# Near Infrared spectroscopy, a quality control tool for the different steps in the manufacture of herbal medicinal products

by

**Magali Laasonen**
**née Grata**

Division of Pharmacognosy
Department of Pharmacy
Faculty of Science
University of Helsinki

ACADEMIC DISSERTATION

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**Supervised by**
Prof. Heikki Vuorela, Ph.D.
Division of Pharmacognosy
Department of Pharmacy
University of Helsinki
Finland

Dr. Tuulikki Harmia–Pulkkinen
Pharmia Oy,
Tuusula
Finland

**Reviewed by:**
Prof. Jouko Korppi–Tommola
Professor of Physical Chemistry
Department of Chemistry
University of Jyväskylä
Finland

Dr. Jukka Rantanen
Department of Pharmacy
Pharmaceutical Technology Division
University of Helsinki
Finland

**Opponent:**
Prof. Rudolph Bauer
Institut für Pharmakognosie
Karl-Franzens-Universität
Graz
Austria


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Front cover: *Echinacea purpurea* and second derivative near infrared spectra of batches of this herb. The *Echinacea purpurea* illustration is reproduced with the permission of the author Tristan Berlund, CA, USA (Tristan Berlund © 1999).

Gummerus Kirjapaino Oy
Saarijärvi 2003

À Marko,

À mon grand-père Jacques

**ABSTRACT**

Near infrared (NIR) spectroscopy is an analytical tool that is still not fully integrated into the pharmaceutical industrial environment. However, its advantages are potentially of considerable benefit for the quality control of herbal medicinal products.

Four methods were developed to demonstrate the ability of NIR spectroscopy as a quality control method in the different steps of the manufacturing process of herbal medicinal products. Qualitative and quantitative methods were established to control the quality of herbal and packaging raw material at reception, and to quantify the active content in the final dosage form. NIR methods showed several clear benefits, such as speed, low analysis costs and environmental friendliness compared to traditional analytical tools. The information obtained from NIR analysis is, however, different to that provided by separative methods such as high–performance liquid chromatography (HPLC). Qualitative NIR techniques can only confirm whether the sample is of required quality or not, and for quantitative measurements NIR intensities have to be calibrated for the sample's properties and do not give any information about any other property. The most labor–intensive part of the NIR analysis is method development. The results of NIR analysis are obtained in less than one minute for a single sample. In contrast, HPLC analysis is time–consuming but very specific, and provides detailed results about the presence or concentration of identity markers.

The use of chemometric tools and the study of factors affecting the spectra during feasibility studies are highly informative. They were used to optimise the calibration set, the regression model and the sample presentation mode, and were found to be critical steps in the development of specific and robust NIR models.

Pharmaceutical guidelines that are currently in force or in preparation were used and compared for the validation of the four NIR methods studied. The validation results proved that the NIR methods were as reliable as the reference analysis methods.

NIR spectroscopy is therefore a very suitable analytical tool for the quality control of herbal medicinal products.

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**ACKNOWLEDGEMENTS**

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Helsinki, April 2003

## LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications referred to in the text by their Roman numeral (I–V). Some unpublished results were also included.

**I** Laasonen M., Rantanen J., Harmia–Pulkkinen T., Michiels E., Hiltunen R., Räsänen M. and Vuorela H. Near infrared reflectance spectroscopy for the fast identification of PVC–based films, *Analyst*, 2001, 126: 1122–1128.

**II** Laasonen M., Wennberg T., Harmia–Pulkkinen T., and Vuorela H. Simultaneous analysis of alkamides and caffeic acid derivatives for the identification of *Echinacea purpurea*, *Echinacea angustifolia*, *Echinacea pallida* and *Parthenium integrifolium* roots. *Planta Med.,* 2002, 68: 568–572.

**III** Laasonen M., Harmia–Pulkkinen T., Simard C. L., Michiels E., Räsänen M. and Vuorela H. Fast identification of *Echinacea purpurea* dried roots using near–infrared spectroscopy, *Anal. Chem.*, 2002, 74: 2493–2499.

**IV** Laasonen M., Harmia–Pulkkinen T., Simard C. L., Räsänen M. and Vuorela H. Development and validation of a NIR method for the quantitation of caffeine in intact single tablets. *Anal. Chem.,* 2003, 75: 754–760.

**V** Laasonen M., Harmia–Pulkkinen T., Simard C. L., Räsänen M. and Vuorela H. Determination of the thickness of plastic sheets used in blister packaging by near infrared spectroscopy: development and validation of the method. *Analyst*, Submitted, December 2002.

**ABBREVIATIONS**

| | |
|---|---|
| EMEA | European agency for the evaluation of medicinal products |
| ESCOP | European scientific cooperative on phytotherapy |
| FT–NIR | Fourier transform near infrared |
| FTS | Fourier transform spectroscopy |
| GC | gas chromatography |
| GMP | good manufacturing practice |
| HMP | herbal medicinal products |
| HMPWP | herbal medicinal products working party |
| HPLC | high performance liquid chromatography |
| LED | light–emitting diode |
| LOQ | limit of quantification |
| MSC | multiplicative signal correction |
| NIR | near infrared |
| OPD | optical path difference |
| PC | principal component |
| PCA | principal components analysis |
| PLS | partial least square |
| RP | reversed phase |
| RSD | residual standard deviation |
| SEP | standard error of prediction |
| S/N | signal–to–noise |
| SNV | standard normal variate |
| ZPD | zero path difference |

# 1. INTRODUCTION

It was more than time…Slowly, but surely, the NIR (near infrared) spectroscopy, previously called the "sleeping technique" (WETZEL 1983), is being accepted by the pharmaceutical industry. Official instances such as the American Pharmacopea and the European Pharmacopea have recently adopted monographs describing this technique (USP 1998, EUROPEAN PHARMACOPOEIA 1997). A draft for the validation of NIR methods is being processed at the European Pharmacopea[1], and the number of NIR pharmaceutical applications has not stopped increasing since the beginning of the 90´s.

It was about time, because NIR spectroscopy was an analytical tool already widely used in the agricultural and food industries in the beginning of the 70´s (BLANCO et al. 1998). The main obstacle to the integration of this technique into the pharmaceutical world has been the regulations governing the introduction of new techniques in quality control laboratories. In contrast, the agricultural industries are not subjected to such strict regulations. Thanks to the regulatory framework that is now being created around the technique, the pharmaceutical industries will soon be able to fully enjoy the several benefits of NIR - speed, flexibility and low running costs.

Herbal medicinal products (HMPs) represent a considerable part of the pharmaceutical market in the world: Europeans are believed to spend more than seven billion US dollars on herbal supplements, and the US market is estimated to grow at about 15% per year (GLASER 1999). In the domain of herbal medicines, a large part of the costs are attributed to quality testing. The wet chemical, spectroscopic and chromatographic methods that are commonly used as quality control tools (SETTLE 1997) are time– and solvent–consuming. The use of NIR was recently investigated for controlling *e.g.* the origin of the drug and quantifying its active or marker substances (WOO et al. 2002, RAGER et al. 2002). It proved to be a very reliable tool compared to traditional methods of analysis. NIR could be more widely used to monitor the complete manufacturing process of the herbal product, *i.e.* from authentication of the plants to the

[1] This draft was adopted in February 2003 (EMEA 2003) after the thesis was written.

quantification of active compounds in the final dosage form. However, the task still remains to develop methods that fulfil pharmaceutical regulations for HMPs.

## 2. REVIEW OF THE LITERATURE

### 2.1. Herbal drugs, herbal drug preparations and herbal medicinal products

#### 2.1.1. Definitions

Herbal medicinal products are medicinal products containing as active substance exclusively herbal drugs or herbal drug preparations (EMEA 2001a, EUROPEAN PHARMACOPEIA 2002).

Herbal drugs are mainly whole, fragmented or cut, plants, parts of plants, algae, fungi or lichens in an unprocessed sate, usually in the dried form but sometimes fresh. Certain exudates may also be considered as herbal drugs.

Herbal drug preparations are obtained when herbal drugs are subjected to treatment such as extraction, distillation, expression, fractionation, purification, concentration or fermentation. They include comminuted or powdered herbal drugs, tinctures, extracts, essential oils, expressed juices and processed exudates (EUROPEAN PHARMACOPEIA 2002). In Europe, herbal drugs are described by monographs from the European Pharmacopea created in 1964 (ARTIGES 1998). They describe general methods of analysis and the appropriate method of storage. In Germany, “Commission E–monographs” have been published by the health authorities since the beginning of the 80´s and describe more than 380 medicinal plants (BLUMENTHAL 1998). A third source of monographs is the European Scientific Cooperative on Phytotherapy, ESCOP, which publishes monographs on individual plant drugs. These monographs highlight the clinical and pharmacological properties of the plants in order to represent a statement on efficacy and safety of a medicinal plant and its preparations (STEINHOFF 1998). Finally, the world Health Organisation (WHO) has also published useful monographs supporting the demonstration of safety and efficacy of herbal medicinal products (EMEA 1999, WHO 1999).

### 2.1.2. Regulations applicable to herbal medicinal products

During the last few years, European regulations concerning herbal medicinal products have been submitted to various modifications, and it is worth mentioning the latest issues. Herbal medicinal products (HMP) are, above all, medicines and therefore fall within the scope of the recent European Economic Community Council directive 2001/83/EC concerning medicinal products for human use, adopted in November 2001 (COUNCIL AND EUROPEAN PARLIAMENT 2001). Directive 2001/83/EC prescribes that no medicinal product may be placed on the market without having obtained a marketing authorisation. Marketing authorisation as a herbal medicinal product is, in principle, granted on the basis of a "full" dossier in terms of proof of quality, safety and efficacy in all Member States, with the exception of Denmark and Finland. In these countries it is only possible to use bibliographic applications for herbal medicinal products (AESPG 1999). In Finland, the National Agency of Medicines has defined herbal medicinal products as products traditionally used for medicinal purposes. These products can be derived from plants, animals, bacteria or minerals, and can contain herbal drugs or herbal drug preparations, but not single purified substances (NAM 2002). In the United States, herbal products are mostly registered as dietary supplements since the Food and Drug Administration does not accept bibliographic evidence of effectiveness, but prefers randomized controlled trials as evidence of efficacy (WHO 1998).

The legal framework concerning medicines, and especially directive 2001/83/EC, is well applicable to certain herbal medicinal products (COMMISSION OF THE EUROPEAN COMMUNITIES 2002), but is not as suitable for the so–called "traditional" herbal medicinal products. These are herbal medicinal products that have been in use for a long period in the European Community in the form of oral, external and/or inhalation preparations, and are designed for use without the intervention of a medical practitioner and in accordance with specified daily doses (EUROPEAN PARLIAMENT 2002). The efficiency and safety of these traditional medicinal products are plausible on the basis of long–term use. For these products, applying for a "full"

marketing authorisation would be irrelevant and too expensive for the pharmaceutical industries. Therefore, in order to simplify the legislation for the traditional HMP, the Commission adopted in January 2002 a proposal for a new directive amending directive 2001/83/EC with respect to traditional herbal medicinal products (COMMISSION OF THE EUROPEAN COMMUNITIES 2002). The European Parliament gave a partial agreement to a modified draft in November 2002. This new directive provides a simplified procedure for the registration, and hence marketing, of certain traditional herbal medicinal products. In other words, for the registration of a traditional HMP, it will no longer be necessary to submit a file containing experimental evidence of the pharmacodynamic, pharmacokinetic and toxicological aspects or clinical evidence proving their therapeutic effects and tolerability in man. However, documentation will have to be submitted providing evidence of quality, efficacy and safety on the basis of experience gained from at least 30 years' traditional use (EUROPEAN PARLIAMENT 2002).

The group of herbal products that is not considered as herbal medicinal products does not have to conform the legislation concerning medicines. These products (BARNES 2002) can be sold as food supplements, as long as no medical claim is made, and their control is in accordance with the food legislation (COUNCIL AND EUROPEAN PARLIAMENT 1989). They are therefore outside the scope of this dissertation.

### 2.1.3. Process manufacturing and distribution

Because HMP are medicinal products, their production must follow the Good Manufacturing Practice (GMP), the principles and guidelines of which are laid down in the European directive 91/356/EEC (COMMISSION DIRECTIVE 1991). A detailed guideline for the manufacture of medicinal products in accordance with these principles was also published recently (RULES GOVERNING MEDICINAL PRODUCTS IN THE EUROPEAN UNION 1998). Moreover, good agricultural and collection practice (EMEA 2002) should be followed in the handling of starting material of herbal origin. This

document is a recent guideline based on a document proposed by the European Herb Growers Association (EUROPAM). It addresses the specific concerns of growing, collecting and primary processing of medicinal plants or herbal drugs. HMP manufacture has different features depending on whether the active substance is in the form of a herbal drug or herbal drug preparation. The basic steps in the most frequently used manufacturing processes for capsule, tablet and liquid dosage forms are described in **Figure 1**.

