceutical formulations.

Sample	**Label value** [a]	**Proposed UV method**				**Official HPLC method**	
		Found [b]	**RSD (%)** [c]	***p*-value (0.05)** [d]	***F*-value (19.00)** [d]	**Found** [b]	**RSD (%)** [c]
A	5.00	4.80±0.04	0.8	0.25	4.00	4.90±0.02	0.4
F	5.00	4.90±0.15	1.2	0.59	1.56	4.80±0.12	2.5
G	5.00	4.80±0.09	0.4	0.53	1.78	4.80±0.12	2.5

[a] Label content for tablets: mg unit $^{-1}$.

[b] Average value ± standard deviation (SD) of three determinations.

[c] Relative standard deviation (RSD) of three determinations.

[d] The figures between parentheses are the theoretical values of *p* and *F*.

In order to investigate the presence of matrix effects and to check the accuracy and precision of the developed method, it was carried out a recovery study. Three different concentrations of folic acid with concentrations of 2.50 µg mL^{-1}, 5.00 µg mL^{-1} and 7.50 µg mL^{-1} were spiked into a known concentration folic acid solution. The recovery test results

are presented in Table 15. The recovery mean values for all samples are within the 100.6% and 101.1% range with RSDs within 0.4% and 1.5%. These values ensure an accurate and precise method, without interference from any excipient present in the analysed tablets (90).

Table 15: Recovery data from three different concentrations of folic acid spiked into a known concentration folic acid tablet previously determined.

Sample	Added (μg mL^{-1})	Found (μg mL^{-1})	Recovery (%)	Mean Recovery [a]
A	2.50	2.55	102.0	$\mu^{a} = 100.6 \pm 1.5$
	5.00	4.95	99.0	
	7.50	7.55	100.7	
F	2.50	2.52	100.8	$\mu^{a} = 101.1 \pm 0.4$
	5.00	5.08	101.6	
	7.50	7.57	100.9	
G	2.50	2.54	101.6	$\mu^{a} = 100.8 \pm 1.1$
	5.00	4.98	99.6	
	7.50	7.60	101.3	

[a] Average ± RSD of three determinations

3.1.2 Determination of folic acid in dissolution tests

The developed UV method for the determination of folic acid in pharmaceutical formulations described above cannot be applied for the quantification of folic acid in dissolution tests since the aliquots retrieved from the dissolution tests are at pH 6. Therefore, to use the same folic acid standards, i.e., folic acid in phosphate buffer at pH 9, to quantify folic acid directly from the dissolution samples, a different strategy was pursued.

To verify the existence of isosbestic points, specific wavelengths at which two chemical species have the same molar absorptivity, and so the absorbance is essentially independent of the pH, a first derivative was applied to the UV-vis spectra of a folic acid

standard solution with a concentration of 17.50 µg mL^{-1} at different pH's (from pH 7.0 to pH 9.5 with increments of 0.5 pH units) (92). In Figure 21 it can be observed that three isosbestic points exist at 253.3, 305.5 and 377.2 nm. All of them were tested to verify if the value of the first derivative of the absorbance was linear against the concentration of folic acid and if they were sensitive enough to perform the quantification.

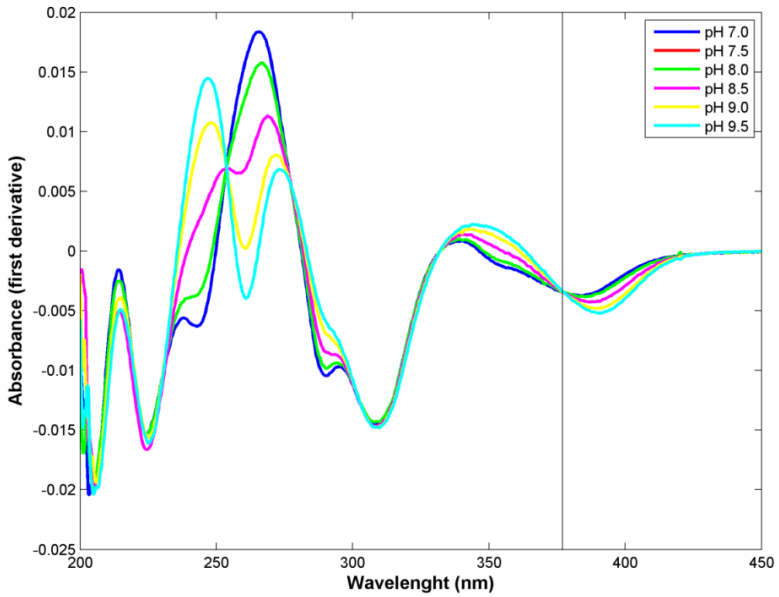


Figure 21: First-derivative UV-vis spectra of a 17.50 µg mL^{-1} folic acid solution at different pH. The gray dash indicates the wavelength used for the calibration curve assessment.

From the tested isosbestic points, the best linear correlation with the best sensitivity was at a wavelength of 377.2 nm in the folic acid concentration range between 1.00 µg mL^{-1} and 17.50 µg mL^{-1}. In Figure 22 can be seen the calibration curve for the determination of folic acid using the first derivative of the absorbance at 377.2 nm.

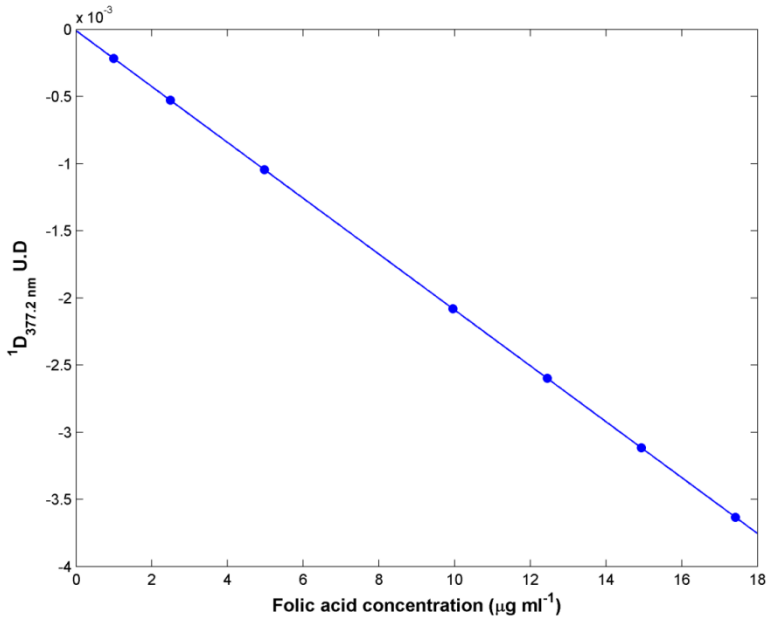


Figure 22: First derivative UV calibration curve for the determination of folic acid during dissolution tests.

The linear regression from the calibration curve equation was:

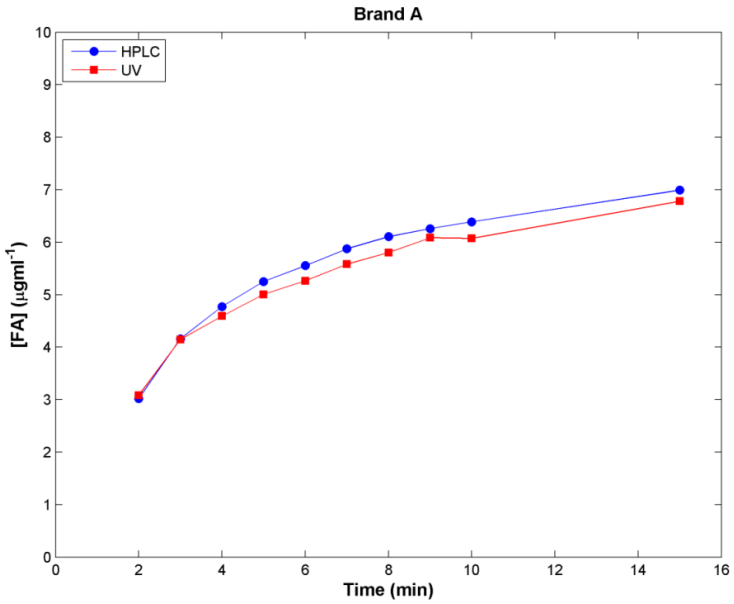
$$^{1}D_{377.2}\,(\text{U.D.}) = -9.21 \times 10^{-6} - 2.08 \times 10^{-4}\,[Folic\ Acid] \qquad \text{(Equation 5)}$$

In Equation 5, $[Folic\ Acid]$ is the concentration of folic acid in µg mL^{-1} and $^{1}D_{377.2}$ is the value of the first derivative of the absorbance at 377.2 nm. Under the described experimental conditions, Beer's law is obeyed in the concentration range from 1.00 to 17.50 µg mL^{-1} of folic acid with an excellent determination coefficient (R^2 = 0.9997).

