Alma Master Studiorum - University of Bologna

PhD Course in Food Sciences (XXIII Cycle)

Study of innovative methods of control in the cereal productive chain for the production of beer and spirits

Presented by:

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Final Exam 2011

DIPARTIMENTO DI SCIENZE DEGLI ALIMENTI

Assessment for admission to the final examination for the degree of PhD in Food Science (XXIII Cycle)

Dr. Valeria SILEONI

Researches performed during the PhD course intended to assess innovative applications of near-infrared spectroscopy in reflectance (NIR) in the production chain of beer. The purpose is to measure by NIR the "malting quality" (MQ) parameter of barley, to monitor the malting process and to know if a certain type of barley is suitable for the production of beer and spirits. Moreover, NIR will be applied to monitor the brewing process.

First of all, it was possible to check the quality of the raw materials like barley, maize and barley malt using a rapid, non-destructive and reliable method, with a low error of prediction.

The more interesting result obtained at this level was that the repeatability of the NIR calibration models developed was comparable with the one of the reference method. Moreover, about malt, new kinds of validation were used in order to estimate the real predictive power of the proposed calibration models and to understand the long-term effects. Furthermore, the precision of all the calibration models developed for malt evaluation was estimated and statistically compared with the reference methods, with good results.

Then, new calibration models were developed for monitoring the malting process, measuring the moisture content and other malt quality parameters during germination.

Moreover it was possible to obtain by NIR an estimate of the "malting quality" (MQ) of barley and to predict whether if its germination will be rapid and uniform and if a certain type of barley is suitable for the production of beer and spirits.

Finally, the NIR technique was applied to monitor the brewing process, using correlations between NIR spectra of beer and analytical parameters, and to assess beer quality.

These innovative results are potentially very useful for the actors involved in the beer production chain, especially the calibration models suitable for the control of the malting process and for the assessment of the "malting quality" of barley, which need to be deepened in future studies.

Dr. Valeria Sileoni has also actively participated for six months in the research of professor Frans van den Berg at University of Copenhagen, Faculty of Life

Sciences, Department of Food Science, Quality and Technology, Spectroscopy and Chemometrics group.

The Board unanimously agrees that Dr. Valeria SILEONI is qualified to sit the final exam for the doctorate degree in Food Science.

Bologna, February 10th 2011   **Coordinator of PhD Course in Food Science**
Prof. Claudio CAVANI

Giudizio di ammissione all'esame finale per il titolo di Dottore di Ricerca in Scienze degli Alimenti (XXI Ciclo)

Dott.ssa Valeria SILEONI

Le ricerca svolta durante questo Dottorato di Ricerca era destinata a valutare le applicazioni innovative della spettroscopia nel vicino infrarosso in riflettanza (NIR) nella catena di produzione della birra. Lo scopo era quello di misurare tramite NIR il "malting quality" (MQ) dell'orzo, di monitorare il processo di maltazione e di sapere in anticipo se un certo tipo di orzo è adatto per la produzione di birra e superalcolici. Inoltre, il NIR è stato applicato al monitoraggio del processo di fermentazione.

Prima di tutto, è stato possibile verificare la qualità delle materie prime come orzo, mais e malto d'orzo con un metodo rapido, non distruttivo, affidabile ed avente un basso errore di previsione. Il risultato più interessante ottenuto a questo livello è stato che la ripetibilità dei modelli di calibrazione NIR sviluppati è stata paragonabile a quella del metodo di riferimento. Inoltre, per quanto riguarda il malto, sono stati utilizzati nuovi tipi di validazione per stimare il reale potere predittivo dei modelli di calibrazione proposti e per capire gli effetti a lungo termine. Inoltre, la precisione di tutti i modelli di calibrazione sviluppati per la valutazione del malto è stata statisticamente e confrontata con i metodi di riferimento, con buoni risultati.

Successivamente, sono state sviluppati nuovi modelli di calibrazione per il monitoraggio della maltazione, misurando il contenuto di umidità ed altri parametri di qualità del malto durante la germinazione.

Inoltre è stato possibile ottenere tramite NIR una stima del "malting quality" (MQ) dell'orzo e di prevedere se la sua germinazione sarà rapida e uniforme e se un certo tipo di orzo sia adatto per la produzione di birra e superalcolici.

Infine, la tecnica NIR è stata applicata al monitoraggio del processo di birrificazione, utilizzando le correlazioni tra gli spettri NIR della birra ed i parametri analitici, ed alla valutazione della qualità della birra.

Questi risultati innovativi sono potenzialmente molto utili per coloro che operano nella catena di produzione della birra, in particolare i modelli di calibrazione sviluppati per il controllo della maltazione e per la valutazione del "malting quality" dell'orzo, i quali dovrebbero essere approfonditi in studi futuri.

La Dott.ssa Valeria Sileoni ha anche partecipato attivamente per sei mesi nella ricerca del professor Frans van den Berg presso l'Università di Copenaghen, Facoltà di

Scienze della Vita, Dipartimento di Scienze degli Alimenti, qualità e tecnologia, spettroscopia e di gruppo chemiometria.

Il Collegio dei Docenti unanime ritiene la Dott.ssa Valeria SILEONI idoneo a sostenere l'esame finale per il conseguimento del titolo.