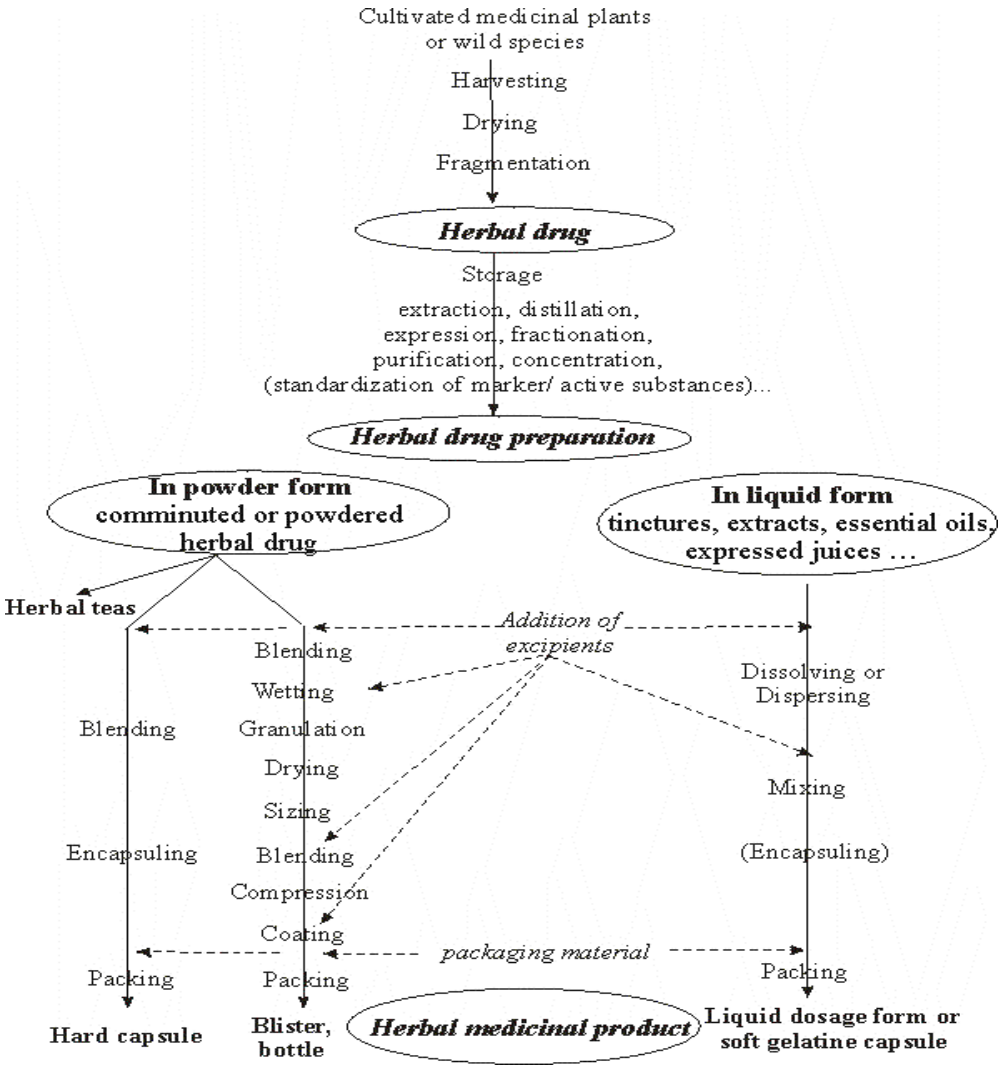


**Figure 1 Basic steps in some of the most widely used manufacturing processes for herbal medicinal products. Manufacturing processes are depicted only for capsule, tablet and liquid forms.**

Herbal drugs are obtained from cultivated or wild plants, and are produced by the following operations: cultivation, harvesting, drying, fragmentation and storage (EUROPEAN PHARMACOPEIA 2002). These operations must follow the guideline on the manufacture of herbal medicinal products (RULES GOVERNING MEDICINAL PRODUCTS IN THE EUROPEAN UNION 1998).

For the production of herbal drug preparations, herbal drugs are subjected to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation (EUROPEAN PHARMACOPEIA 2002). They can be in liquid form (*e.g.,* extracts, tinctures, and essential oils) or in powder form. Powdered herbal drug preparations may be supplied in bulk form or as a sachet, *e.g.* for herbal teas, prepared extemporaneously by the patient. They consist of one or more herbal drugs prepared by means of infusion, decoction or maceration (EUROPEAN PHARMACOPEIA 2002). Infusions are prepared by pouring boiling water over the dried herbal drugs, in chopped form and, after being allowed to draw for 5–10 minutes, strained (BISSET 1994). Decoctions are prepared by pouring cold water on the drug placed in a saucepan, bringing it to the boil, simmering for about twenty minutes, and then sieving the suspension (CHEVALLIER 1996). Maceration consists of pouring water onto the herb and leaving it to stand overnight (CHEVALLIER 1996). HMPs are prepared with herbal drugs or herbal drug preparation as active substances. Excipients are added to give the finished product, as for any other medicinal product. The finished products are usually in solid (*i.e.* tablet or capsule), liquid (*i.e.* oral solutions, drops), or semisolid (*i.e.* gel, unguent or cream) forms. The dosage form of the 84 HMPs marketed in Finland (NAM 2002) are shown in **Figure 2**. In Finland, HMPs are also called semi–medicinal products because they are marketed with a simplified marketing authorisation based on bibliographical application.

The retail sale of herbal medicinal products is usually restricted to pharmacies in Belgium, France, Greece, Ireland, Italy, Luxembourg, Portugal and Spain, but it is also permitted in other outlets – at least for certain HMPs – in Austria, Denmark, Finland, Germany, the Netherlands, Sweden and the United Kingdom (AESPG 1999). In Finland, 44 % of the HMPs are sold only in pharmacies, the other 56% being distributed in pharmacies and in general food stores (NAM 2002).

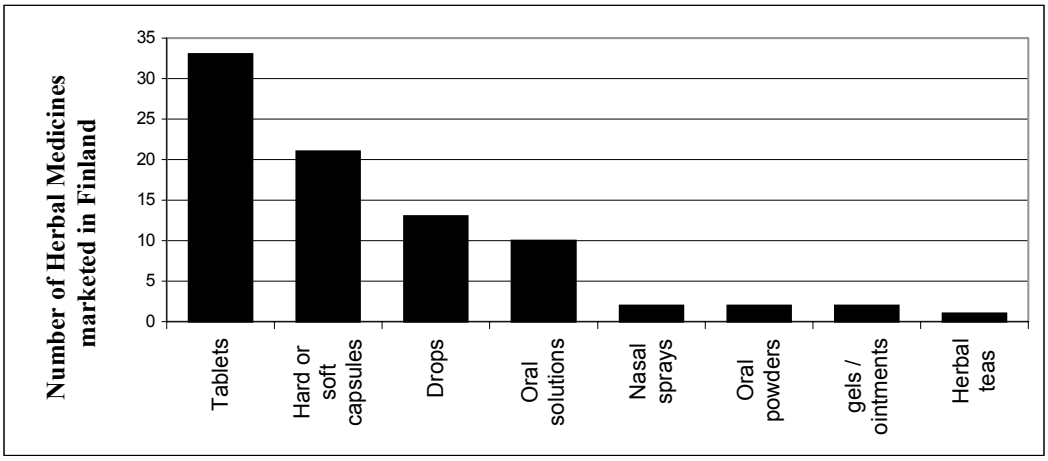


**Figure 2 Distribution of the dosage forms of the 84 herbal medicinal products marketed in Finland.**

## 2.2 Quality control of herbal medicinal products

### 2.2.1. Quality control tests during the manufacturing of herbal medicinal products

In May 1997, a Working Party on herbal medicinal products (HMPWP) was formed at the EMEA to prepare guidance for the mutual recognition in marketing authorisations for herbal medicinal products (EMEA 1999). These guidelines concern regulations, quality, efficacy and safety of the herbal products, and were prepared because there were assessment differences on these topics in the individual European Union countries. So far, two guidelines under the topic of "Quality" have been adopted (EMEA 2001a and 2001b). They aim at providing a uniform set of specifications for herbal drugs and HMPs to support marketing authorisations. The specifications consist of a list of tests and acceptance criteria designed to verify the suitability of the herbal drug preparation or the herbal medicinal product for its intended use (EMEA 2001a). They define the product quality and are therefore useful in ensuring the safety and efficacy of HMPs. The general monographs *Herbal drug* and *Herbal drug preparation* should be used to interpret the specification requirements (EUROPEAN PHARMACOPOEIA 2002).

Specifications applied to herbal drugs usually include (EMEA 2001a): definition, characterization, identification, tests and assay. Identification testing should consist of

three or more of the following tests: macroscopic characters, microscopic characters, chromatographic procedures and chemical reactions. Tests include foreign matter, particle size, water content, inorganic impurities and toxic metals, microbial limits, mycotoxins, pesticides and fumigation agents. Content assays are applied in the case where the constituents with therapeutic activity are known, otherwise an assay of marker substances is required.

Specifications applied to herbal drug preparations include (EMEA 2001a): definition, characterization, identification, tests and assay. Identification testing should be specific and discriminatory with respect to substitutes or adulterants that are likely to occur. A combination of chromatographic tests is recommended for this purpose. The tests include residual solvents, water content, inorganic impurities and toxic metals, microbial limits, mycotoxins, pesticides and fumigation agents. Content assays are required for known constituents with therapeutic activity, as well as herbal drug content assays when possible.

The specifications applied to herbal medicinal products include (EMEA 2001a): description of dosage form, identification, assays (the same as for herbal drug preparation), impurities, microbial limits and specific tests. Specific tests depend on the dosage form. For tablets and hard capsules, dissolution/disintegration, hardness/friability, uniformity of dosage units, water content and microbial limit tests should be performed. For oral liquid: uniformity of dosage units, pH, microbial limits, antimicrobial preservative content, antioxidant preservative content, alcohol content, dissolution, particle size distribution, redispersibility, reconstitution time and water content should be performed among others.

The specifications are, nevertheless, only a part of the global quality scheme to be applied to HMPs. The overall quality control procedure should also include control of the raw materials and excipients, in–process testing, process evaluation and validation, stability testing and testing of batch consistency (EMEA 2001a). Analytical procedures not described in a Pharmacopea should be validated according to the ICH guidelines in order to prove that they are suitable for their intended purpose. The two ICH guidelines are a worldwide basis for both the regulatory authorities and industry (ERMER 2001). They are called ”Validation of analytical methods: Definitions and terminology” (EMEA

1994) and "Validation of analytical procedures: Methodology" (EMEA 1996). They prescribe that identification tests should be validated with respect to their specificity, and that assays should be validated with respect to their accuracy, precision, specificity, linearity and range.

### 2.2.2. Traditional quality control tools

The quality control of bulk herbal drugs and finished products is mainly performed in the quality control laboratory with conventional spectroscopic, chromatographic, titrimetric, or other wet chemical analytical methods. However, these procedures are time–consuming and expensive because they require the use of environmentally unfriendly chemicals and personnel with a relatively high level of training. A sample preparation step is frequently required before performing the analysis of the herbs. Recent sample–preparation techniques for the extraction, clean up, and concentration of analytes from herbal materials include solid–phase microextraction, supercritical–fluid extraction, pressurised–liquid extraction, microwave–assisted extraction, solid–phase extraction, and surfactant–mediated extraction (HUIE 2002). The most widely applied sample–preparation techniques are nevertheless selective solvent extraction, filtration and precipitation (HOSTETTMANN et al. 1998, SNYDER et al. 1997).

The usual qualitative analysis techniques are infrared spectroscopy (IR), ultraviolet/visible (UV/VIS) absorption spectrometry, thin layer chromatography (TLC), and microscopic identification. The most common tools for quantitative analyses are high performance liquid chromatography (HPLC) for a wide variety of compounds, and gas chromatography for volatile organic compounds (SETTLE 1997). HPLC is one of the most widely used analytical tools for qualitative and quantitative measurements. It is also used for the isolation of pure natural compounds from an extract (HOSTETTMAN et al. 1998). An HPLC chromatograph consists of the following devices: solvent reservoir, high–pressure pump, packed column, detector and recorder. The most widely used detectors are UV detectors. The two main modes of HPLC analysis are liquid–solid and liquid–liquid chromatography. They are also called adsorption (normal phase) and partition (reverse phase) modes, respectively. The reverse phase HPLC separates

compounds according to their hydrophobicity, and is widely used for all types of biomolecules.

## 2.3. Near infrared (NIR) spectroscopy

### 2.3.1 Historical and physicochemical basis

The principles of NIR spectroscopy are linked to the development of Fourier Transform spectroscopy (FTS) as early as in the middle of the 19$^{th}$ century. In 1862, Fizeau constructed the first variable path interferometer for wavelength measurement and it is considered to be the ancestor of the actual Fourier interferometers (CONNES 1984). Michelson used this idea to develop his two–beam interferometer (MICHELSON 1890, 1891a, 1891b). Michelson, however, never realised the greatest potentiality of his technique and faced several limitations. For example, the only detector that Michelson used was his own eye (CONNES 1984). Therefore he was not able to record precisely the fringe intensity. Moreover, he did not realise that multiplexing could be used to measure all frequencies simultaneously, and had no concern about the energy throughput advantage. Multiplexing, a technique also used in telephone engineering to send a large number of messages simultaneously, was first applied to the interferometric device in the middle 50´s by Fellgett during his doctoral studies (FELLGETT 1984). He was also the first to derive a spectrum from the interferogram by using Fourier transformation. At approximately the same time, Jacquinot discovered the throughput advantage (JACQUINOT 1984). FTS was seldom used until the advent of computer technology that allowed rapid and economical Fourier transformation of an interferogram into a spectrum. A major step in the acceptance of this technique by industry was the development of an algorithm by Cooley and Tukey in 1965 that significantly increased the speed of the Fourier transformation computation and led to Fast Fourier Transform equipment (PERKINS 1986). Since then, the number of practical applications of the FTIR and FT–NIR spectroscopy has increased dramatically (BLANCO et al. 1998).

The NIR region, discovered by William Herschel in 1800, is situated between the visible and the IR region of the electromagnetic spectrum, and ranges from approximately 780 nm to 2500 nm (DAVIES 1998), corresponding to a frequency range of 4000 $cm^{-1}$ to 12 800 $cm^{-1}$ (EUROPEAN PHARMACOPOEIA 2002) (see **Figure 3)**.

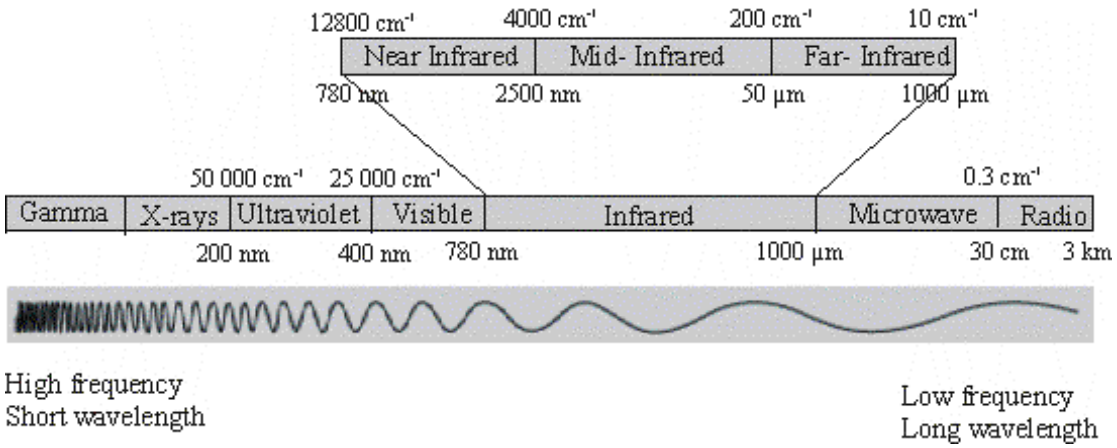


**Figure 3 Electromagnetic spectrum and positioning of the spectral regions.**

At temperatures above absolute zero, all the atoms in molecules are continuously vibrating. Two of the major types of vibration are stretching and bending, as illustrated for a non-linear group $CH_2$ in **Figure 4**.