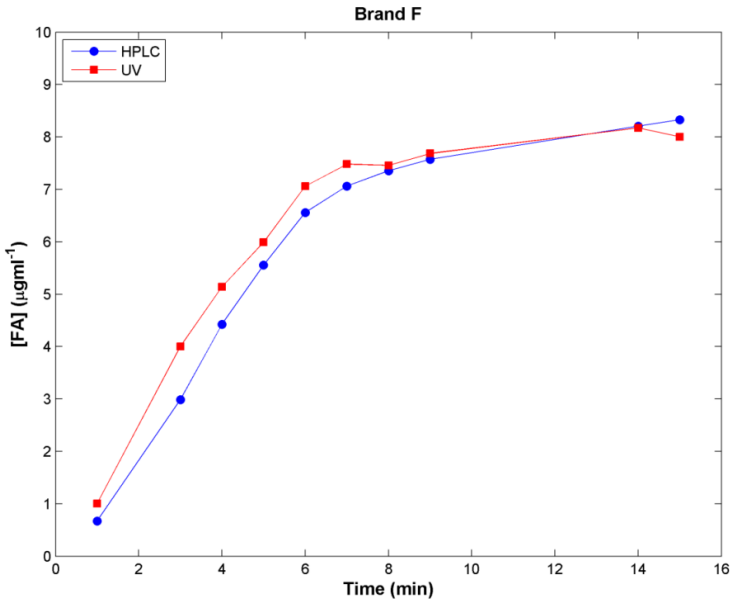
Therefore, by applying a first derivative to the UV spectra, it was possible to determine folic acid in solutions retrieved directly from the dissolution tests with pH around 6 with a calibration curve performed with standards at pH 9.

The three commercial brands already mentioned were subjected to dissolution tests and aliquots of 3 mL were retrieved during the dissolution tests to be analysed by HPLC and UV. Table 16 depicts the concentration of folic acid determined by the reference method (HPLC) and by the first derivative UV method. The concentrations presented in Table 16 are corrected for the decrease of volume that results from the removal of 3.0 mL in each sampling point. Some of the dissolution points do not have results because some experimental problem (probably sample contamination) occurred during the HPLC measurements and/or UV measurements and so the points were considered outliers.

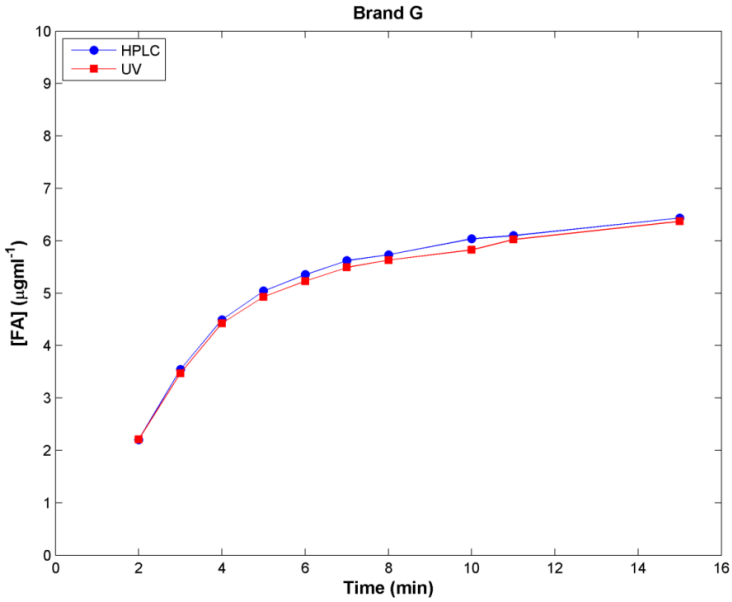
As can be seen in Figure 23 a), b) and c), the dissolution profiles determined with the UV first derivative method and the HPLC reference method for the three commercial brands are very similar.



a)



b)



c)

Figure 23: Dissolution profiles of folic acid (FA) tablets of the three commercial brands: a) brand A, b) brand F, c) brand G) determined by HPLC and by the first derivative UV method.

To perform a statistical comparison between both analytical methods, the determination coefficient (R^2) and a regression analysis considering the 95% confidence level were performed (see Table 17). Linear regression data may be used for calibrating a new method against an established one or validating the utility of a method in relation to analytical quality specifications. The correlation coefficient indicates the magnitude of total random error of the method comparison, including nonlinearity, drift or shift, total analytical imprecision and sample-related effects. The methods are considered statistical equal if the confidence interval (CI) for the slope includes the value 1 and if the confidence interval for the intercept includes the value 0 (93) (94). For the three commercial samples both criteria were met with the exception of the slope for sample A. However, analysing the determination coefficients it can be seen that for the three cases values are always above 0.99 (the defined threshold).

Table 16: Dissolution profiles of folic acid in commercial tablets.

Time (min)	Dissolution											
	Sample A				Sample F				Sample G			
	UV 1st derivative method		Official HPLC method		UV 1st derivative method		Official HPLC method		UV 1st derivative method		Official HPLC method	
	µg mL^{-1}	(%)	µg mL^{-1}	(%)	µg mL^{-1}	(%)	µg mL^{-1}	(%)	µg mL^{-1}	(%)	µg mL^{-1}	(%)
1	----	----	----	----	1.0	10.2	0.7	7.3	----	----	----	----
2	3.1	32.3	3.0	30.6	----	----	----	----	2.2	22.9	2.2	22.9
3	4.1	42.7	4.2	42.9	4.0	40.8	3.0	31.3	3.5	36.5	3.5	36.5
4	4.6	47.9	4.8	49.0	5.1	52.0	4.4	45.8	4.4	45.8	4.5	46.9
5	5.0	52.1	5.2	53.1	6.0	61.2	5.6	58.3	4.9	51.0	5.0	52.1
6	5.3	55.2	5.5	56.1	7.1	72.4	6.6	68.8	5.2	54.2	5.4	56.3
7	5.6	58.3	5.9	60.2	7.5	76.5	7.1	74.0	5.5	57.3	5.6	58.3
8	5.8	60.4	6.1	62.2	7.4	75.5	7.4	77.1	5.6	58.3	5.7	59.4
9	6.1	63.5	6.2	63.3	7.7	78.6	7.6	79.2	----	----	----	----
10	6.1	63.5	6.4	65.3	----	----	----	----	5.8	60.4	6.0	62.5
11	----	----	----	----	----	----	----	----	6.0	62.5	6.1	63.5
14	----	----	----	----	8.2	83.7	8.2	85.4	----	----	----	----
15	6.8	70.8	7.0	70.6	8.0	81.6	8.3	86.5	6.4	66.7	6.4	66.7

Table 17: Comparison between the HPLC reference method and the proposed first derivative UV method for the determination of folic acid concentration in dissolution tests (HPLC reference method (x) and first derivative UV spectrophotometric (y)).

Sample	R^2	Slope		Intercept	
		Value	95% CI	Value	95% CI
A	0.995	0.912	0.860 to 0.965	0.278	-0.011 to 0.567
F	0.999	0.993	0.973 to 1.014	-0.130	-0.264 to 0.005
G	0.999	0.973	0.946 to 1.001	0.040	-0.100 to 0.181

3.2 Development of a NIRS method for dissolution testing monitoring

The goal of this work was the assessment of a novel possibility involving the use of NIRS to monitor *in-situ* dissolution tests in order to expand surrogate methods in QC control in the pharmaceutical industry and to develop a more effectively understanding of CQA.

For that end, in a first approach, dissolution tests were performed using laboratory scale tablets to verify the possibility of the NIRS method be sensitive to process variations. Then, the use of NIRS and multivariate calibration for high-throughput monitoring and control of dissolution tests was evaluated using the immediate release commercial formulation (brand F) as model.