If the fundamental frequency of a specific vibration, ν, is equal to the frequency of the radiation impinging on the molecules, and if the molecule undergoes a change in its dipole moment during the vibration, then the radiation is absorbed and excites a vibrational transition in the molecule. Molecular vibrations are often described by means of the harmonic oscillator model. The basic assumption is that the shift of an atom is directly proportional to the force opposing the shift. For a harmonic model, only transitions between two consecutive vibrational energy levels are allowed. The energy difference between the two levels is $h\nu$, where ν is the fundamental frequency. In practice, molecular vibrations tend to follow an anharmonic model. This leads to the appearance of additional allowed transitions with energy differences of two, three *etc*., times the fundamental frequency (BLANCO et al. 1998). These higher transitions are called overtones and they are observed beyond the fundamental vibration region in the

near infrared region. In the NIR region also combination vibrations are observed. The most common group frequencies seen in the NIR region are –OH, –CH, –NH, and –SH overtones. Overtone and combination transitions are much less likely than the fundamental transitions (BLANCO and VILLARROYA 2002a). This explains why the intensities of the generally broadly overlapping NIR bands are weaker than the intensities of the fundamental IR bands, by a factor of 10 to 1000 (MACDONALD and PREBBLE 1993).

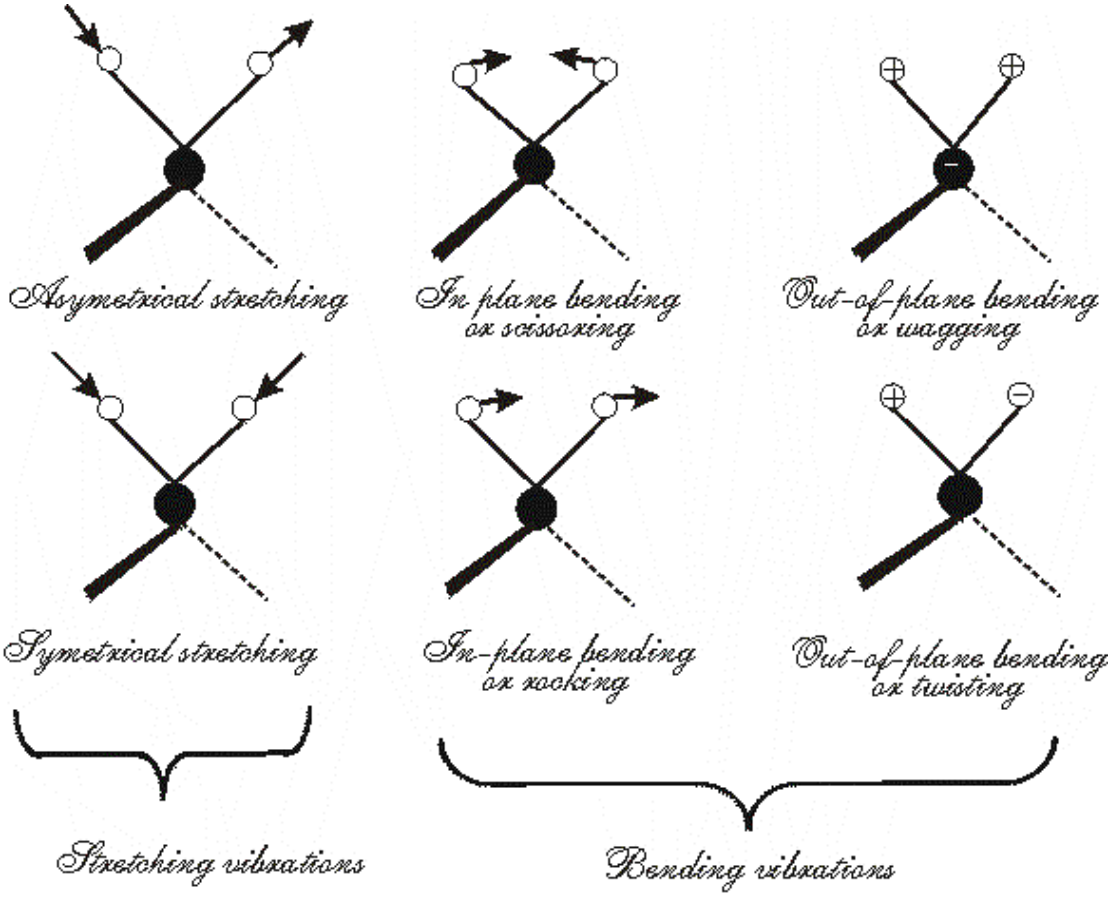


**Figure 4 Main vibrational modes of a nonlinear $CH_2$ group. A + sign indicates a motion from the plane of the page to the reader, and a — sign indicates motion from the plane away from the reader. Modified from SETTLE 1997.**

The weakness of absorption bands in the NIR region is the key to the main advantage of the technique: it allows for longer path lengths (1 mm–1 cm) to be used than in the Mid–IR region (often < 0.1 mm for liquids (SETTLE 1997)). The samples are thus often analysed without sample pretreatment.

### 2.3.2. NIR spectrophotometers

The essential features of NIR spectrophotometers are: a source of radiation, an operating contrivance and a detector. The NIR source produces radiation spanning a large or a narrow range of frequencies in the NIR region. They can be thermal or non–thermal sources. Thermal sources consist of an incandescent filament producing thermal radiation, *e.g.* the Nernst filament, which is a heated ceramic filament containing rare–earth oxides (ATKINS 2001), or quartz–halogen lamps (BOMEM 1994). Non–thermal sources usually consist of light–emitting diodes (LED), laser diodes, or lasers that emit much narrower bands of radiation than thermal sources (OSBORNE et al. 1993).

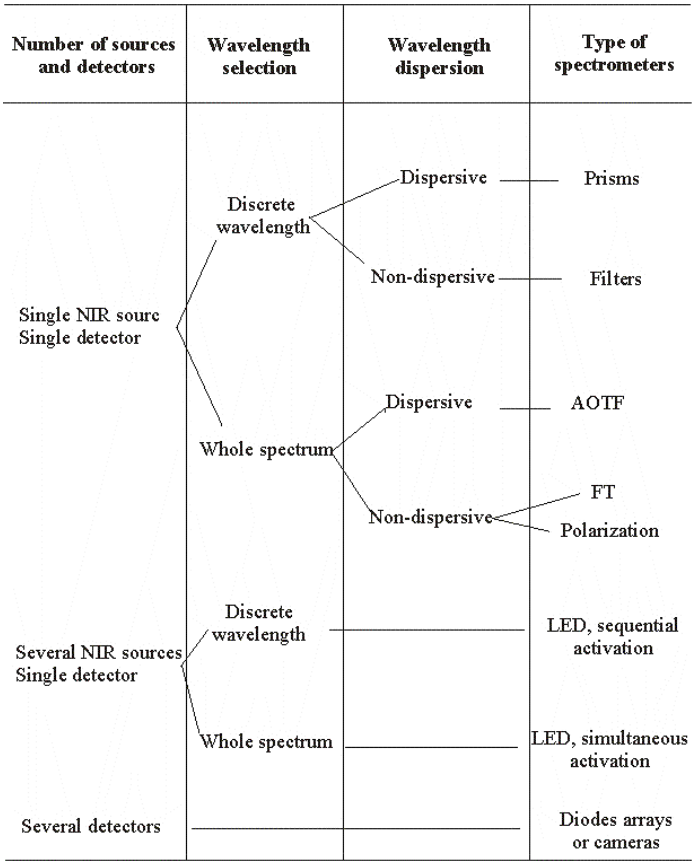


**Table 1 Classification of NIR spectrophotometers as a function of the number of sources and detectors, the wavelength selection device and the type of wavelength dispersion (Modified from BERTRAND 2002).**

NIR spectrophotometer can be divided into three groups (BERTRAND 2002): those with one source and one detector, those with several sources and one detector, and those with several detectors (see **Table 1)**. One of the main parts of the operating contrivance is the wavelength selection device. It may be a discrete absorption device, *i.e.* only a narrow area of the spectral range is measured at once, or a whole spectrum device that measures the

information from several wavelengths simultaneously. The NIR spectrometer optical system can be either a dispersive or non–dispersive device.

The dispersive optical systems or monochromators of single source and single detector spectrometers separate the radiation of different frequencies into different spatial directions. An exit slit is used to select a narrow range of wavenumbers to strike the detector. Prisms were the simplest monochromators used in spectrometers (ATKINS 2001) and are still in use, but they give poor dispersion. They are made from glass or quartz and utilize the variation of the refractive index as a function of the frequency as a separating tool.

Non–dispersive optical systems based on filter devices may include up to 20 filters on a carousel (BERTRAND 2002). This type of instrument is robust and still in use for routine analysis.

Acousto–Optic Tunable Filters (AOTF) have been incorporated in NIR spectrophotometers in recent years. This technique uses acousto–optic diffraction of light in an anisotropic crystalline medium as the separation device (OSBORNE et al. 1993, BERTRAND 1998 and BLANCO and VILLARROYA 2002a). The absence of moving parts in AOTF ensures good wavelength stability, and provides a rugged, cost–effective instrument with a high–signal–to–noise ratio. The resolution of AOTF instruments is approximately 5 nm (SWEAT and WETZEL 2001).

Fourier Transformed instruments are based on interferometers that are widely used in modern spectrometers (OSBORNE et al. 1993). The Fourier Transform technique is based on the use of an interferometer (mostly of the Michelson -type) that is able to detect intensities of several spectral frequencies in a composite signal. The Fourier Transform of the recorded interferogram is the infrared spectrum. The purpose of an interferometer (SMITH 1996 and BERTRAND 2002) is to split a beam of light into two beams and to introduce a difference in their respective travelling distances. The optical path difference is denoted as δ. The interferometer shown in **Figure 5** consists of four arms, one for the source, the second having a moving mirror M2, the third a fixed mirror M1, and the last one is open. The beamsplitter is used to transmit half of the radiation obtained from the source to the moving mirror and to reflect the other half of the

radiation to the fixed mirror. The beamsplitter usually consists of a very thin film of germanium covered on both sides by a potassium bromide (KBr) substrate (PERKINS 1986). The two separated beams respectively strike M1 and M2 and are reflected back to the beamsplitter. They are then recombined and exit the interferometer in the direction of the sample and detector. M2 is moving longitudinally back and forth. When $\delta = 0$, both mirrors are equidistant from the beamsplitter. This is called the "zero path difference" (ZPD). When the interferometer is in the position of ZPD ($\delta = 0$) or when $\delta = n\lambda$, the two recombined beams are in phase with each other and the intensity of the detector signal will thus be maximum.

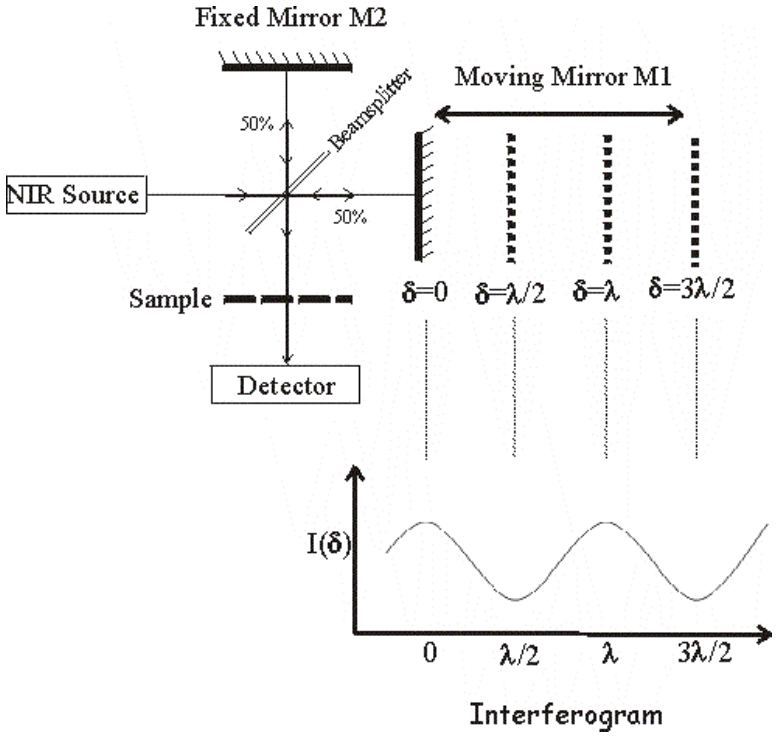


**Figure 5 Principle of a Michelson interferometer and example of an interferogram obtained from a monochromatic source (Adapted from PERKINS 1986).**

These states are called constructive interference. Destructive interferences are obtained when $\delta = (n + 1/2)\lambda$, and in these cases the resulting beam intensity is zero. Intermediate intensities are obtained at intermediate positions of $\delta$. The plot of the intensity versus the optical path difference is called an interferogram. **Figure 5** shows an interferogram obtained with a monochromatic source. When the source is polychromatic (SMITH

1996), radiation of different wavelengths undergoes destructive and constructive interference at different optical path differences. Each wavelength of light leads to an interferogram with a specific path difference, resulting in intensity typical of their frequency that can be measured by the detector. The signal passing through the sample is the sum of each specific interferogram and therefore contains intensity information about all the wavelengths contained in the band passing the sample. The interferogram, an "intensity versus time" function, is then Fourier transformed to obtain the final NIR spectrum, which is an "intensity versus frequency" function. FT–NIR spectrophotometers can be obtained from several suppliers, including Bomem Inc., Bran + Luebbe, Brücker Instrument, Büchi Labotecknik or Perkin Elmer. The newest features on FT instruments include, for example, an imaging -system providing pictures of the samples showing the chemical distribution at the microscopic level.

Polarization or crystal spectrometers are also "whole spectrum" techniques, but they are not as well known as FT techniques (BERTRAND 1998). They are based, as is the case with FT spectrometers, on the interference of two light beams travelling a slightly different distance. A birefringent crystal is used to split the incoming beam into two beams of different polarisation. The difference in the optical path is due to the fact that the two beams have different refraction indices.

The group of spectrometers containing several sources includes non–thermal optical designs, such as LED or laser diodes, and selection of the wavelength is inherent in the narrow emitting range of the source. LED spectrometers (BERTRAND 1998) contain several LEDs, each coupled to a narrow band optical filter. The LEDs are activated one after the other in a sequence and, because all the measurements are focused on the same channel, only one detector is needed. LEDs can also be activated simultaneously and the instrument functions as a multiwavelength device (BERTRAND 2002).