NIRS results were correlated with results obtained with the optimized first derivative UV method, hereby adopted reference method for quantification of folic acid. This method was preferred since it is faster and simpler when compared with the HPLC method and provides equivalent results as demonstrated in the previous sections.

Preliminary studies were performed to evaluate the capacity of NIRS to distinguish folic acid solutions from pure water. Two different folic acid solutions with concentrations of 5.00 µg mL^{-1} and 10.00 µg mL^{-1} were measured by NIRS in the same experimental conditions as the dissolution tests. In Figure 24 can be seen that the NIR spectra from the two folic acid solutions and the NIR spectra from pure water can be distinguished in the region between 6200 cm^{-1} and 5600 cm^{-1}.

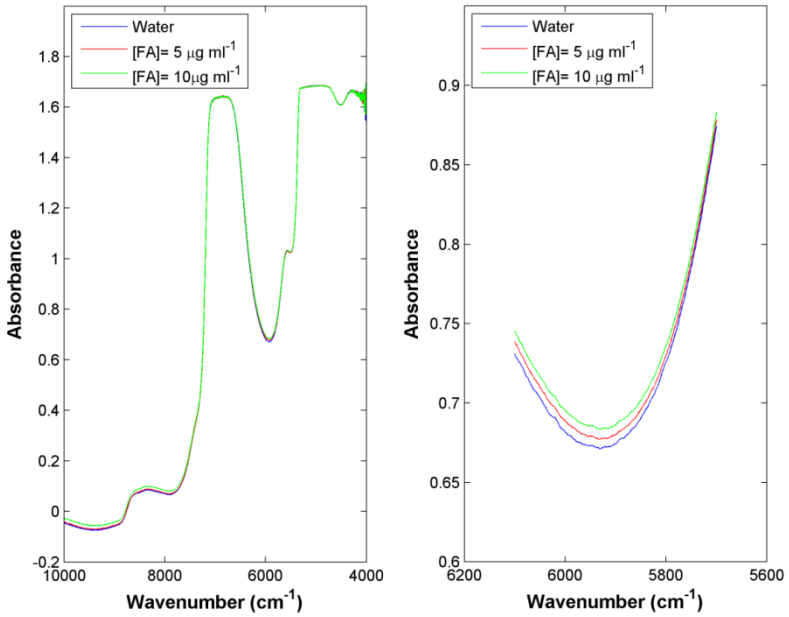


Figure 24: Left: NIR spectra of pure water and two folic acid (FA) solutions with concentrations 5.00 and 10.00 µg mL^{-1}. Right: Amplified region (6200-5600cm^{-1}).

3.2.1 Laboratory scale tablets

In order to characterize the manufactured tablets a hardness test was done (see Table 18). As expected, the tables that were produced with a lower compression force (batch A, B and C) gave lower hardness values, between 37 N and 41 N. The tablets produced with a higher compression force (batch D, E, and F) have a higher hardness values, between 81 N and 91 N. Tablets form batch G and H that were produced with Ludipress® gave higher values of hardness when compare with the tablets without Ludipress®. The reason for that is that the lactose was different from the Ludipress® lactose that is much more compressible, affecting in this way the hardness values.

Table 18: Hardness values for the 8 produced batches.

Batch	Compression force (ton)	Hardness (N) [a]
A	2	41
B	2	37
C	2	41
D	5	91
E	5	84
F	5	81
G	2	86
H	5	122

[a] Average of ten determinations

NIRS in reflectance mode was also used to characterize the laboratory scale tablets. Each tablet (20 per batch) was measured in both sides and in triplicate by NIRS.

A PCA was made with all spectra (6 spectra per tablet) to see if there was differences in the spectra from the different batches. Figure 25 shows the PCA score plot from the first component (PC1) against the second component (PC2). Each point in the score plot is a single spectrum. The spectra were pre-processed with a first derivative followed by a SG filter of 15 points fitted with a second order polynomial and mean centered prior to PCA.

The first PC captured 87.2% of variability in the sample set. The greatest source of variability in the spectra was the found to be related with compaction pressure. The way that particles are packed in the tablet affects the NIRS measurement because the light travels differently depending on the particles arrangement in the tablets.

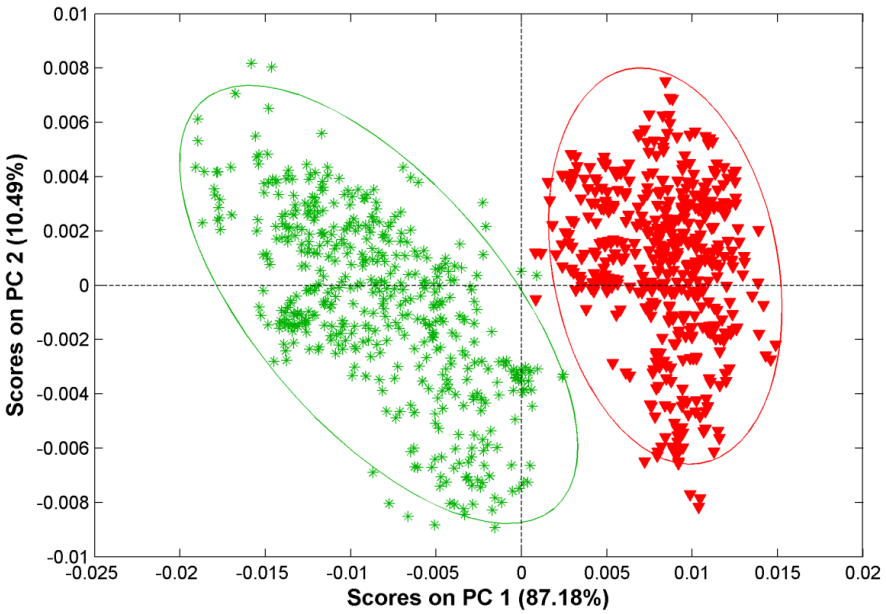


Figure 25: PCA score plot from a model calibrated with NIR spectra (10000 cm^{-1} – 4000 cm^{-1}) obtained in reflectance mode of tablets produced under laboratory conditions. Legend: ▼spectra from tablets produced with a compression force of 2 tons, ✱ spectra form tablets produced with a compression force of 5 tons.

In Figure 26 one spectrum from a tablet of batch A and one spectrum from a tablet of batch D are presented pre-processed in the same way as the spectra form the PCA analysis. The only difference in these two batches is the compression force, and as can be seen in the entire spectra differences they are only related to differences in the compression force.

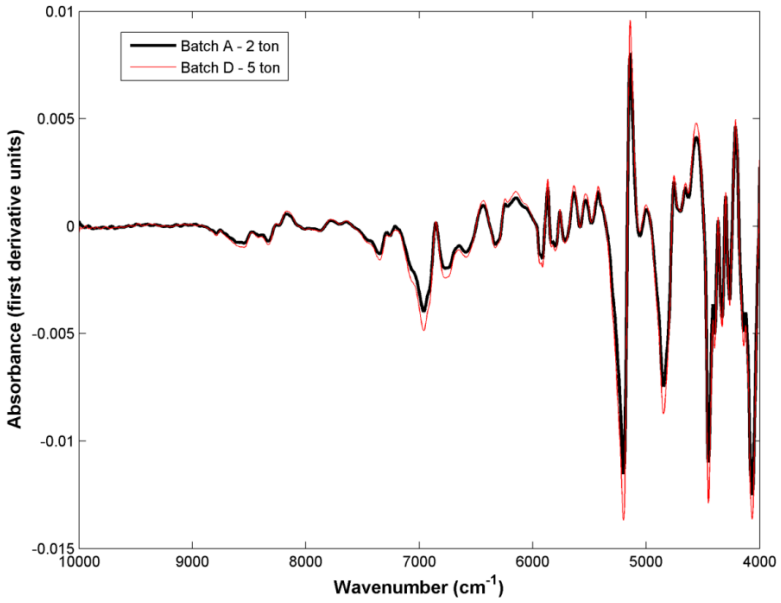


Figure 26: Pre-processed first derivative reflectance NIR spectra from batch A (compression force of 2 tons) and batch D (compression force of 5 tons).

After the characterization of the laboratory scale tablets, one tablet per batch in a total of eight tablets were subjected to dissolution testing as described in the experimental section.

Regarding the reference UV spectroscopic method, for each day of analysis a new calibration curve was made with new standards (see Table 19 for calibration curve data).