The last group of spectrophotometers contains several detectors and they are called multichannel spectrometers. The operating principle is based on diode arrays or cameras that can measure many wavelengths simultaneously (BERTRAND 1998). This type of

instrument is available from Büchi Labotecknik, Perten Instruments or Multichannel Instruments. However, single detector instruments are normally used.

Concerning detector technology, silicon–based photodetectors are recommended for the short–wavelength infrared range (700–1000 nm or 14286–10000 $cm^{-1}$). For lower energies and longer wavelengths (1100–2500 nm or 9090–4000 $cm^{-1}$), semiconductors such as lead sulphide (PbS), indium gallium arsenide (InGaAs) or indium arsenide (InAs) can be used as detectors (USP 2002, BOMEM 1994).

### 2.3.3. Advantages and disadvantages of Fourier Transform spectrometric techniques compared to traditional analytical methods

The advantages of FT transform techniques over dispersive instruments have resulted in almost total replacement of the dispersive instruments in spectroscopy.

**The Multiplex or Fellgett Advantage:** In a dispersive spectrometer, wavenumbers are observed sequentially. In an FT–IR and FT–NIR spectrometer, all the wavenumbers of light are observed simultaneously. Therefore, when spectra are collected under identical conditions the signal–to–noise (S/N) ratio of the FT–IR spectrum will be greater than that of the dispersive IR spectrum (HILL et al. 1997).

**The Throughput or Jacquinot Advantage:** In FT–IR instruments there is no need to limit the beam width in order to obtain an adequate resolution. In fact, a circular optical aperture is used in FT instruments, and the beam area is 75 to 100 times larger than the slit area of dispersive instruments (SETTLE 1997). As a consequence, there is an advantage of increased beam intensity going through the sample and therefore a much higher throughput with a FT–IR than with a dispersive instrument (JACQUINOT 1984).

**Wavenumber accuracy or Conne´s advantage:** In FT instruments, *e.g.* in FT–IR Bomem spectrometers, a wavelength accuracy of 0.04 $cm^{-1}$ can be obtained (BOMEM FT–IR REFERENCE MANUAL 2001), which is much higher than the traditional wavelength accuracy obtained with dispersive instruments (only about 1–5 $cm^{-1}$) (SETTLE 1997). This difference is due to the fact that the frequency-stabilised helium–neon laser is used as internal wavelength standard. Therefore the frequency precision is

determined by the frequency stability of the laser, which leads to precise and reproducible wavelengths (PERKINS 1987, FROST et al. 1993).

**High and constant resolution:** Spectral resolution is a measure of how well a spectrometer can distinguish closely spaced spectral features. Filter instruments cannot offer high resolution because, in dispersive instruments, resolution decreases as lower frequencies are scanned. In FT–IR, the resolution depends on the optical path difference (OPD) that can be achieved. Thus it is constant across the scanning range (WILKS 1986, PERKINS 1987).

**Practical and powerful data station:** FTIR or FTNIR instruments are normally equipped with a powerful computer capable of carrying out the Fast Fourier Transformation needed to obtain the spectrum. Additionally, the instrument computer uses software that can perform data processing (SETTLE 1997) such as baseline correction, smoothing, derivatisation or library searching, and therefore improve data information.

The most attractive advantage of FT–NIR spectroscopy over traditional analytical tools and any other spectroscopic method is probably that the measurements are non–destructive, and non–invasive, and that it is possible to use solid samples without pre–treatment and therefore without solvents. This leads to a large increase in the analysis speed compared to traditional analysis methods, and decreases the risk of errors due to weighing and dilution operations (TRAFFORD et al. 1999, HAN and FAULKNER 1996).

The variety in sampling technologies is another attractive feature of NIR spectroscopy. Several accessories are adaptable to a number of situations, and can be used with different scanning modes. For instance, fibre optic probes were used already ten years ago for real–time analysis (WILLIAMS and MAC PETERS 1991) and are nowadays often used in the diffuse reflectance mode for routine qualitative and quantitative applications (BLANCO et al. 1999a, BLANCO et al. 2000a and 2000b). Diffuse reflectance is also easily used for off-line analysis for samples contained in simple glass vials (WARGO and DRENNEN 1996, FRAKE et al. 1998). The transmittance mode is more and more widely used for recording spectra from intact

tablets (SCHILLING et al. 1996). It gives results with a better repeatability and a smaller prediction error than reflectance measurements (CORTI et al. 1999, THOSAR et al. 2001). The transflectance mode, which is a variant of the diffuse reflectance mode, has also been investigated recently. In this case, incident light crosses the sample, is reflected by a reflectance material such as stainless steel or PTFE (Polytetrafluoroethylene) located on the opposite side, and travels back through the sample before reaching the detector (BLANCO and ROMERO 2002b).

An important property of the NIR signal is that, because it depends on both the chemical composition and the physical properties of the sample, analysis of these two characteristics can be performed by the same technique (CHEN and SØRENSEN 2000). NIR also has the potential to be used for developing on–line methods, leading to real–time control systems (RANTANEN et al. 2000b). This advantage can be well used in the pharmaceutical or chemical industry to give real–time information about processes.

On the other hand, there are three main disadvantages of NIR spectroscopy over traditional techniques. First, the development of a NIR method is time–consuming because it is necessary to analyse several representative samples by a time–consuming reference analysis method (HPLC or Karl Fisher titration for example). Secondly, NIR methods lack robustness: calibrations often need to be updated, *e.g.* when a sample is provided by a new supplier, or when the manufacturing process of the sample is modified (CANDOLFI and MASSARD 2001). This is especially problematic with raw material whose quality may vary from time to time leading to false identification of the material. Furthermore, NIR spectroscopy is not very sensitive and it can usually be satisfactorily applied to major components (BLANCO and VILLARROYA 2002a) but not to impurities or low–dose substances.

Other minor disadvantages are the following: First, in contrast to IR spectra, NIR raw spectra exhibit low specificity. They do not show clear peaks characteristic to a specific compound of interest. Thus, extensive statistical calculations are required to extract useful qualitative or quantitative information (LOWRY et al. 2000). Second, NIR spectroscopy methods are developed using the reference analysis results of the calibration samples. Thus, the accuracy of the NIR method cannot be better than the accuracy of the

reference method. Furthermore, the transferability of NIR methods from one instrument to another is limited due to the frequent need for updating calibrations after routine maintenance or repair of the instrument (WANG et al. 1998). Finally, the absence of NIR training in pharmacy schools is one of the major obstacles to the acceptance of NIR spectroscopy by pharmacists. The specialised vocabulary used in the chemometrics world makes things even less accessible for pharmacists.

### 2.3.4. Use of chemometrics

Chemometrics is a chemical discipline that utilizes mathematics and statistics to design optimal measurement procedures and experiments and to provide maximum relevant chemical information by analysing chemical data (MASSART et al 1988). Traditional applications of chemometrics often involve data pre–processing for enhancing analytical measurements to obtain chemically or physically relevant information from the sample (LAVINE 1998) and to reduce the irrelevant variability that arises from the effect of instrument changes over time or physical phenomena, such as temperature, or scattering.

Of the number of existing signal–preprocessing techniques, only the most widely used mathematical tools will be described here. In the reflectance mode, NIR spectra are subjected to large baseline shifts introduced by the spectrometer or sample especially in the case of solid powdered samples with a large particle size distribution, because scattering of the light is strong (ISAKSSON and NAES 1988, CANDOLFI et al. 1999a). Baseline effects can also be due to a number of reasons such as detector drift, changing environmental conditions *e.g.* temperature and humidity, and sampling accessories. One of the best methods for removing baseline effects is to use derivative spectra. A constant background can be removed by transforming the original spectra into first–derivative spectra, while the linear background can be removed by taking second–derivative spectra (CANDOLFI et al. 1999a). The second derivative is more often used because it increases the selectivity of interesting bands (STORDRANGE et al. 2002) and thus simplifies the data interpretation. However, as derivation amplifies the spectral noise, it is necessary to smooth the data before derivation (CANDOLFI et al. 1999a). The most widely used

differentiation method is the Savitzky and Golay algorithm (SAVITZKY and GOLAY 1964), which combines smoothing and differentiation and thus removes the noise.

The use of Standard Normal Variate (SNV) transformation leads to the removal of the major effects of light scattering and particle size. The SNV algorithm normalises each spectrum by dividing the difference between the transmittance and average transmittance by the standard deviation of transmittance (CHAMINADE et al. 1998). De–trending is also a baseline correction method. It removes offset and curves linearity, which often occurs in the case of powdered, densely packed samples. The baseline is modelled as a function of wavelength and subtracted from the spectrum. Normally, de–trending is carried out in combination with SNV transformation (CANDOLFI et al. 1999a).

Multiplicative signal correction (MSC) can be used (LAVINE 1998) to resolve the problem of a varying background due to differences in optical path length and to compensate for different scatter and particle sizes from sample to sample. The principle is that MSC establishes a linear regression between spectral variables and the average spectrum. The slope and offset values of the regression spectrum are then removed from the original spectrum in order to give a corrected spectrum (ISAKSSON and NAES 1988, CHAMINADE et al. 1998).

Multivariate calibration remains, by far, the fastest growing area of chemometrics (LAVINE 1998). This procedure is used to relate the analyte concentration or the measured value of a physical or chemical property to a measured response.

PLS is now dominating the practice of multivariate calibration, because of the quality of the calibration models produced and the ease of their implementation (LAVINE 1998). This algorithm was developed by WOLD and MARTENS in the beginning of the 80´s (TENENHAUS 1998, ERIKSSON et al. 2000). Industrial problems can frequently be described on the basis of an input/output system: **X** are the input variables, and **Y** the output variables being observed. The PLS regression is a linear regression technique that can be used to understand and explain the relationship between **X** and **Y** (TENENHAUS 1998).

The PLS regression principle (WOLD et al. 2001) is to find new variables to estimate the latent or underlying **X** variables. The new variables are called **X**–scores and

denoted by **T**. The **X**–scores are used to model **X** and to predict **Y** (response variables). The **X**–scores are orthogonal and restricted in number and are linear combinations of the original variables **X** with the coefficients, or weights **W**. The relationship between the matrices **X, Y, T** and **W** are shown in **Equations 1–5** and in **Figure 6**. The explanation of the abbreviations are as follow: **X** is the (**N** x **K**) matrix of the predictor variables, **Y** is the (**N** x **M**) matrix of the response variables, **N** is the number of observations, **k** is the index of the **X** variables, **W** is the **X** weight matrix, **W*** is the matrix of the **X** weights transformed to be independent between components, **A** is the number of components in the PLS model, **C'** is the transposed Y weight matrix, **T** is the **X**–score matrix (**N** x **A**), **U** is the **Y**–score matrix (**N** x **A**), **P'** is the transposed loading matrix, **E** is the matrix of the **X** residuals, and **F** is the matrix of the **Y** residuals.

The notation employs uppercases for the matrix (*e.g.*, **X**), and lowercases for their corresponding values (*e.g.* $\mathbf{x}_a$).

$$\mathbf{T = XW^*} \qquad \textbf{(1)}$$

**X**–scores, multiplied by the loadings **P**, are a good estimation of **X** providing that the residues **E** are small.

$$\mathbf{X = TP' + E} \qquad \textbf{(2)}$$

**Y**–scores, multiplied by the weights **C**, are a good estimation of **Y** providing that the residues **G** are small.

$$\mathbf{Y = UC' + G} \qquad \textbf{(3)}$$

The **X**–scores are good predictors of **Y**, providing that the residues **F** are small.

$$\mathbf{Y = TC' + F} \qquad \textbf{(4)}$$

Therefore the summarising equation is in the form of a multiple regression, with **XW*** as the PLS regression coefficients:

$$\mathbf{Y = XW^*C' + F} \qquad \textbf{(5)}$$

If the predictive power of this regression is too weak when only one component is calculated, then a second component is calculated (TENENHAUS 1998). This iterative procedure can be continued to calculate as many components or factors as are predictively significant.

The geometric interpretation (**Figure 6**) of the PLS model is a projection of the **K**–dimensional **X** matrix down on an **A**–dimensional plane (**A**<**K**). Each plane has a direction corresponding to a PLS component, **A**. The direction of each plane is described by its slope, $\mathbf{p}_{ak}$ (loadings). Each point projected on the plane is characterised by its co–ordinates, also called scores **t.** The plane satisfactorily approximates **X** and, at the same

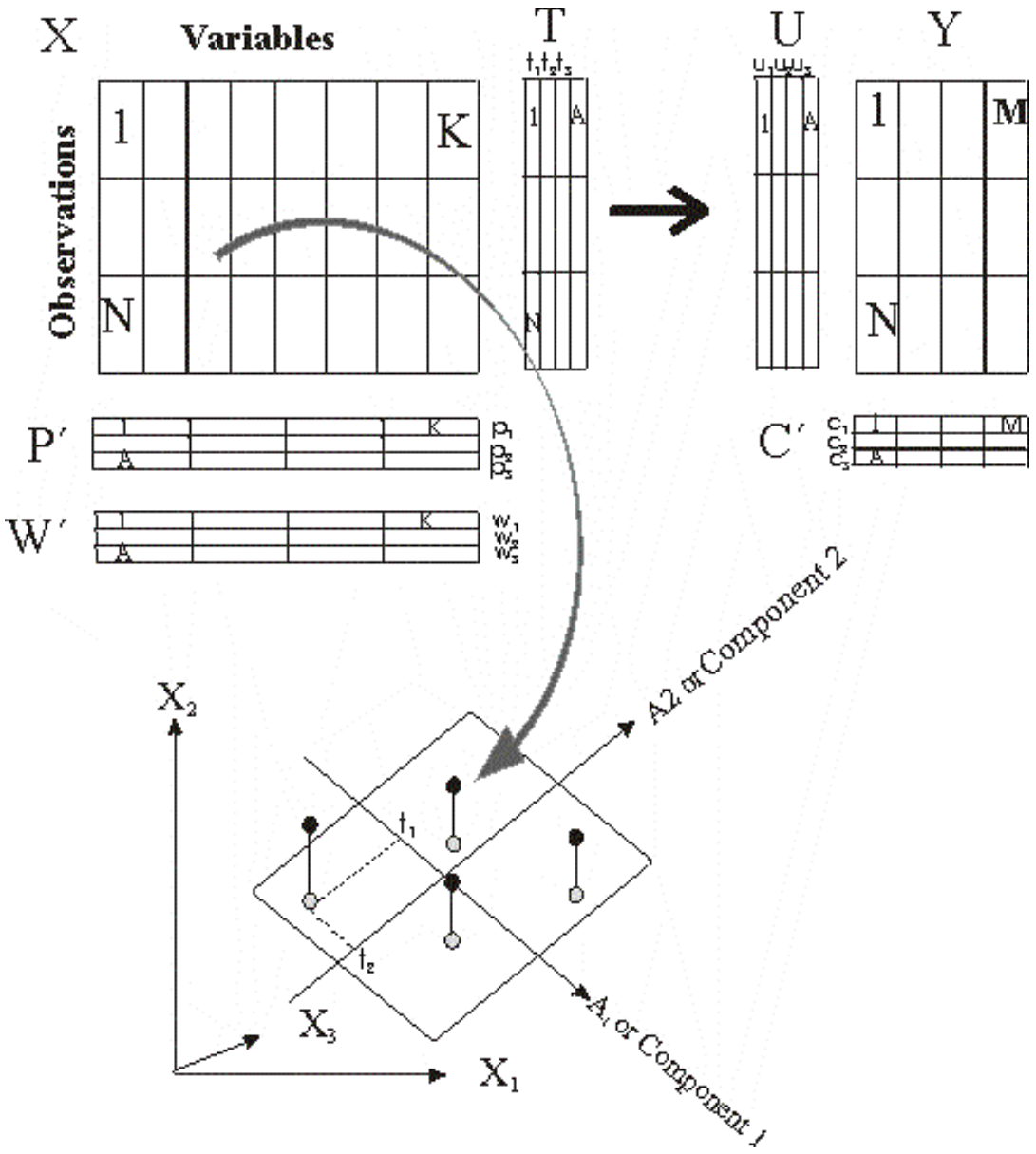


**Figure 6 Matrix and geometric representation of a PLS model. The geometric representation exemplifies the case of an X matrix projected onto a two–dimensional plane (two-component model). Adapted from WOLD et al. 2001.**

time, the positions of the projected data points on this plane (scores **t**), are related to the responses, **Y** (WOLD et al. 2001).