Table 19: Parameters of the three calibration curves for the determination of folic acid.

Calibration Curve	Date	Batch	Slope	Intercept	R^2
1	04/06/2013	A	0.0582	0.0018	0.9999
2	05/06/2013	B	0.0590	0.0072	0.9995
3	06/06/2013	C to H	0.0567	-0.0032	0.9999

In Table 20 is the folic acid concentration during dissolution tests for each of the tablets, measured by the first derivative UV spectroscopic method. The concentrations presented are already volume corrected. These results will be used to calibrate the NIRS method when applying PLS method.

Table 20: Folic acid concentration obtained from dissolution testing of one tablet of each batch of the laboratory scale tablets measured by the first derivative UV spectroscopic method.

Sampling time point (min)	Laboratory scale tablets							
	Folic acid concentration (μg mL^{-1})							
	A	B	C	D	E	F	G	H
1	2.92	2.84	2.95	4.64	4.71	4.31	1.76	1.18
2	4.51	4.47	5.28	5.61	5.70	5.69	3.83	2.69
3	5.69	5.33	6.48	6.10	6.13	6.28	5.41	4.29
4	6.95	6.66	7.02	6.27	6.44	6.65	6.53	5.64
5	7.28	7.13	7.33	6.53	6.72	7.00	7.02	6.20
6	7.70	7.63	7.63	6.70	6.83	7.12	7.40	6.52
7	8.00	7.90	7.82	6.74	7.01	7.28	7.68	6.75
8	8.20	8.08	8.00	6.86	7.05	7.41	7.94	6.84
9	8.39	8.24	8.15	6.99	7.13	7.47	8.00	6.96
10	8.34	8.33	8.30	6.96	7.17	7.61	8.19	6.98
11	8.54	8.43	8.39	7.06	7.33	7.53	8.12	7.15
12	8.72	8.57	8.42	7.07	7.35	7.60	8.38	7.16
13	8.74	8.72	8.46	7.17	7.41	7.69	8.42	7.21
14	8.84	8.81	8.45	7.20	7.45	7.83	8.46	7.19
15	8.78	8.82	8.61	7.20	7.46	7.87	8.51	7.25

The transflectance raw NIR spectra acquired during dissolution tests for all the batches of the laboratory scale tablets are shown in Figure 27. Spectral bands are highly overlapping and it is very difficult to identify the chemical constituents of the samples, excipients and API, or to distinguish similar features between them. In the spectra it is clear the water absorption peaks at 6900 cm^{-1} and 5200 cm^{-1}, originating saturated bands. These spectral zones in which the spectra is saturated are not suitable to use in PLS model, so the two remaining areas marked in the Figure 27 as Zone 1 (between 10000 cm^{-1} and 7200 cm^{-1}) and Zone 2 (between 6310 cm^{-1} and 5540 cm^{-1}) were tested in the PLS model.

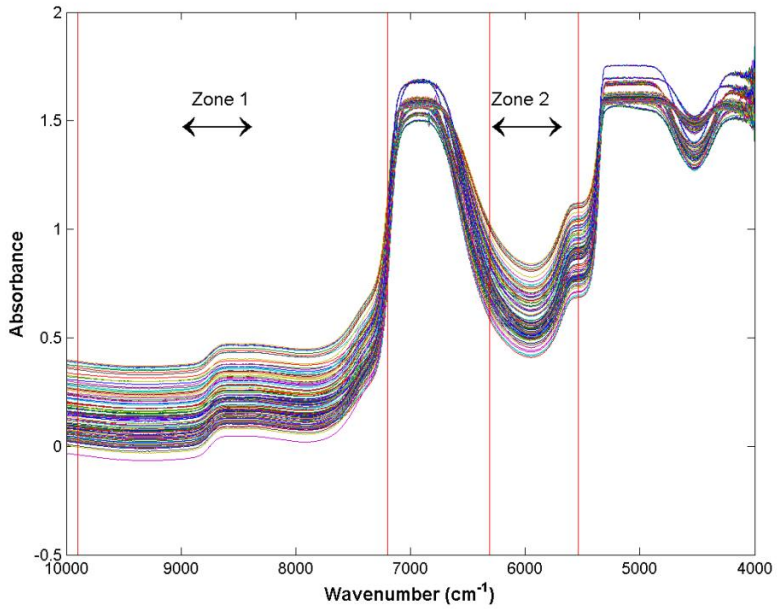


Figure 27: Transflectance NIR spectra obtained from the eight dissolution tests performed with one laboratory scale tablet for each batch.

Besides the two different spectral zones, several pre-processing methods were tested, namely SG filter with a first or second derivative, and three normalization methods (SNV, MSC and normalize). The best results (lower RMSECV) were obtained using the normalize function and the spectral zone 2. The normalize function normalizes the variables to area =.1 and is used mostly to correct pathlength variations (68). Since the excipients present in the formulation are not soluble in water, is possible that pathlenght variations occurred when particles of the excipients cross the transflectance probe, and thus this pre-processing method minimizes these variations that will affect the NIR spectra and consequently the PLS model. In Figure 28 is present the same spectra as in Figure 27 but pre-processed with the normalize function.

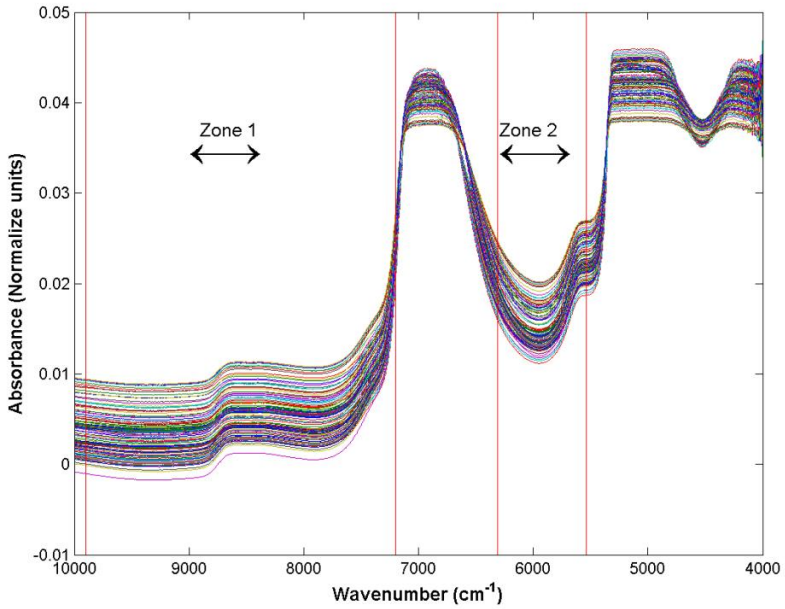


Figure 28: Transflectance pre-processed NIR spectra obtained from the eight dissolution tests performed with one laboratory scale tablet for each batch.

In Figure 29 the pre-processed spectra corresponding to the spectral zone used in the PLS model (zone 2) for one dissolution test can be seen. The dissolution can be followed over time; this is an evidence of the NIR sensitivity for the changes in the dissolution medium over the dissolution test.

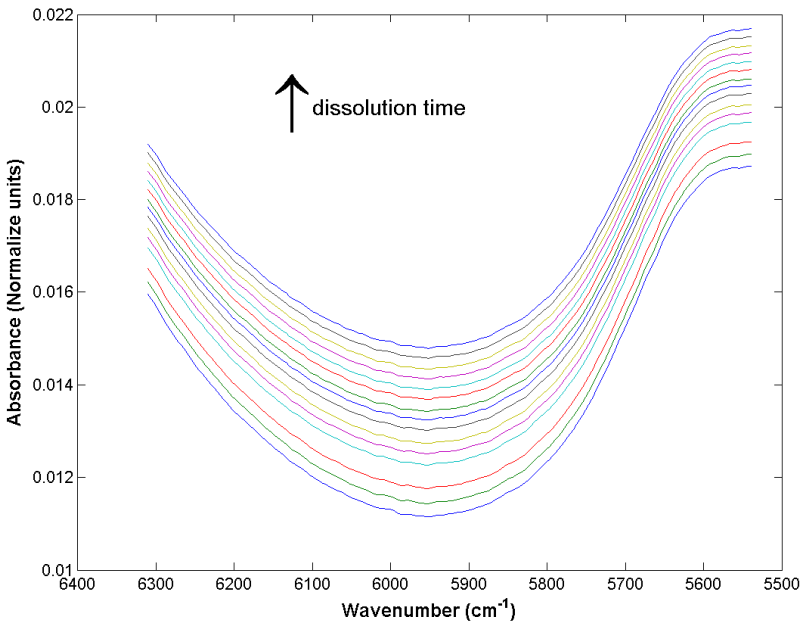


Figure 29: Pre-processed NIR spectra corresponding to the spectral zone used in the PLS model (Zone 2). The spectra in this figure correspond to one dissolution test performed with one laboratory scale tablet.

The first dissolution points correspond to a very low concentration of folic acid (lower than 6.5 µg mL^{-1}) and also the presence of the excipients in the dissolution medium makes very difficult to quantify these first sampling points by NIRS. For this reason, to build the calibration model the first 6 sampling time points were excluded.

In Figure 30 is shown the 15 sampling point's raw NIR spectra for the dissolution tests for each batch. As can be seen there are some batches in which the spectra is almost overlapped, e.g., batch A and B, and some others that the spectra is more dispersed, e.g., batch C to G. After some considerations it was concluded that the spectral dispersion was due to the non-soluble excipients present in the dissolution medium that dispersed the light and affect the spectra. The dispersion is not always the same depending in how the tablet starts to desegregate. Nevertheless, the light scattering effect was pronounced and had a linear relation with the increasing folic acid concentration. Additional studies are being made in order to understand this phenomenon but due to the time restrains it was not possible to include those studies in this thesis. Due to the experimental constrains explained, the spectra from the batches A, and B were removed from further calculations.

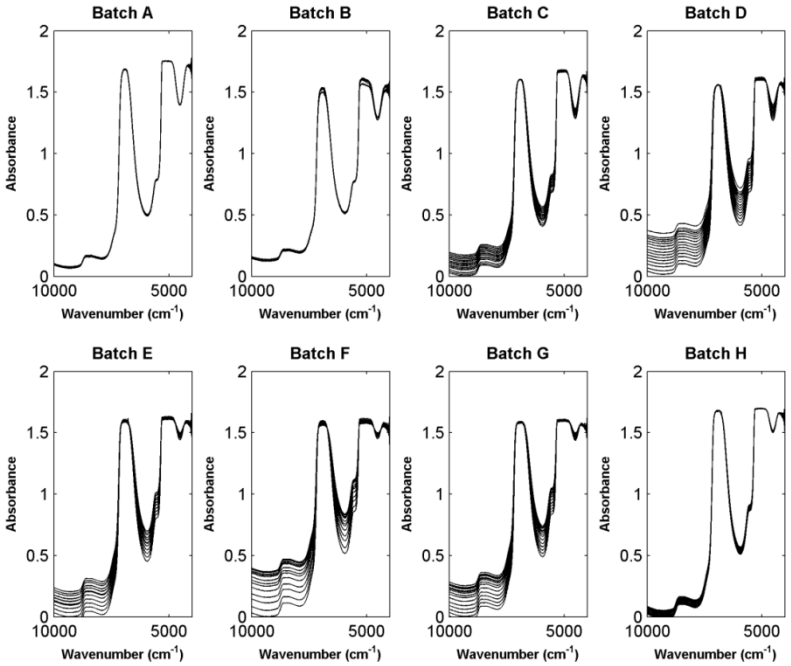


Figure 30: NIR spectra corresponding to the dissolution tests performed with eight batches of the laboratory scale tablets.

As already stated, the regression technique used to relate the NIR spectra with the concentration of folic acid determined by the first derivative UV method was PLS with internal cross-validation, using the leave-one-out method.

The optimal number of LV was chosen based on the lowest RMSECV value according to the leave-one-out cross-validation strategy. The model error was also assessed by using the RMSECV. For 6 LV a RMSECV of 0.15 µg mL^{-1} was obtained corresponding to a relative standard error (RSE) of 2.08% with a cross-validation R^2 of 0.95, which is indicative of a good prediction model (Table 21). These results show a good correlation and low levels of RMSECV between the dissolution percentage predicted by NIR and the dissolution percentage determined by UV for each batch.

Table 21: PLS model results for the determination of folic acid during dissolution tests by NIRS using laboratory scale tablets.

Pre-processing	**Spectral Range**	**LV**	**RMSECV µg mL^{-1}**	**RSE_{CV} (%)**	**R^2_{CV}**
Normalize and MNCN	6310 cm^{-1} to 5540 cm^{-1}	6	0.15	2.08	0.95

The folic acid concentrations measured by the first derivative UV method were plotted against the respective concentration estimated by the NIR method for batch D (Figure 31). The highest variation between the reference method values and the ones predicted by NIRS are in the beginning of the dissolution, where the concentration of folic acid is very low.

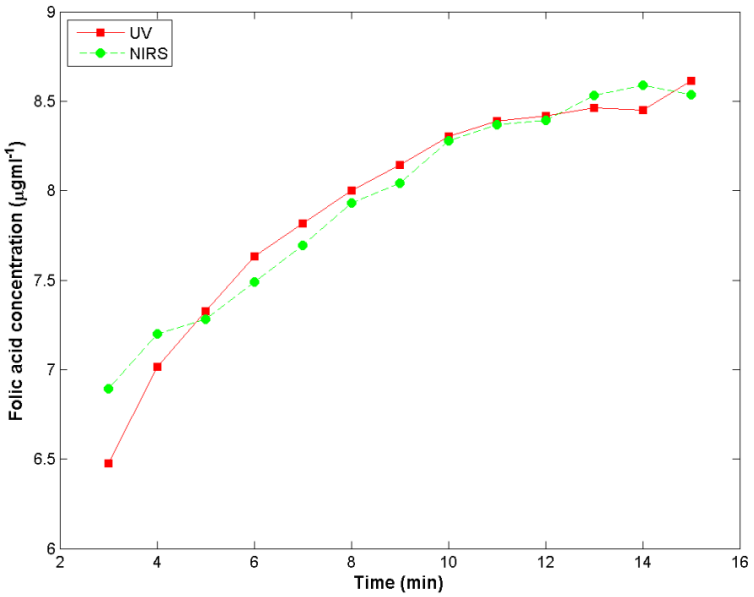


Figure 31: Dissolution profile determined with the first derivative UV reference method and with the NIRS method of batch D tablet.

Figure 32 plots the folic acid concentrations determined by the UV method against the predicted NIR concentrations. Ideal predictions should lie on a 45° line.

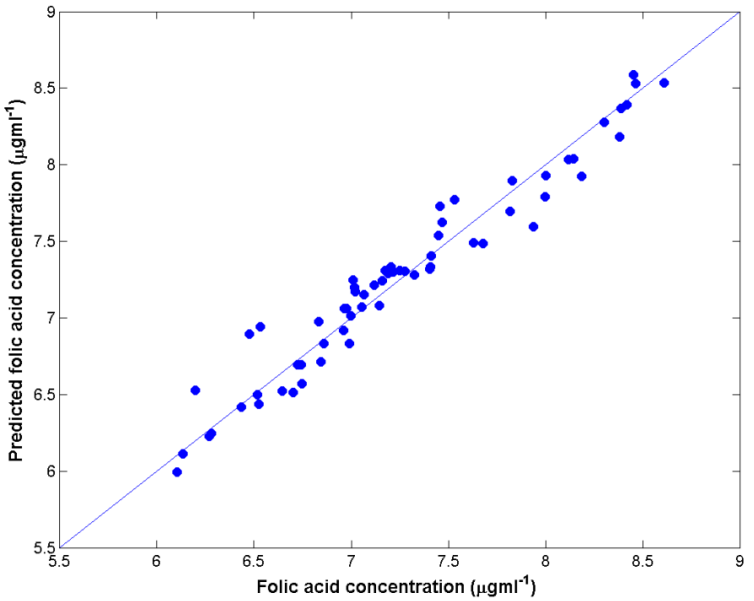


Figure 32: UV determinations versus NIRS predictions of folic acid concentration for all dissolution sampling points.

3.2.2 Commercial tablets

Ten commercial tablets of the brand F belonging to the same batch were subjected to dissolution testing with the conditions already described in the experimental section.
Samples collected over the dissolution tests were measured according to the optimized first derivative UV method. All dissolution tests were monitored by NIRS according to the conditions previously described in the last section.
Table 22 presents the parameters of the calibration curve for the quantification of folic acid by the first derivative UV method.