The number of factors to be retained can be evaluated in several ways, often based on cross–validation. The principle of cross–validation is to remove one sample or a group of samples from the calibration set and then to calculate the model with the remaining samples (HAALAND and THOMAS 1988, TENENHAUS 1998, WOLD et al. 2001). The model will be different depending on which sample is removed and on the number of factors included. The removed sample is predicted by each model that includes a successive number of factors. The cross–validation is repeated by omitting another sample, and so on, until each sample from the calibration set has been removed once. Then, the differences between the actual and predicted Y values are calculated for the deleted data. The sum of squares of these differences (Predictive Residual Sum of Squares or PRESS) gives an estimation of the predictive ability of the model. The number of significant PLS components is usually calculated to be the minimum number for which the PRESS value is not significantly different from the lowest PRESS value, as described by HAALAND and THOMAS (1988). If the number of factors (or components) is too high, the risk of overfitting the model is increased. An overfitted model has little or no predictive power (WOLD et al. 2001), because it includes factors that are not related to the constituent of interest but instead to the noise.

Principal Component Regression is another widely used multivariate calibration method, dominated by the use of a compression technique, Principal Component Analysis (PCA). PCA also allows data visualisation by means of data dimensionality reduction, (DASZYKOWSKI et al. 2003).

PCA is the most popular linear projection method. It projects multidimensional data onto a few directions called principal components (PCs). PCs are a linear combination of the original variables that describe the data variance (WOLD 1987). They explain successively decreasing amounts of variance in the matrix X (STORDRANGE et al. 2002). Thus, the first PC is the direction that best approximates (minimised least square error) the original data (DASZYKOWSKI et al. 2003) and explains the maximum variance of the data. The second PC improves the approximation, and so on for the further PCs. The number of extracted components equals the number of rows in the

original data matrix, but all components with small eigenvalues are considered as data noise and are eliminated.

There are several other important chemometrics methods, such as multiple linear regression (MLR) which is another widely used multivariate calibration method (BLANCO et al. 1998), quantitative structure activity relationship (QSAR), pattern recognition, multivariate process modelling (WOLD and SJÖSTRÖM 1998) or artificial neural systems (ZURADA 1992).

### 2.3.5. Applications of NIR spectroscopy in pharmaceutical technologies and in herbal medicinal products

In addition to applications in the food (IWAMOTO and KAWANO 1992, BENSON 1996, REEVES and ZAPF 1999), textile (CLEVE et al. 2000), biological (SOWA et al. 1999, SASIC and OZAKI 2001), petroleum (PARISI et al. 1990) and chemical industries (WILLIAMS and MAC PETERS 1991), pharmaceutical technology is one of the main application fields for NIR spectroscopy. The current applications concern a large part of the pharmaceutical operations shown in **Figure 1**.

In the initial stage of pharmaceutical processes, NIR is used for the identification of raw materials: active substances (MONFRE and BRIMMER 1996, GERHÄUSSER and KOVAR. 1997) and excipients (SVENSSON et al. 1997, EBUBE et al. 1999, KRÄMER and EBEL 2000, CANDOLFI et al. 1999b). The physical properties of the raw material are also determined by near infrared spectroscopy, *e.g.* the particle size of drugs or excipients (O´NEIL et al. 1998, FRAKE et al. 1998). In the following step of the manufacturing process, the monitoring of blending processes can be performed successfully with NIR (MACDONALD and PREBBLE 1993, SEKULIC et al. 1996, HAILEY et al. 1996). NIR can provide real–time information about the blending, which is often not the case with traditional analysis methods. When tablets are manufactured, wet granulation is often the next process phase. NIR has frequently been used to monitor the granulation process, for example, to quantify a drug during the different steps of the granulation process (HAN et al. 1996), to measure the particle size (RANTANEN et al. 1998), or to follow the moisture content during granulation (RANTANEN et al. 2000a

and 2000b). After granulation, the tablets are sometimes coated. Here, as well, NIR can be applied *e.g.* to monitor the film coating process (ANDERSSON 1999) and to perform a final identification test of the active compound in the final dosage form (DEMPSTER et al. 1995). The identification of active substances through blister packaging can also be performed *e.g.* for discriminating between active tablets and placebos during clinical trials (MACDONALD and PREBBLE 1993). One of the most important steps in tablet quality control is the quantitative assay for the active substance. Currently, mostly other spectroscopy methods, and chromatographic, titrimetric and wet chemical methods, are used to analyse finished products. However, these tools are destructive for the sample, while NIR is not. Quantitative measurements are nevertheless not the major application for NIR spectroscopy in the pharmaceutical industry, probably because it requires more extensive work than qualitative methods, and also because of the lack (nearly fulfilled nowadays!) of adapted guidelines for the validation of such procedures (CIURCSAK 1998). However, a large number of publications reflect the advantages of the NIR assay of active substances in semi–finished or finished products such as granules, cores or tablets (HAN and FAULKNER 1996, BERTHA–SOMODI et al. 1996, TRAFFORD et al. 1999, BLANCO 1999a, 1999b, 2000a and 2000b, RAMIREZ et al. 2001). Quantitative measurements for the determination of moisture in finished products are also a popular application of NIR spectroscopy (MACDONALD and PREBBLE 1993, LAST and PREBBLE 1993). Very few papers have described the use of NIR spectroscopy to monitor the quality of a pharmaceutical product during the different steps of its manufacture (HAN and FAULKNER 1996, BLANCO et al. 2000a).

Concerning herbal medicinal applications, NIR spectroscopy has, during the past few years, become a useful tool for the non–destructive analysis of plant species and herbal products. An electronic search using the SciFinder Scholar (American Chemical Society, version 2002) database showed that there are approximately twenty applications in this field in the literature. In fact NIR spectroscopy has primarily been used to identify or classify herbal drugs and herbal drug preparations, but seldom to control the quality of herbal medicinal products. Literature applications report significant improvements in terms of speed and flexibility of NIR analysis compared to the conventional or traditional

**Table 2 NIR applications for herbal drugs, herbal drug preparations and herbal medicinal products**

| Type of application | Description of the application | Literature |
|---|---|---|
| **NIR Classification of herbal drugs** | Screening and authentication of Chinese herbal drugs (reflectance) | LI et al. 2001 |
| | Differentiation of unprepared crude seeds of fennelseed and hemlock. (reflectance). | KUDO et al. 1997 |
| | Authentication of coffee bean variety (diffuse reflectance). | DOWNEY and BOUSSION 1996 |
| **NIR Classification of herbal drug preparations** | Classification of cultivation area of ginseng (reflectance). | WOO et al. 2002 |
| | Discrimination of Astragali Radix, Ganoderma, and Smilacis Rhizoma according to geographical origin (reflectance). | WOO et al. 1999a |
| | Fast identification of very similar species: Ginseng Radix, Austragali Radix, and Smilacis Rhizoma (reflectance) | WOO et al. 1999b |
| | Classification of olive oils as a function of their geographical origin (transmittance). | BERTRAN et al. 2000 |
| | Differentiation of essential oils as a function of their type, source and batch.(reflectance). | WATT 1999 |
| **NIR Quantification of substances in herbal drugs or in herbal drug preparation** | Quantification of hyperforin and I3, II8–biapigenin in St. John's wort extracts (reflectance). | RAGER et al. 2002 |
| | Quantitation of echinacoside in Echinacea roots (reflectance) | SCHULTZ et al. 2002 |
| | Analysis of fibre content in flax stems (reflectance). | BARTON et al. 2002 |
| | Determination of nootkatone and aldehyde contentsin citrus oils (transflectance) | STEUER et al. 2001 |
| | Determination of glycyrrhizin in radix Glycyrrhizae and ginsenosides in radix Notoginseng (reflectance). | CHEN and SØRENSEN 2000 |
| | Determination of ginsenosides in American Ginseng (reflectance). | REN and CHEN 1999 |
| | Quantification of alkaloids and phenolic substances in tea leaves (diffuse reflectance). | SCHULTZ et al. 1999 |
| **NIR quantification of substances in herbal medicinal products** | Determination of Sennoside content directly from granulates (diffuse reflectance). | MOLT et al. 1997 |
| | Quantification of caffeine in milled tablets | ALLEN et al. 1974 |

analysis of drugs (RAGER et al 2002, SCHULTZ et al. 2002). However, none of these applications were validated according to pharmaceutical regulations. These methods can therefore not be used as such in the pharmaceutical industries. **Table 2** gives a list of randomly selected typical literature applications concerning the NIR analysis of herbal drugs, herbal drug preparations and herbal medicinal products, and does not attempt to be complete.

### 2.3.6. Regulatory requirements for the use of NIR spectroscopy in pharmaceutical industries

Although there are still no monographs in which the use of near infrared spectroscopy is mandatory, the regulations concerning NIR spectroscopy are nowadays better adapted to its practical use in the industry. The first sign of the acceptance of NIR spectroscopy was the publication of the European Pharmacopoeia monograph on NIR (EUROPEAN PHARMACOPOEIA 1997) describing the control of instrument performance and the establishment of a spectral reference library for qualitative purposes. Then appeared the revision in 1998 of the USP general chapter of Spectrophotometry and Light–scattering (USP 1998), and the publication of a guideline for the qualification of NIR instruments (PHARMACOPEIAL FORUM 1998).

However, the European Pharmacopoeia monograph on NIR (EUROPEAN PHARMACOPOEIA 1997) does not give extended details on the way to develop and validate a qualitative application or any information about the development or validation of quantitative methods. In fact, up until now, quantitative NIR methods were validated according to the ICH guidelines Q2A and Q2B (EMEA 1994, EMEA 1996) which, nevertheless, address traditional method validation requirements more than NIR method validation. The reason for this is that these guidelines were planned for separative techniques such as high–performance liquid chromatography or gas chromatography, and are not as suitable for non–separative procedures such as direct spectroscopic methods. The lack of specific validation guidelines led several authors to attempt to adapt the ICH guidelines to the purposes of their own NIR quantitative application. (PLUGGE and VAN DER VLIES 1993, FORBES et al. 1999, TRAFFORD et al. 1999, BLANCO 2000a

and 2000b, MARK et al. 2001, MOFFAT et al. 2000). The Pharmaceutical Analytical Sciences Group (PASG) even proposed its own guideline (BROAD et al. 2002). This guideline covers the design, development and validation of NIR methods, whilst remaining complementary to ICH Q2A and Q2B. In the meanwhile, the EMEA published draft guideline for the use of near infrared spectroscopy in the pharmaceutical industries (EMEA 2001c). This document aims at regulating the validation processes of qualitative and quantitative NIR methods, and provides some recommendations about the qualification of NIR spectrometers. The draft is under modification and will greatly contribute to the acceptance of this technique as an analysis tool in the European industries. After acceptance of this draft, the pharmaceutical industries will have the regulations required to implement NIR spectroscopy as a routine analysis tool in their laboratories.

## 3. AIMS OF THE STUDY

Near infrared spectroscopy has often been applied for herbal drugs but, paradoxically, there are no published studies describing the application of pharmaceutical guidelines to validate NIR methods for the quality control of herbal medicinal remedies. Thus, the main target of this study was to demonstrate the applicability of NIR spectroscopy to monitor the quality of herbal medicinal products according to pharmaceutical regulations.

In this context, the specific aims were:

1) To develop new quality control applications for NIR spectroscopy in the joint domain of the pharmaceutical and the herbal drug industries.
2) To investigate the use of chemometrics tools for the optimization of NIR method development.
3) To study the factors affecting the robustness of NIR analysis methods.
4) To compare the advantages and disadvantages of NIR spectroscopy with one of the most widely used traditional analytical methods, high performance liquid chromatography or HPLC.
5) To investigate several ways of adapting in–force and draft guidelines to the validation of NIR methods.

# 4. EXPERIMENTAL

## 4.1. Material

### 4.1.1. Plant material

The plant material (**II, III**) consisted of 18 batches of *Echinacea purpurea*, 23 batches of *Echinacea angustifolia*, 10 batches of *Echinacea pallida* and 2 batches of *Parthenium integrifolium*. The samples were obtained in the form of dried roots from Heinrich Klenk (Germany), Martin Bauer (Germany), Frantsilan Yrttitila (Finland), Alfred Galke (Germany), Richters (Ontario, Canada), Bioforce, (Switzerland), Ian and Linda Grossard´s farm (Manitoba, Canada), Medicinal herb farm (Kelowna, Canada), Nutrilite farm (California, USA) and Teardrop farm (Kansas, USA).