Table 22: Parameters of the calibration curve for the determination of folic acid by the UV method with first derivative**.**

Calibration Curve	Date	Batch	Slope	Intercept	R^2
1	29/06/2013	1 to 10	0.0587	-0.0002	1.000

In Table 23 is presented the folic acid concentrations measured by the first derivative UV spectroscopic method during dissolution for each of the tablets. The concentrations presented are already volume corrected. These results will be used to calibrate the NIRS method when applying PLS method.

Table 23: Folic acid concentration obtained from the dissolution testing of each tablet of the brand F commercial immediate release formulation.

Sampling time point (min)	Commercial tablets									
	Folic acid concentration (μg mL^{-1})									
	1	2	3	4	5	6	7	8	9	10
1	0.97	1.08	0.95	1.09	1.12	0.98	1.00	1.04	1.13	1.34
2	2.69	3.08	2.71	2.79	2.83	2.51	2.52	2.52	2.50	2.89
3	4.75	5.24	4.97	4.58	4.49	4.02	4.29	4.19	4.47	4.57
4	6.70	7.17	6.80	6.68	6.53	6.22	6.20	5.63	6.50	6.09
5	7.62	8.00	7.84	7.96	7.92	7.93	7.47	7.19	7.92	7.17
6	8.40	8.44	8.36	8.35	8.59	8.29	8.13	8.20	8.44	7.85
7	8.87	8.81	8.64	8.56	8.77	8.58	8.59	8.50	8.84	8.48
8	9.07	8.94	8.97	8.91	9.04	8.82	8.97	8.76	8.96	8.61
9	9.27	9.14	9.14	8.96	9.25	9.02	9.17	8.97	9.06	8.94
10	9.40	9.19	9.30	9.22	9.35	9.15	9.30	9.13	9.27	9.02
11	9.50	9.41	9.32	9.38	9.42	9.31	9.41	9.21	9.39	9.08
12	9.64	9.41	9.45	9.44	9.74	9.30	9.43	9.22	9.45	9.20
13	9.71	9.46	9.51	9.44	9.64	9.43	9.64	9.31	9.51	9.28
14	9.79	9.56	9.49	9.51	9.72	9.58	9.66	9.42	9.57	9.28
15	9.86	9.58	9.62	9.64	9.67	9.51	9.70	9.39	9.60	9.38

The transflectance raw NIR spectra acquired during dissolution tests for all the commercial tablets are shown in Figure 33. The spectra are very similar to the spectra form the laboratory scale tablets, as expected. The same two spectral regions, between 10000 cm^{-1} and 7200 cm^{-1} and between 6310 cm^{-1} and 5540 cm^{-1} were tested in the PLS model.

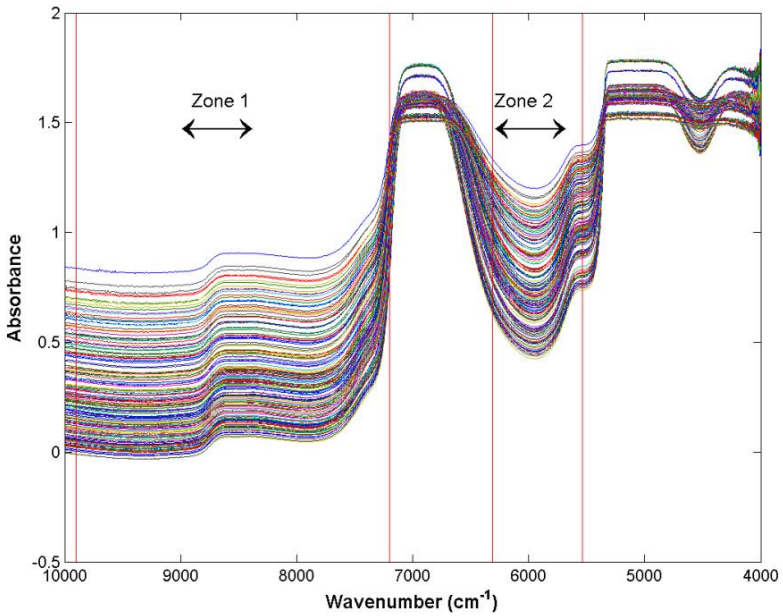


Figure 33: Transflectance NIR spectra obtained for the ten dissolution tests performed with the commercial tablets.

The same pre-processing methods (SG filter with a first or second derivative, SNV, MSC and normalize) were tested for this case. The best pre-processing method was normalize and the spectral area that gave the best results was zone 2 (between 6310 cm^{-1} and 5540 cm^{-1}). In Figure 34 are shown all the spectra for the dissolution of the 10 tablets pre-processed with the normalize function and in Figure 35 is shown for one tablet (tablet 8) the spectra correspondent to a dissolution test for spectra zone 2. The discussion done for the case of the laboratory scale tablets is also applied here.

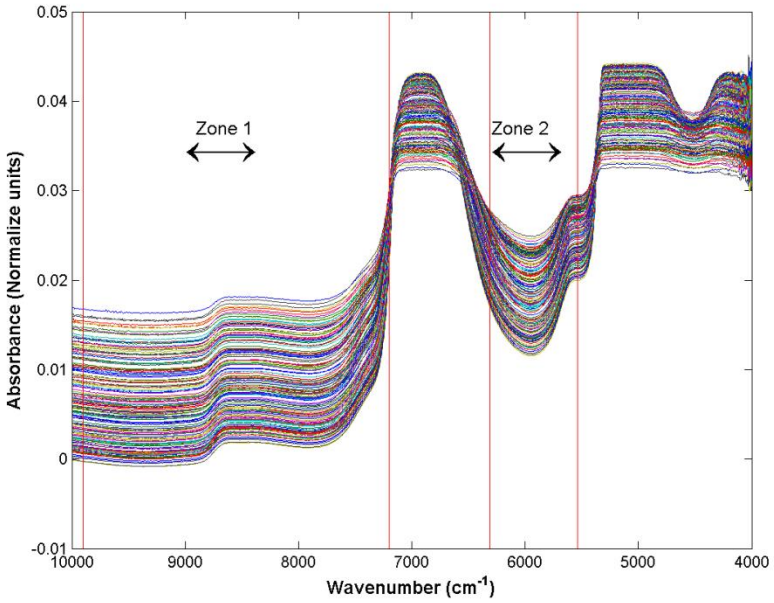


Figure 34: Transflectance pre-processed NIR spectra for the ten dissolution tests performed with the commercial tablets.

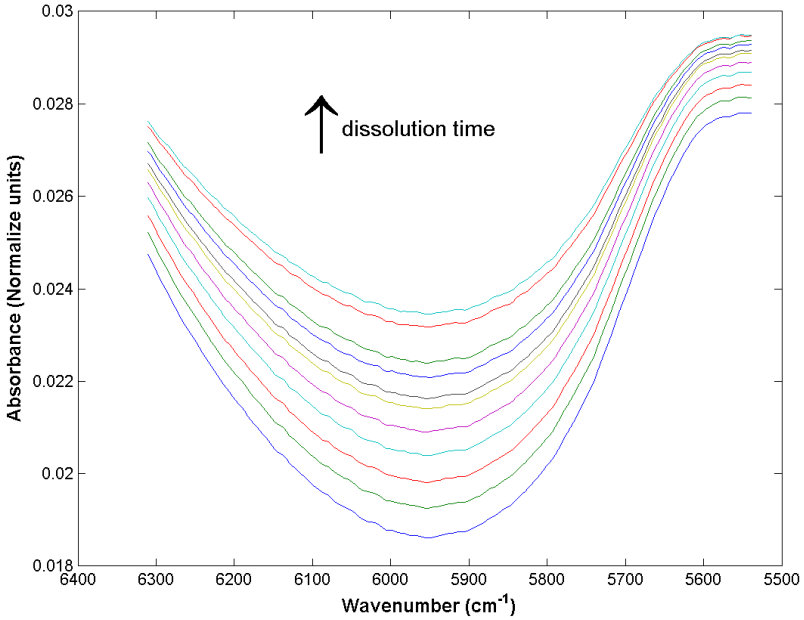


Figure 35: Pre-processed NIR spectra corresponding to one dissolution test of a commercial tablet zoomed in the spectral zone used for the PLS model (zone 2).