### 4.1.2. Plastic raw material

The plastic films (**I, V**) were collected over a period of two years from a large number of batches, several suppliers and several countries. The suppliers were: Perlen Converting AG (Switzerland), Aerni–Leuch AG (Switzerland), Paskel International SA (Mexico), Solvin SA (Belgium) and Riflex Film AB (Sweden). The different types of sample differed with respect to their polymeric composition, colour and thickness:

**A type:** Clear transparent rigid 250 μm PVC (Poly(vinyl chloride)) film.

**At type:** Identical to the A type except for the thickness of the PVC layer (other than 250 μm).

**B type:** Clear transparent rigid 250 μm PVC film coated with 40 g $m^{-2}$ of PVDC (Poly (vinylidene dichloride)), with a nominal thickness of 273 μm.

**Bt type:** Identical to the B type except for the thickness of the PVC layer and / or the amount of PVDC coating (nominal thickness other than 273 μm).

**C type:** Clear transparent rigid 250 μm PVC films coated with 5 g $m^{-2}$ of TE (Thermoelast®) and 90 g $m^{-2}$ of PVDC, with a nominal thickness of 308 μm.

**Ct type:** Identical to the C type except for the thickness of the PVC layer and / or the amount of TE and PVDC coating (nominal thickness other than 308 μm).
**D type**: Rigid plastic films of a different polymer composition than the A, B or C type samples. For example, PP (Polypropylene) or PVC/PE (polyethylene)/PVDC films were included in this group. The films were of several different thickness and colour.
A total of 193 batches of plastic film were collected.

### 4.1.3. Finished herbal medicinal product

The pharmaceutical product studied was the commercially available, 170 mg caffeine tablet produced by Pharmia Oy, Finland (**IV**). The active principle is caffeine, or 1,3,7–trimethylxanthine, a purine alkaloid. This alkaloid occurs naturally in tea leaves, coffee beans, cocoa beans and maté leaves. The product is not marketed as a herbal medicinal product in Finland because its pharmacological activity is based on a purified single substance (NAM 2002), but it is marketed as a medicinal product with a "full" marketing authorization. However, this product is a herbal medicinal product according to the European directive (COUNCIL AND EUROPEAN PARLIAMENT 2001) because its pharmacological activity is based on an alkaloid from vegetal sources.

The tablets contained 58.82% m/m anhydrous caffeine, and an excipient mass consisting of α-lactose monohydrate, cellulose dry powder, starch and lubricants. Twenty–two production batches were supplied by Pharmia Oy, and eleven laboratory–made batches were prepared in order to broaden the caffeine concentration range. These batches were obtained by overdosing or underdosing the samples by adding caffeine or excipient mass during the mixing process prior to tableting. The caffeine concentration of the laboratory batches covered the range from 0 (excipient tablets) to 100% m/m (pure caffeine tablets). Two other laboratory batches were prepared using the same formula as for the production batches, except that caffeine was replaced by two other alkaloids with rather similar structures to caffeine: theobromine (3,7–dimethylxanthine) or theophylline (1,3–dimethylxanthine).

### 4.1.4. Computer programs (I–V)

The spectrometer was equipped with the software package from ABB Bomem, Inc, (Quebec, Canada) including Grams 32 version 4.04 for spectral acquisition, PLSPlus/ IQ version 3.03 for spectral processing and chemometrics analysis, and AIRS (Advance Infrared Software) version 1.54 for routine qualitative analysis. Statistical calculations and PCA was performed using Systat version 9 and 10 (SPSS, Chicago, IL). The HPLC method for Echinacea analysis (**II, III**) was developed using a computer–assisted optimisation programme, Dry Lab 2000 (LC Resources, Walnut Creek, California).

### 4.2. Methods

#### 4.2.1. High performance liquid chromatography (HPLC) (II, III, V)

The analytical equipment consisted of a Perkin Elmer Series 200LC pump and autosampler with 200 µl loop (Norwalk, CT, USA), a Perkin Elmer LC 235 C Diode Array detection system (Norwalk, CT, USA), and a PE Nelson 600 Series link (Norwalk, CT, USA).

For the analysis of *Echinacea* species (**II, III**), the samples were analysed using an RP–18 analytical column (Luna 3µ $C_{18}$, 100x4.60 mm i.d. Phenomenex, USA) with a fitted security guard cartridge ($C_{18}$ ODS (Octadecyl) 3x4 mm, Phenomenex, USA). The mobile phase flow rate was 1.0 ml/min for a total run time of 20 minutes, with UV detection at 255 nm. The mobile phase consisted of A = water +0.1 % ortho–phosphoric acid 85 % and B = acetonitrile. The gradient profile was developed using the computer–assisted optimization program Dry Lab 2000. Identification of peaks was performed by comparison with reference compound retention times and UV spectra recorded by photodiode array detection.

For determination of the caffeine content (**IV**), the isocratic reversed–phase HPLC method used a methanol:$NaH_2PO_4$ 0.05M (30:70) mobile phase, a Lichrocart 125–4, Lichrospher 5µm RP–18 Merck column, a flow rate of 1 ml/min, and a run time of 12 minutes. The UV absorbance was measured at 275 nm.

### 4.2.2. Characterization of the herbal drugs (**II, III**).

The moisture content of the herbal drugs, milled to powder with a grinder (Ika Labortechnik, type A10, Staufen, Germany), was determined using an infrared dryer (Sartorius Thermocontrol YTCOL, Sartorius GmbH, Göttingen, Germany). This dried the samples at 110°C until the loss in weight was less than 0.1% during 50 seconds. The particle size distribution of the samples was also evaluated by vibrational sieve analysis (Analysette, Fritsch, Germany).

### 4.2.3. Near infrared reflectance spectroscopy (I, III–V)

The spectra were recorded on a FT–NIR MB160 spectrometer (ABB Bomem, Inc, Quebec, Canada) fitted with a Powder Samplir reflectance accessory, a Quartz–Halogen lamp and a cooled Indium Arsenide detector. The reflectance standard package was supplied by Labsphere Inc. (North Sutton, New Hampshire, USA). The diffuse reflectance mode was used for the three types of sample (powder, plastic, tablet). Each spectrum was an average of 60 or 64 interferograms co–added at 16 $cm^{-1}$ resolution, in the range of 10000–4000 $cm^{-1}$. For spectral acquisition, the sample presentation was optimized according to the type of sample. For plastic samples (**I, V**), each film was placed in the beam of the Powder Samplir Accessory with a Spectralon 99% reflective standard located on top of the film. The radiation was scattered through the film, reflected by the standard disk, passed back through the film, and finally picked up by the detector. For *Echinacea* samples (**II, III**), the herbal powder was poured without tapping into a 20 ml borosilicate scintillation glass vial (Kimble glass, NJ, USA). The vial containing about 5 ml of powder was placed in the beam of the Powder Samplir accessory. For intact tablet samples (**IV**), a "tablet holder" was constructed in order to prevent light scattering from the beam due to the small nominal diameter (7 mm) of the tablets. The tablet holder consisted of a 3 mm–thick piece of metal, with a 6 mm–diameter aperture in its centre. Tablets were placed on the "tablet holder" located in the centre of the beam, and scanned on both faces.

### 4.3. Data analysis

#### 4.3.1. Second–derivative spectra

A second–derivative spectrum is the rate of change of the slope of the spectrum tangent. They can be used to remove the linear background from raw spectra (OSBORNE et al. 1993). Spectra derivation was used during the feasibility studies in order to select the spectral regions (**I, III–V**) and to correlate the spectral data with the property of interest in the quantitative studies (**IV, V**). Moreover, it was always used as a pre–treatment option during the construction of calibration models in order to improve their selectivity and robustness (**I, III–V**). The 9-point Savitzky and Golay algorithm was used to perform the derivations (SAVISTZKY and GOLAY 1964).

#### 4.3.2. Hierarchical analysis

Hierarchical clustering (MASSART et al. 1988) was used (**IV**) as a tool for data visualization. Similarity between pairs of second–derivative spectra was evaluated by measuring the strength of the linear relationship between two spectra (Pearson distance). The linkage distances between clusters were evaluated by the average of the distances between all the points in the clusters. Spectra were then linked in one hierarchical classification system, represented by a dendrogram.

#### 4.3.3. Principal component analysis (PCA)

PCA analysis (WOLD 1987, DASZYKOWSKI et al. 2003) was performed during the feasibility studies for the qualitative analyses (**I, III**) as a tool for data visualisation and to improve our knowledge of the sample spectral features. PCA was primarily performed on second–derivative spectra in order to improve the selectivity of the analysis. The PCA analysis results were not published in the first article (**I**), but will be described in this dissertation.

### 4.3.4. Pre–treatment options

Several pre–treatment options were selected, before performing the PLS regression, to remove the spectral variability between samples due to irrelevant physical differences (**I, III–V**). The pre–treatment options were chosen case by case, by the trial–and–error method, as a function of the spectral variations found in the calibration data. The algorithms used were among the following chemometric tools: mean centering, second–derivative of Savitsky–Golay, SNV (standard normal variate) correction, and MSC (multiplicative scatter correction).

### 4.3.5. Partial least square (PLS) algorithm

A partial least square algorithm was used as a calibration model for the qualitative and quantitative methods. This algorithm was chosen because of the quality of the calibration models produced and because of the adaptability of PLS software to pharmaceutical regulations (validated software, data security, batch analysis mode).

For the qualitative analyses (**I, III**), binary coefficients were assigned to spectra as a function of their identity according to the reference analysis results. Samples of the required quality (true samples) were assigned the theoretical value of 100, and samples to be rejected (false samples) were assigned the value of 0. The model was then calculated using pre–treatment options. The number of significant PLS factors was chosen as defined by HAALAND and THOMAS (1988). Three parameters were used as criteria to discriminate true samples from false samples: predicted value, spectral residuals and factor scores. Acceptance limits for each criterion were first set at 3 standard deviations around the mean value obtained for this criterion by the true calibration samples. The acceptances limits were later optimized during the internal validation step, *i.e.* when calibration samples are tested against the method to control the performance of the calibration model. If the sample results fall within these acceptance limits, then the sample is considered as “accepted” and the quality control laboratory can conclude that the sample fulfils the specifications. If the results are outside the limits, then the sample is “rejected”.

For quantitative analysis (**IV, V**), spectra were assigned the value found by the reference method for the parameter of interest. Acceptance limits for this parameter are those defined by the material specifications. The quality of the developed models was evaluated by performing a validation of the methods according to pharmaceutical regulations already in force or in preparation.

# 5. RESULTS AND DISCUSSION

## 5.1. New applications of NIR spectroscopy

During the course of this study, new applications were developed for NIR spectroscopy in the field of pharmaceutical quality control for herbal medicinal products. These applications have never been published before, and they concern the control of raw material at reception and the assay of the active principle in the final dosage form. They were fully validated according to current pharmaceutical regulations or those in preparation.

Three methods were developed for the quality control of the raw material at reception (**I, III, V**). They can be used to ensure that the raw material sent by the supplier fulfils the required specifications. The first one was a method that controls the identity of the pharmaceutical plastic sheets for blistering (**I**). It can be successfully combined with the NIR method (**V**) for the determination of the thickness of the plastic sheets. Quality control of primary packaging is of prime importance in the case of herbal medicinal products because natural products are especially sensitive to moisture. The two methods are optimally used when a spectrum is simultaneously processed with both models. The other identification model developed concerned the confirmation of the species identity for *Echinacea purpurea* root (**III**), which is of importance to avoid any risk of adulteration of the herb.

The last method was developed to show the possibility of using NIR as a quantitative method for the assay of a compound of natural origin in intact solid dosage form (**IV**). This method is very flexible. It can be used to determine the caffeine content of a batch, as well as the content uniformity of caffeine tablets, and to validate the manufacturing process, *e.g* by controlling content deviations or comparing the caffeine content of tablets pressed by the different punches of the press.

## 5.2. What are the benefits of using chemometric analysis during feasibility studies?

Chemometrics tools can be optimally used for fast visual control of spectral differences between samples before developing an NIR regression model because it enhances the relevant chemical information from the samples (MASSART et al 1988, LAVINE 1998). The output from the chemometric analysis is a range of useful information that can be used to optimise the development of the targeted NIR method.

PCA analysis, for example, is very informative (WOLD 1987, DASZYKOWSKI et al. 2003). It was performed on second-derivative spectra from plastic samples (**Figure 7, unpublished results**) before developing the identification method (**I**). Principal components 1 and 2 (**Figure 7a**) make a clear difference between samples containing Thermoelast® (C Type samples) and samples not containing this compound (A and B types). Principal components 3 and 4 (**Figure 7b**), in contrast, exhibit clear evidence of spectral differences between the three main types of plastic (A, B and C types). This

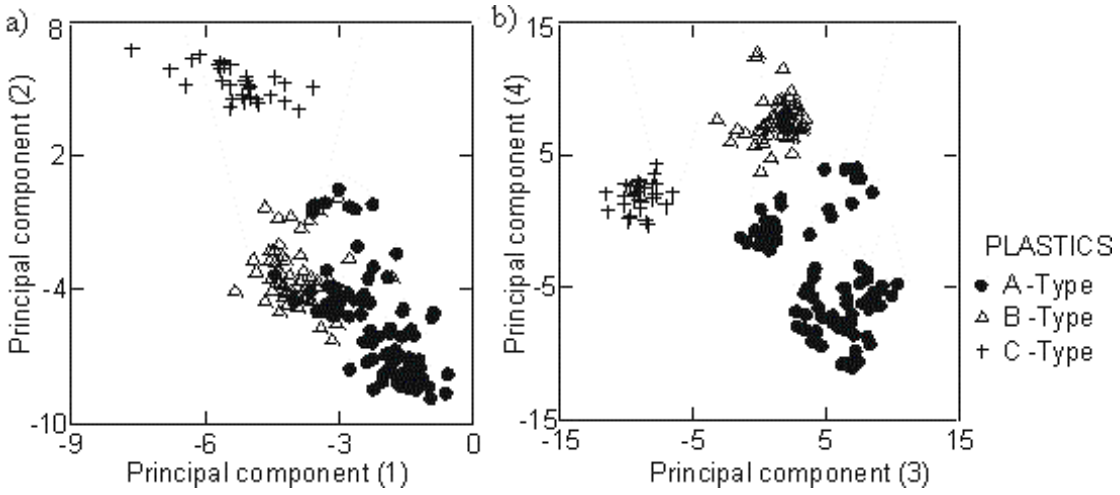


**Figure 7 Principal component analysis (PCA) of a set of samples composed of A–, B–, and C–type plastic sheets. This plot displays the 1st, 2d (a) and 3d, 4th (b) principal components. Variance explained by the four components explains 55% of the data set variation including 26% for the first principal component (Unpublished results).**

result is a good sign that a robust identification method can be developed based on spectral differentiation. Moreover, PCA is useful to select a representative calibration set and to favour the most specific spectral region. **Figure 7** demonstrates that spectral differences are larger between the C type and the two other types than between the A and B types. Therefore, the spectral region to be included in the regression model must be chosen in order to emphasise the spectral variability between the A and B types of plastic.