Figure 36 represents the spectra from the dissolution tests of the 10 tablets of the commercial brand F. As can be seen the same scattering effects that also affected the spectra of the laboratory scale tablets were also present here. For the same reasons

already explained the batch correspondent to tablet 1, 2 and 6 were removed from the calculations. Also, the first sampling points with folic acid concentration lower than 6.5 µg mL^{-1} were also removed from the calculation for the same reasons stated before.

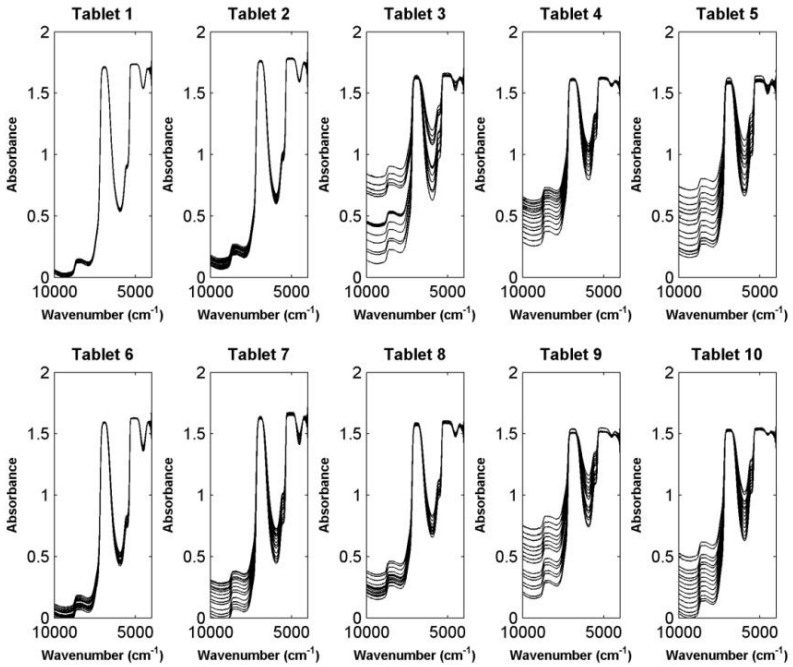


Figure 36: NIR spectra corresponding to the dissolution tests performed with the ten commercial tablets (brand F) containing folic acid.

Even after removing three batches, it was still possible to use six of the remaining batches to calibrate and one to assess the prediction ability of the method. So, batch 4 was not used in the calibration set and was used to test the model. PLS with cross-validation was used to relate the NIRS spectra with the folic acid concentrations measured by the first derivative UV method. The model error was assessed by the cross-validation error and model robustness was evaluated in terms of the RMSEP.

Table 24: PLS model results for the determination of folic acid during dissolution tests by NIRS using commercial tablets.

Pre-processing	Spectral Range	LV	RMSECV µg mL^{-1}	RSE$_{CV}$ (%)	R^2_{CV}	RMSEP µg mL^{-1}	RSE$_P$ (%)	R^2_P
Normalize and MNCN	6310 cm^{-1} to 5540 cm^{-1}	6	0.24	0.84	3.2	0.34	3.8	0.87

The leave-one-out strategy indicated that the best number of PLS LV was 6. For this number of LV the RMSECV was 0.24 µg mL^{-1} corresponding to a RSE of 3.2 % and to a R^2 of 0.84 (See Table 24). These results are evidence of a good prediction model.

The folic acid concentrations obtained with the UV method were plotted against the respective NIR concentration estimations for a commercial tablet 8 (Figure 37). As can be seen there are a good agreement between the reference method values and the valued predicted by the NIR method.

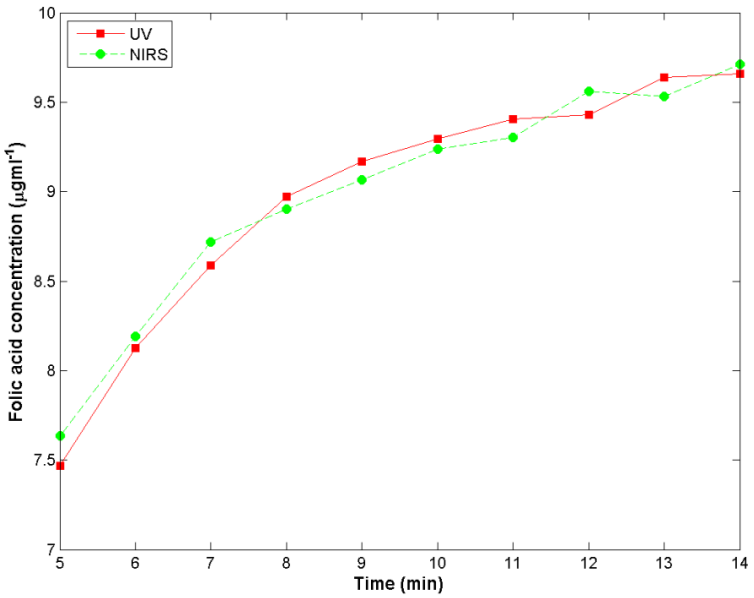


Figure 37: Dissolution profile determined with the reference (UV) and NIRS methods of one commercial tablet (number 8). NIRS predictions were obtained by leave-one-out cross-validation.

Figure 38 represents the NIR predicted concentrations for the tablet 4 and the actual concentrations obtained with the reference first derivative UV method.

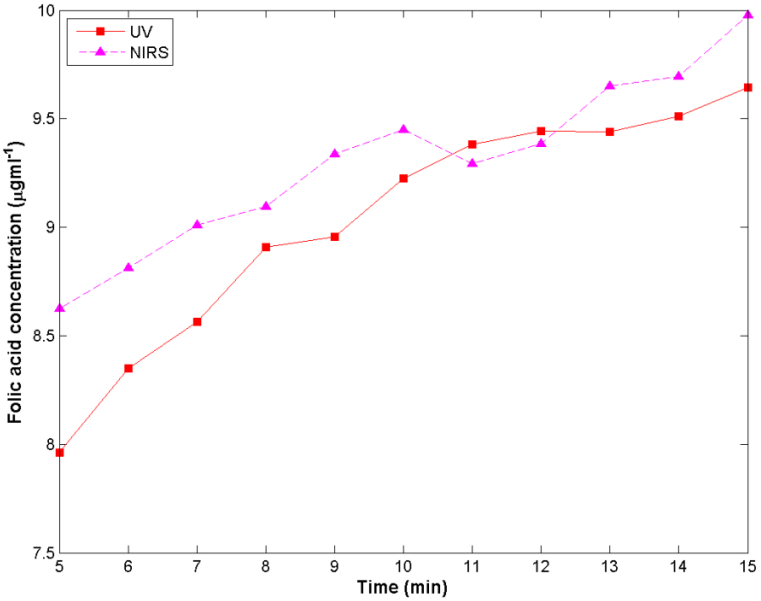


Figure 38: Dissolution profile determined with the reference method (UV method with first derivative) and with the NIR method for commercial tablet number 4.

The figures-of-merit for the PLS test were: RMSEP=0.34 µg mL^{-1}; RSE= 3.8% and Rp^2: 0.87 (Table 24). The results of the correlation between the predicted and experimental values are higher than 0.80, which confirm a good model predictive ability with a low RSE (lower than 5%) (68).

Figure 39 represents the folic acid concentrations determined by the UV method against the predicted NIR concentrations. The blue points correspond to leave-one-out estimations for the 6 dissolution tests included in the calibration and in pink the projected dissolution test 4. The closer the values approach the 45º diagonal line, the better the prediction is.

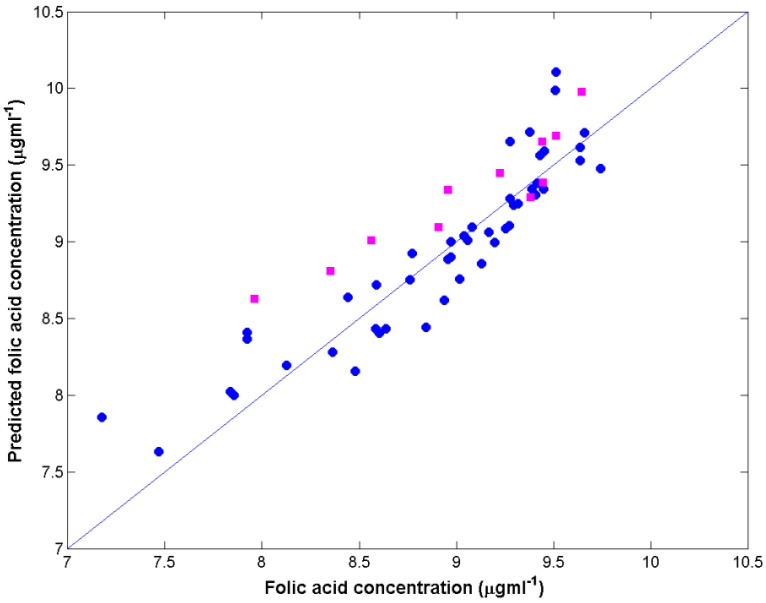


Figure 39: Folic acid concentration obtained by UV spectroscopy versus NIRS predictions for all dissolution tests used in the PLS model for the commercial samples (• calibration samples, ■ test samples).

It was already mentioned that the dissolution profile of a tablet depends not only on the dissolution conditions, but also on the physical and chemical composition of the tablet, namely, the porosity, particle size distribution, tablet hardness, among other factors (32, 33). NIR spectra are able to collect all physical and chemical information of folic acid tablets, which can be linked to the dissolution behaviour (53).

This work was highly affected by the different disaggregation of the individual tablets, adding variability to the results. The light scatter caused by the undissolved excipient particles in the dissolution vessel, decisively influence the NIR spectra, increasing the error in estimating the analyte concentrations.

The non-linear behaviour observed for lower concentrations may also affect the study. This analysis is limited by the low levels of API presented in the tablet, the immediate release formulation and the sensitivity of the *in-situ* fibre optic probe.

However, this study demonstrated the versatility of this novel application of NIRS, the method has excellent potential to be improved and optimized by:

- selection of a more sensitive probe;
- developing a NIR transflectance probe with a mechanism for preventing the disturbance caused by the undissolved particles; and
- by applying a major calibration with more sample sets to understand the scientific background of these results.

In this study, a PLS calibration model was established for NIR quantitative analysis based on percentage of drug dissolution with an FT-NIR spectrophotometer, thus overcoming the disadvantages of off-line analysis and complexity using UV spectrophotometric and HPLC methods and making on-line/in-line dissolution determination of folic acid tangible.

Although the work here presented requires further studies and improvements, the results concerning the experimentally designed tablets as well as the commercial ones indicate that NIR in the transflectance mode combined with the PLS is a potential method for the dissolution assessment in the QC of a pharmaceutical industry laboratory, or even in the dissolution profile assessment during investigation of a potential drug. Also, FT-NIR showed great potential in the study of drug dissolution processes.

As already mentioned in chapter 1, item 1.6 (application of NIRS to dissolution tests) several studies on the development of a dissolution testing monitoring method with NIRS have been reported, but all of them use NIRS in diffuse reflectance mode, and none uses transflectance. Therefore, this work is considered pioneer in respect of working with dissolution testing monitoring in NIR transflectance mode.

4. Conclusions

4.1 Development and validation of a UV spectrophotometric method for the determination of folic acid

In this first part of the work an analytical method based on UV spectroscopy was developed for determining folic acid in pharmaceutical formulations and validated in conjunction with the reference method (HPLC).The present method is found to be simple and more sensitive than most of the already reported spectrophotometric methods. The statistical parameters and the recovery study data clearly indicate the accuracy and precision of the method. Analysis of commercial samples containing folic acid showed no interference from common additives and excipients in general.

For the determination of folic acid in dissolution tests a modification in the UV method was needed due to pH differences between standards and samples collected from the dissolution vessel. A first derivative UV spectrophotometric method was developed.

For both cases the statistical comparison between the reference method (HPLC) and the proposed method, showed that it is suitable to quantify folic acid in pharmaceutical formulations and in dissolution tests. These results along with the fact that it is a simple, fast and resources saving efficient method make it an important surrogate for HPLC, which is an expensive and time consuming technique.

4.2 Development of a NIRS method for dissolution testing monitoring

The main goal of this work consisted in the assessment of the applicability of NIRS as an efficient PAT tool to monitor *in-situ* dissolution testing of an immediate release tablet formulation.

Preliminary studies were made to evaluate the ability of NIRS to distinguish API from water. Results showed that not only NIR was capable of distinguish the API from the water, but also it could be sensitive to different folic acid concentrations.

Laboratory scale tablets were manufactured in the laboratory with different characteristics in order to create a robust model. A PCA model evidenced that NIRS is able to detect differences in tablets produced at distinct compression forces.

Dissolution tests of eight batches (1 tablet for each batch) were performed and the dissolved API percentage was measured by first derivate UV method. NIR spectra were

correlated with reference measurements obtained by UV using PLS. Models were optimized regarding the spectral range, spectra processing and number of LV (through leave-one-out cross-validation). Models quality was very good which can be demonstrated by the excellent agreement between NIR predictions and the reference method estimations for the API concentration over the dissolution test.

Finally, 10 tablets of one commercial brand, of the same batch were subjected to dissolution tests, in the same conditions as the manufactured ones. A new calibration model with good correlation was achieved. Then, an external data set was used to confirm models' accuracy.

Results clearly showed that NIRS along with multivariate analysis is a potential analytical technique candidate for the in-line study of drug dissolution. This study demonstrates that it is possible to use *in-situ* NIRS to monitor dissolution tests. This methodology can easily be adapted to other solid forms since its application is straightforward.

While some goals were fully achieved, this work opened the door to several important issues that still need to be addressed. A better interpretation of the influence of undissolved particles in the NIR spectra over the dissolution test is required to stabilize NIR predictions. Either by interpreting correctly the effect of the particles or by developing a strategy to overtake the light scattering caused by the undissolved excipient particles, some action is required before attempting to implement this methodology in practice. This could be the objective of a future work since it is certainly a missing element in this work.

There are other issues that will need future optimization and improvement such as an improvement of the probe sensitivity, conducting more dissolution tests ideally from experimentally designed tablets with many variables to improve NIR models' accuracy and robustness.

In summary, this study indicated that NIRS has excellent potential as an analytical method for dissolution tests *in-situ* monitoring. This was demonstrated for the immediate-release tablet formulation based in folic acid but can be expected to have excellent potential for other solid dosage forms.

These results expand the NIRS applications portfolio for pharmaceutical solid oral dosage forms.

5. Future work and perspectives

The work developed in this thesis opened new perspectives in the implementation of NIRS for dissolution methods monitoring. The approach followed in this work revealed to have some advantages but also some drawbacks were identified. The most interesting feature of the proposed method is to promote an insight into what is happening in the dissolution vessel in real time and the associated low-cost per monitored dissolution.
While some of the initial targets were accomplished by this work, essentially the demonstration of feasibility, many issues remain open for research and therefore can be subject of continuation works. Among the most problematic issues faced in this particular work that need a careful attention is the identification of the real effect of light scattering caused by particles in suspension on the near-infrared spectra that cannot be removed by mathematical processing. This must be accomplished by monitoring a series of dissolutions with the *in-situ* probe and periodically performing some sampling, filtering and measuring again with the probe now without the particles interference. This study should be accompanied by a deep characterization of particles distribution over the dissolution course to allow the establishment of a relation between the presence of particles and spectral features observed when the probe is monitoring inside the vessel.

The following items summarise some of the work that is still essential to consolidate the knowledge initiated with this thesis:

- applicability range consolidation: develop the method from experimentally designed tablets with different designed variables; the goal is the identification of CQA and to identify the technique applicability range;
- test the methodology for the same API but with tablets made of different excipients; identifying the effect of different excipients on this methodology is absolutely critical;
- consolidate the methodology using an extensive number of samples: designed tablets and tablets from different lots of the same manufacturer and different manufacturers;
- verify the impact of the probe configuration on the quality of monitoring results (e.g., optical pathlength, probe configuration, placement inside the vessel);
- incorporate validation parameters such as linearity, range, accuracy precision and robustness into this strategy; perform tasks related with analytical method development and validation requirements in accordance with the ICH guidelines;
- validate the methodology with other solid dosage forms working on a common framework for dissolution testing monitoring with NIRS; and

- identify the possibility to use the same methodology using dispersive instrumentation which is less expensive and therefore more prone to be implemented in practice by pharmaceutical companies.

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