Moreover, the spectral variability within the A type cluster is larger than that within the other two clusters. Therefore care must be taken to choose samples representative of this variability when creating the calibration set. On the other hand, as the spectral variability is small within the C type spectra, the choice of calibration samples can be made randomly.

PCA can also be used to detect non–representative samples and to avoid selecting them as calibration samples. For instance, in the NIR analysis of *Echinacea* (**III, Fig. 3**), a PCA plot was used to visualise the data and allowed the detection of a physical (different particle size distribution) and a chemical (different chemical profile than other *E. purpurea* samples) outlier within the *E. purpurea* samples.

Second derivatisation of the spectra is a convenient way to conduct the spectral region selection because it increases the selectivity of interesting bands (CANDOLFI et al. 1999a), and eliminates any baseline offset and slope changes due to irrelevant physical effects on the samples. Thus, the correlation between spectra and the chemical property to be quantified is much sharper. Differences between non–derived spectra and second–derivative spectra are well demonstrated by **Figure 8.** Correlation between the caffeine concentration and the spectra is evident for the derived spectra, although it is unclear for

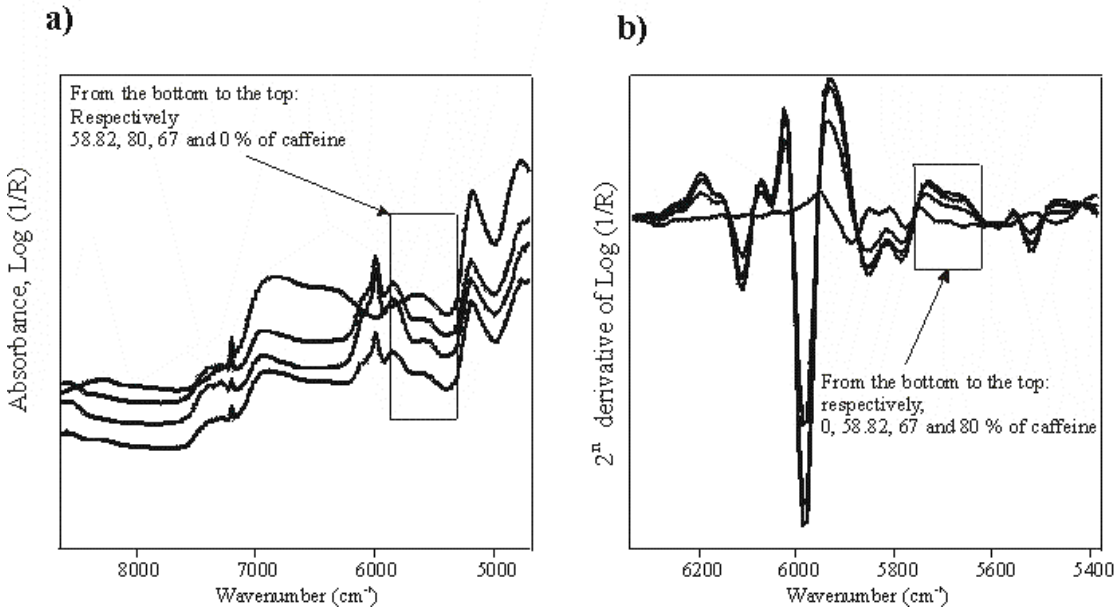


**Figure 8 Recorded spectra (a) and second–derivative spectra (b) of tablets containing various concentrations of caffeine.**

raw spectra because of the physical differences between tablets (*e.g.* hardness, density, and position on the tablet holder). However, when the property to quantify is a physical characteristic of the sample such as the thickness of the plastic film (**V, Fig. 1. and 2.**), the improvement is not so significant because derivatisation does not remove variability arising from chemical differences. Second–derivative spectra are thus useful to optimise the method specificity (STORDRANGE et al. 2002). They help to select precisely the spectral region having the highest correlation with the property of interest (**IV, Fig. 1**), as well as the region interfering the least with excipients or closely related compound features (**IV, Fig. 1. and 4**).

Hierarchical clustering is another way to classify the spectral differences between samples (MASSART et al. 1988). In this study it was used to distinguish the differences between spectra recorded from the two faces of caffeine tablet (**IV**). Similarity between pairs of second–derivative spectra was evaluated by measuring the strength of the linear relationship between two spectra (Pearson distance). The linkage distances between clusters were evaluated by the average of the distances between all the points in the clusters (MASSART et al. 1988). All the spectra were then linked in one hierarchical classification system, represented by a dendrogram. The resulting dendrogram (**IV, Fig. 2**) exhibited two clear clusters: one containing spectra from the front face of the tablet, the other one containing the spectra from the back face. It was thus obvious that the tablet sides had different spectral features, which could lead to quantitative errors when applying the PLS regression. The spectral difference was explained by density variations between the two faces of the tablet as a result of compressional force differences between the upper and the lower punch in the rotary machine (**IV)** (TRAIN 1956, AULTON 1988). This information led to the decision to modify the spectral acquisition mode, which originally consisted in recording the spectrum from only one face of the tablet. The tablets were therewith scanned on both faces and the two spectra then averaged to give the tablet spectrum.

### 5.3. Factors affecting the robustness of NIR analysis methods

Lack of robustness is one of the main disadvantages of NIR spectroscopy compared to other techniques (BLANCO and VILLARROYA 2002a). Several parameters can affect NIR spectral features and some of them were directly identified during this study. They can lead to misidentification of a sample or to imprecision in the quantitative results, and thus should be investigated prior to developing the model.

The chemical composition of the samples modifies the spectral features. The case of *Echinacea purpurea* identification is a good example. Chemical variations in this species (**II, Table 1**) are large and attributable to the place and season of harvest, drying methods, age of the plant, storage methods etc. Within the collected samples, one *E. purpurea* batch, with the code P2, had a relatively different chemical profile compared to the other samples within this species: its Dodeca–2E, 4E, 8Z, 10E/Z–tetraenoic acid isobutylamide contents were nearly undetectable. This distinction was reflected by the sample spectra and depicted during the feasibility study by the PCA analysis (**III, Fig. 3**). In order to prevent this sample from being rejected by the NIR method, samples with a large range of isobutylamide content (*e.g.* varying location of growth, climate) were included in the calibration set. As a result, this sample was accepted by the developed NIR model.

The physical properties of samples can also affect the method specificity. Again, during *Echinacea* identification method development (**III**), PCA analysis showed a spectral outlier, P1, which was found to have a much narrower particle size distribution than the other samples (**III, Fig. 4**). The reason for this was that it was received directly in milled and sieved form, although the other samples were milled at reception and not sieved. Attempts to include this sample in the calibration set led to failures as it was depicted as an outlier by the *F*–test. Thus, as it is assumed that all future samples will be milled at reception, no special actions were taken to improve the calibration set. As a result, this sample was not accepted by the method and was thus labelled as a "false negative" sample. Nevertheless, it gave an indication that all samples should be milled in the same way in order to avoid false negative identification. Another example was demonstrated by the study on caffeine tablets. Hierarchical analysis (**IV, Fig. 2**) revealed

a difference in the spectral features of the two faces of a tablet. This was explained on the basis of the variation of the caffeine density due to differences in the upper and lower punch compressional forces during tableting. The concentration difference between the two faces of the tablet was quantified by NIR and found to be between 1.4 and 1.0% m/m of caffeine for two batches (**IV**).

Replacement of the NIR quartz halogen source can cause frequency variation and thus spectral shifts. In our case, the frequency is calibrated according to the expected position of a water vapour band (BOMEM 1994): *i.e.* 7299.87 ± 0.1 $cm^{-1}$, which is a combination of symmetrical stretching and asymmetrical stretching ($\nu_1 + \nu_3$) of O–H vibrations (NIELSEN 1942). Maintenance tests are performed each day of analysis to control the frequency calibration. However, if calibration spectra were recorded at a precise frequency, a small deviation from this value can induce a significant spectral shift in the recorded spectrum and lead to misidentification of the spectra. As an example, during our study the NIR source was replaced but not precisely realigned, which induced significant deviation of the water band frequency up to 7299.81 with a day–to–day variation of approximately 0.02 $cm^{-1}$. This value was, however, still within the frequency acceptance limits. As the calibration model for the plastic identification (**I**) was created at a very stable frequency (approximately 7299.87 ± 0.01 $cm^{-1}$), the spectra were affected by a significant shift and were thus no longer identified correctly by the method. To solve the problem, the NIR source had to be realigned so that the frequency reaches the same value as previously (**unpublished result**). In contrast, two batches of caffeine tablets (**IV**) were analysed before and after the source replacement, prior to the source realignment. The results were not found to be significantly different ($n = 6$, $P = 0.19$ and 0.73 respectively). Therefore, the effect on the spectral results depends on the type of model developed: if the acceptance criteria are very tight, such as in the plastic identification model (**I**), the effect may be significant.

Variation in sample presentation has always been known as a factor affecting spectral features (YOON and MOFFAT 1998). However, it is highly dependent on the situation. In the plastic thickness analysis method, robustness versus the position of the

reflectance standard was evaluated (**V**). The results were found to be unaffected if the reflectance standard does not fit properly on the optical window due to a curling form of the sheet. However, changing the reflectance standard from a routine standard to a newer, cleaner standard was found to affect the spectra and resulted in a prediction error of 3.8 μm when measuring the thickness of the plastic film (**V**). However, this result is of little practical significance because it is lower than the standard error of prediction of the NIR method itself (SEP = 4.3 μm). In the case of caffeine tablets (**IV**), the results were not affected ($P = 0.30$, $n = 6$) when the tablet holder was not fitted properly in the center of the optical window. The repeatability of the results was, however, poorer (Relative standard deviation RSD = 1.1 %) when the tablet holder was located off–center than when it was placed correctly in the center of the beam (RSD = 0.78 %). Thus the correct position of the tablet holder is a critical point in the NIR analysis of tablets.

Change of supplier or a change in the manufacturing process of the samples can also lead to slight spectral modifications, which may affect the method specificity. As the identification model of plastic samples (**I**) was used routinely for quality control purposes, a sample was received and not correctly identified by the NIR method even though it was positively identified by the reference IR method. The supplier was contacted and it was shown that the manufacturing process of this sample had been slightly modified. In this case, the only way to solve the problem is to collect other samples of the new quality, to include them in the calibration set, and to update the regression model.

Therefore, in order to ensure the robustness of an NIR method, the potential factors likely to affect the results must be investigated, such as the chemical and physical characteristics of the sample or sample presentation. Once these factors are known, two different types of decision can be taken. The first one is to make sure that these variations will not appear during the creation of calibration and the routine analysis of the samples. For example, solid herbal drugs should always be milled in the same way, and the frequency should not be subjected to large variations. On the other hand, when it is difficult to control these factors, it may be better to include them in the calibration set as

normal variations of the sample. For example, if herbal drugs were susceptible to be received in solid or in powder form, then it would be better to introduce samples milled at reception and pre–milled samples in the calibration set. In this case, this variation will be recognised as a normal variation of the samples, and thus samples will be correctly identified by the PLS method irrespective of their particle size distribution. In the same way, calibration could be created over a large period of time in order to include large frequency variations. The resulting model will thus not be so susceptible to slight frequency variations.

### 5.4. Comparison between NIR analysis and HPLC outputs

In this study, a comparison of the outputs obtained with NIR and traditional analysis methods was performed by analysing *Echinacea purpurea* roots by both high–performance liquid chromatography (HPLC) (**II**) and NIR spectroscopy (**III**). The aim was to confirm the identity of *Echinacea purpurea* roots and enable the detection of possible mislabelled samples or adulterants, e.g. other *Echinacea* species or *Parthenium integrifolium* (BAUER and WAGNER 1991). In traditional HPLC analysis of *Echinacea* species, hydrophilic and lipophilic fractions are usually separated by means of different sample preparation methods and different HPLC settings (BAUER and WAGNER 1991). This results in an analysis time of usually over 40 minutes without including sample preparation. In order to increase the speed and limit the costs of HPLC analysis, a fast HPLC method was developed (**II**). This method combines the analysis of hydrophilic and lipophilic compound during the same run, *i.e.* using the same extract for both types of compound and a single injection in the column. This enables the compounds of interest to be separated within 20 minutes, *i.e.* about twice as fast as for traditional HPLC analysis. The time needed to perform the HPLC analysis for five duplicate *Echinacea purpurea* samples was approximately 18 hours, including sample preparation, establishment of the three calibration curves, analysis runs and cleaning of the HPLC column.

As a comparison, the NIR analysis is able to verify the identity of *Echinacea purpurea* in 30 minutes, including grinding of the samples, calibration of the instrument, analysis of

the five duplicate samples, and cleaning the scintillation vials. The analysis is performed on solid material, without the need for solvent. This explains why speed and environmental friendliness are most probably the main advantages that spring to mind when comparing NIR spectroscopy with other analytical techniques.

The information obtained from the two techniques is, however, quite different. HPLC (**II**) gives specific and simultaneous results about the identity of the sample and the concentration or presence of marker substances. HPLC chromatograms are thus specific enough to determine the identity of the sample whatever its species (*E. purpurea*, *angustifolia* or *pallida),* and can very easily detect the presence of the potential adulterant *Parthenium integrifolium*. On the other hand, the developed NIR analysis (**III**) only gives an answer to the question: is this sample an *Echinacea purpurea* root sample or not? If the answer is no, then it is almost impossible to determine the identity of the sample due to the poor specificity and selectivity of spectral features in NIR spectroscopy (**III, Fig. 1**). In the pharmaceutical industry, however, it is more important to make sure that the sample is of the required quality, and it is of secondary importance to know the real identity of the sample in case it does not fulfil specifications. Furthermore, the method does not give any indication of the marker level of the sample. However, it would be possible to overcome this drawback, as demonstrated recently by Schulz and co-authors (SCHULZ et al. 2002), who described a quantitative NIR method for the determination of the echinacoside content in *Echinacea* roots. NIR quantitative methods are nevertheless able to quantify only the parameters they have been calibrated for and do not give indication on any other parameters. In addition, more extensive development work is required for developing a quantitative method than that for qualitative methods. The last output is that routine NIR analysis can be performed by an almost untrained person. In contrast, HPLC analysis requires highly trained personnel.

Thus, if assessing the exact identity of the sample or determining precisely its chemical profile is the target to be reached, then the HPLC method is more suitable than NIR for the analysis. But, if controlling that the sample is of the required identity and quantifying a few marker substance is the target, NIR is the suitable technique and the advantages in terms of time and costs saved during the analysis are huge (fast analysis, no solvent, no highly trained personnel).

## 5.5. Validation of NIR methods

In this chapter, the way to validate NIR methods will be discussed. Two types of document were followed in creating the validation plans. ICH guidelines (EMEA 1994 and 1996) are the document currently in–force for the validation of analytical methods, but are not well adapted to non separative methods such as NIR spectroscopy. The recent draft guideline (EMEA 2001c) is targeted for the validation of NIR methods.

### 5.5.1. Validation of qualitative NIR methods

According to the ICH guidelines (EMEA 1994 and 1996), identification methods should only be validated for their specificity, *i.e.* their ability to discriminate compounds of closely related structure. Specificity should be assessed by confirming that positive results are obtained from samples containing the analyte (called true samples in the field of NIR spectroscopy) and that negative results are obtained from samples not containing the analyte or containing material structurally closely related to the analyte (false samples). These criteria are just as applicable to the NIR method as to separative methods such as HPLC. The two qualitative NIR methods developed (**I, III**) were validated by adapting these two guidelines. An internal validation was first performed to control the performance of the model with the calibration set samples, and the external validation was then used to control the specificity of the model versus samples completely independent of the calibration set. Both the calibration and validation sets contained true samples and false samples (**I, Tables 3, 4, 5 and III, Table 1**) so that the ICH criteria for assessing specificity were fulfilled. The validation of qualitative NIR methods was clarified in the recent draft guideline (EMEA 2001c). Specificity should be assessed in two steps, *i.e.* internal validation and external validation, but robustness should also be demonstrated (investigation of the effect induced by possible variations such as temperature, humidity, sample presentation, or changing lamps). In qualitative NIR

methods (**I, III**), only the robustness versus the change of raw material supplier was assessed and both methods proved to be robust regardless of the supplier of the products.[2]

### 5.5.2. Validation of quantitative NIR methods

One of the first steps in the validation of an NIR quantitative method should be to control the performance of the calibration model. This was not included in the specifications of the ICH guidelines (EMEA 1994 and 1996), but it is of primary importance before any further validation steps. EMEA has now included this specification in the draft for the validation of NIR methods (EMEA 2001c). The chosen model should be presented in detail, including the equation, correlation coefficient, slope, and *y* intercept of the equation. However, this draft does not mention anything about the importance of the model residuals. Graphical analysis (**V**) of the residuals could be conveniently performed even at the method development stage in order to check that the model is based on correct statistical principles.

A simple probability plot can confirm that the residuals are normally distributed random variables with a mean of 0 (**V, Fig. 3 a**), and a studentized residual plot (**V, Fig. 3 b**) can show that the residuals have a constant variance and are independent of the concentration. These results give the insurance that the model is statistically valid. For the model validation, the EMEA also recommends to assess the adequacy of the calibration by calculating the standard error of calibration (SEC). However, it was found that the formula given for the calculation of SEC is applicable to multiple linear regression (MLR), but not to PLS regression (**V**). The main reason for this is that the spectra are usually mean centred before performing the PLS regression, which takes one degree of freedom. Thus in this case, the divisor (*n-p-1)* should be used instead of (*n-p)* in the SEC formula given by the EMEA guideline (**Eq. 6**).

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[2] The draft for the validation of NIR methods was adopted on February 20, 2003 (EMEA 2003), after this thesis was completed. The main modification brought by the adopted document concerning the validation of qualitative methods was as follows. Internal validation is no longer part of the validation parameters for qualitative methods. This means that specificity testing of the batches included in the reference library no longer needs anymore to be included in the validation report. However, it remains a crucial step to test the method validity and improve the acceptance limits as demonstrated in (**I**).

$$SEC = \sqrt{\frac{\sum_{i=1}^{n}(y_i - Y_i)^2}{n - p}}, \qquad \textbf{(6)}$$

Where $n$ = the number of batches, $y$ = the reference method value, $Y$ = the NIR predicted values, and $p$ = the number of coefficients used in the calibration model. Therefore it was suggested (**V**) that the SEC formula in the final EMEA guideline should be more detailed in order to take into account the differences between the MLR techniques and the PLS regression techniques.

According to the ICH guidelines, accuracy, precision, specificity, linearity and range should be demonstrated during quantitative method validation. The NIR method (**IV**) developed to determine the caffeine content in tablets was validated according to these specifications. Some specifications had to be adapted because they were not directly applicable to NIR methods.

Accuracy was found to be rather difficult to assess using only the prescribed specifications. In fact, as the NIR calibration range has to be expanded by introducing laboratory batches with a larger variation of active principle concentration, accuracy is affected by the physical differences between laboratory samples and production samples. The difference between the accuracy calculated only from production samples or from production and laboratory samples, was found to be relatively large: 99.4 and 98.9 %, respectively, of mean recovery from the reference HPLC value (**IV**). This is why accuracy was also validated by other parameters such as standard error of prediction, prediction bias and *t*–test between the NIR and reference values (**IV**). In the EMEA draft for the validation of NIR methods (EMEA 2001c), accuracy assessment is now much simpler: accuracy should be studied by determination of the standard error of prediction (SEP) and the number of outliers of the validation set. SEP of the NIR method should not be larger than 1.4 x SEL (standard error of laboratory of the reference method). The thickness determination method (**V**) was validated according to this draft, and SEP was found to be similar to SEL of the reference method (4.3 and 5.0 µm, respectively).

Precision was easy to evaluate using the specifications given by the ICH guidelines. In fact, no modifications have been included in the new EMEA draft to assess the

precision of NIR methods. In the NIR methods developed, repeatability was found to be well below the normally accepted criteria for analytical methods (RSD = 1%): RSD < 0.75% (**IV**) and RSD < 0.65% (**V**). Similarly, intermediate precision was found to be well below the normally accepted criteria (RSD = 2%): RSD < 0.65 % (**IV**) and RSD < 1.05 % (**V**).

Specificity assessment has been modified considerably in the EMEA draft compared to the ICH guideline currently in–force. The specificity of the caffeine quantitative method (**IV**) was assessed by adapting the ICH criteria described in Chapter 5.5.1. It was proved that the absorption peaks that correlated with the caffeine concentration did not interfere with the excipient peaks (**IV, Fig. 1**). This result was also quantified by a comparison of the spectral residuals obtained with three production batches and one laboratory batch containing only excipients. The acceptance criterion (mean spectral residual + 3 standard deviations) was calculated from the results of production batches from the calibration set. One–sample *t*–tests confirmed that the mean residual of the excipient batch was significantly different ($t = 24.8$, $P < 2\ 10^{-5}$, $n = 6$), and 35 times higher than the acceptance criterion. The mean spectral residuals from the production batches were within the acceptance criterion. The ability of the method to discriminate tablets containing caffeine from tablets containing theobromine or theophylline, two alkaloids with structures closely related to caffeine, was also evaluated. Their spectra and second derivatives (**IV, Fig. 4**) showed significant visual differences between caffeine and theobromine or theophylline tablets. Thus this method is able to discriminate caffeine tablets from tablets not containing the analyte, and from tablets containing closely related compounds. Specificity was therefore validated. The specificity of the thickness determination method (**V**) was evaluated according to the EMEA draft. As the quantitative method was used simultaneously with the NIR identification of plastic samples, specificity was assessed by challenging an updated version of the identification method (**I**) with samples of different polymer combination, thickness or colour. The NIR method gave 2.0, 4.5 and 0.0 % of false negative (or type I) error, and 1.7, 2.1 and 1.0 % of false positive (or type II) error, for the identification of A, B and C types of plastic, respectively. Therefore the method is specific enough to discriminate samples with different polymer combination, thickness or colour.

There is no significant difference between the two guidelines in the way linearity is to be assessed. For the caffeine (**IV**) and the thickness determination (**V**) methods, linearity was assessed by providing a plot of the NIR–predicted value versus the reference method value, together with the correlation coefficient and equation of the regression line. Moreover, it was confirmed that the confidence interval for the slope and for the *y* intercept included one and zero, respectively. Finally, a *t*–test was used to prove that the *y* intercept did not differ significantly from zero, and analysis of the variance demonstrated that the slope also did not differ significantly from one. Therefore the linearity of the two methods was validated.

According to the ICH guideline, establishing that linearity, accuracy and precision are validated is sufficient to validate the range of application of the methods. The range of the two quantitative methods was validated therewith (**IV, V**). Nevertheless, according to the recent EMEA guideline, this criterion no longer needs to be validated for NIR methods.

The quantification limit is not a parameter suggested for the validation of an assay by the ICH guideline, and neither by the draft EMEA guideline. However, it is convenient and interesting to calculate this parameter with a simple approach based on the standard deviation of the response and the slope, as recommended by the ICH guideline (EMEA 1996) when impurities are to be quantified. Using a simple calculation that involves no further laboratory work, the limits of quantitation were calculated for both quantitative methods and were found to be 13.7 % m/m of caffeine in the tablet (**IV**), and 41 μm of thickness for plastic sheets (**V**). These values confirmed that the range of the methods were valid.

As the criteria for robustness provided by the ICH were not applicable to NIR methods, specific criteria were created for this purpose during the validation of the caffeine NIR method (**IV**). Robustness was assessed by evaluating the effect of changing the position of the sample holder in the beam and replacing the NIR source (**IV**). The EMEA draft guideline is more detailed about robustness evaluation and recommends that the effect of temperature, humidity, different position of the sample in the optical window, different sampling presentation devices or instrumental variations *e.g.* changing lamps, reflectance standard, should be evaluated. Robustness for the thickness

determination method (**V**) was thus validated by evaluating the effect of an incorrect position of the reflectance standard and of a change in the reflectance standard. The results of robustness evaluation were discussed already in Chapter 5.3. [3]

[3] The draft for the validation of NIR methods was adopted on February 20, 2003 (EMEA 2003), after the thesis was completed. The main modifications brought by the adopted document concerning the validation of quantitative methods were as follows.

The performance of the calibration model now takes into account the difference between multiple linear regression (MLR) and PLS regressions as suggested in (**V**). The modification, however, brings a different solution to the one suggested in (**V**). MLR and PCR methods should indeed be evaluated by the determination of the standard error of calibration SEC, and PLS methods should be evaluated by the determination of the standard error of cross validation SECV (**Eq. 7**).

$$SECV = \sqrt{\frac{\sum_{i=1}^{n}(y_i - Y_i)^2}{n}} \qquad \textbf{(7)}$$

Where $n$ = the number of batches in the calibration set, $y$ = the reference method value, $Y$ = the NIR predicted values. This suggestion solves, as does the one proposed in (**V**), the problem of the non-suitability of SEC formula for PLS regression. This, however means that the calibration model performance should be evaluated by different parameters depending on the type of regression used. The suggestion proposed in (**V**) had the advantage of keeping the same parameter (SEC) for all types of regression and thus facilitating their comparison.

It is also suggested to add information concerning specificity of the method, *e.g.* a comparison of the NIR bands of the analyte of interest with those from the matrix or a comparison of the factor loading with the analyte bands. The relevancy of this information was already demonstrated during the validation of the caffeine quantitative method (**IV**).

# 6. CONCLUSIONS

The present study demonstrates that NIR can successfully be used to monitor the quality of herbal medicinal products at different steps of the manufacturing processes. This was illustrated by the development of four previously unpublished NIR methods. Three of the methods target the quality control of raw material at reception. They can be used routinely in the quality control laboratory to ensure that the raw material sent by the supplier fulfils the required specifications. One of the methods aims at confirming species identity for *Echinacea purpurea* root and at avoiding any risk of adulteration of the herb. Another method controls the identity of the pharmaceutical plastic sheets used for blistering. It can be successfully combined with the NIR method developed for the determination of the thickness of plastic sheets. The time gained is optimal when a sample is analysed simultaneously by both methods. The fourth method was developed for the assay of caffeine, an alkaloid of natural origin, in intact tablets.

The importance of carrying out a feasibility study prior to the development of an NIR model was brought to the fore. It was shown that the use of a suitable chemometrics analysis during feasibility studies leads to a better knowledge of the spectral variability of the samples and allows pertinent choices to be made prior to development of the model. Principal component analysis, second–derivative and hierarchical analysis proved to be especially helpful in choosing representative calibration samples, a specific spectral region, and an improved spectral acquisition mode. Moreover, the study of factors affecting the spectra was found to be crucial to optimise the variability of parameters that should be included in the calibration set (frequency, supplier, particle size) and the sample presentation mode.

Chemometrics analysis and a robustness study are thus highly critical steps for the development of specific and robust NIR models.

When comparing the outputs and benefits of NIR compared to traditional analysis methods, HPLC was found to give more informative composition results as compared to NIR spectroscopy. Thus, when the analysis aims at assessing the exact identity of the

sample or precisely determining its chemical profile, then HPLC is a more suitable analysis technique than NIR spectroscopy. However, controlling that the sample is of the required identity and quantifying a small number of marker substances are often the goals for the pharmaceutical industry. In these cases, NIR is a technique of choice for the quality control of herbal medicinal remedies. In addition, it was shown that the advantages in terms of time and costs saved during the analysis are considerable compared to HPLC: NIR routine analysis is more than an order of magnitude faster, environmental friendly, and does not require highly skilled personnel.

The methods were validated according to pharmaceutical regulations and all the criteria met the recommended specifications. Qualitative methods were validated for their specificity, and quantitative methods were validated for their accuracy, precision, specificity, linearity, range, limit of quantitation and robustness. In–force validation guidelines and those in preparation at that time were compared for this purpose. The draft validation guideline for NIR methods was shown to be a much more suitable regulatory framework than the guidelines in force at that time. A number of potential improvements for the validation draft guideline were, nevertheless, suggested during the completion of this work. They were found to be very similar to the modifications actually brought to the adopted version of this document.

This demonstration of the applicability of NIR spectroscopy as a quality control tool for herbal medicinal remedies and the implementation of the final validation draft will hopefully facilitate the acceptance of NIR spectroscopy in pharmaceutical laboratories.

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