Bologna, 10 Febbraio 2011   **Il Coordinatore del Dottorato di Ricerca in Scienze degli Alimenti**

TABLE OF CONTENTS

AIM OF THE PROJECT AND ABSTRACT

The near-infrared spectroscopy in reflectance (NIR) is a non-destructive, low cost, rapid and effective technology for predicting simultaneously multiple components in food products. Because of their characteristics, cereals are extensively investigated by means of NIR spectroscopy and many calibrations to assess their composition are been developed. Moreover, due the capability of NIR spectroscopy to measure quickly, easily and reliably the amount of water and organic molecules, such as starch, proteins, oils, fibres, ashes, acids, sugars and ethanol, this analytical technique is considered suitable to be applied to beer production chain. In particular, concerning the production of beer, NIR spectroscopy has found implementation in the analysis of raw materials, intermediates finished products, and in process control. Many papers deal with the applications of NIR spectroscopy to quantitative analyses on raw materials such as malt and barley, designed to quantify the amount of moisture, nitrogen, amino acids, proteins, extract [1, 2] and β-glucans [3]. These substances increase proportionally with endosperm modification, and therefore constitute an index of quality of malt. Concerning the intermediate products, there are many papers about the applications of NIR spectroscopy to quantitative analyses on wort, and in particular to extract, total carbohydrates, total nitrogen, fermentability and α-free-amine nitrogen (FAN) on laboratory and industrial wort [4].

Some recent papers deal with the applications of NIR to the finished product, beer, aiming at determining real extract, ethanol, nitrogen and polyphenols [5]. Finally, concerning the control of the production process, it is possible to constantly monitor through NIR spectroscopy the mashing phase and the evolution of fermentation [6].

This PhD thesis research project is aimed at assessing innovative applications of near-infrared spectroscopy in reflectance (NIR) in the production chain of beer. The purpose is to measure, through this rapid, non-destructive and reliable method, the "malting quality" (MQ) parameter of barley, to predict whether if its germination will be rapid and uniform, to monitor the malting process and to know if a certain type of barley is suitable for the production of beer and spirits. Moreover, NIR will be applied to monitor the brewing process, by finding correlations between NIR spectra of beer and analytical parameters.

Concerning the analysis of raw materials reasonable calibration models have been developed to determine the parameters of interest on malting barley and maize.

About the **maize**, NIR spectra of grist and flour samples were acquired and correlated with the **moisture and fat content** by PLS algorithm (Partial Least Squares). The calibration models were validated by both cross-validation leave-one-out (CV loo) and test set validation by eliminating 33% of samples in calibration and using these samples to calculate the error of prediction (TS 33% out). In addition, these calibration models were validated following the guidelines of the international organizations of accreditation. In Italy, the organization is ACCREDIA, which belongs to EA (European co-operation for Accreditation)., therefore the standard deviations calculated on the 10 predicted values were compared using Chi square's test with the reference methods' one, with good results (**PAPER I and II**).

Concerning **malting barley**, NIR spectra were acquired on 40 samples of whole grains and used to develop calibrations able to correlate these spectra with the parameters of **moisture and total nitrogen content**, with satisfactory results.

Then, a process control using NIR spectroscopy applied to **malting process** has been implemented. During malting process, the spectra were collected daily on samples of germinating barley at-line (i. e. outside the production line but during the process, in real time), and a calibration model was developed to relate the spectra acquired on germinating barley with their **moisture**. (**POSTER I**). Furthermore, calibration models were developed to monitor other important parameters to evaluate the performance of malting barley by NIR spectra. These models were developed on the assumption that some important parameters for the assessment of malt quality, which analytically are determined on the dried malt, are "visible" to the NIR already on the green malt. Then, the spectra of green malt collected during the last day of germination were correlated with some analytical parameters determined on the corresponding malts after drying by PLS algorithm. The obtained calibration models were validated through cross-validation (CV) and allowed a good prediction performances, showing the possibility of using NIR control of the germination process, in order to assess how they are evolving the most important quality parameters of malt. This possibility would be extremely important for maltsters, which could change the process causing acceleration or deceleration of germination varying the parameters of humidity and temperature.

Subsequently, several calibration models were developed to allow a full assessment of the **malt** quality by NIR spectroscopy. The spectra were acquired on flour and whole grains of more than 200 samples of malt and used to develop calibrations for determining the classic parameters of interest on malt, such as **moisture and total nitrogen content**. The predictability of the calibration models

obtained was good, and also the repeatability of the methods developed was comparable with the one of the primary methods following the ACCREDIA guidelines (**PAPER I and II**).

In addition, various calibration models have been developed for the determination of other malt quality parameters. Compared to the models of the first year of PhD (**POSTER II**), the calibrations have been improved implementing new spectra acquired during 2008, 2009 and 2010 and especially using advanced chemometric methods that have allowed refinement of the models, mainly concerning the selection of the spectral bands (interval-PLS algorithm) and new spectral pretreatments (Extended Multivariate Scattering Correction). These techniques have been acquired during the stage abroad, at the University of Copenhagen, Faculty of Life Sciences, Department of Food Science, Quality and Technology, spectroscopy and chemometrics group. The calibration models were developed through PLS algorithm, using the software MATLAB 7.6 R2008a. The different pretreatments or combinations of them were compared manually. The choice of spectral range has been performed applying the interval-PLS algorithm (PLS-toolbox). The identification of outliers was conducted using four different tests. Once developed, the various models have been validated through cross-validation leave-one-out.

In addition, the acquisition of a greater number of spectra allowed dividing the calibration data set into groups, including the spectra acquired during the different years. In this way, it was possible to exclude from time to time samples belonging to each year and calculate the corresponding error of prediction. This strategy allows obtaining a true estimate of the real predictive power of the method applied on unknown samples in future years (**POSTER III and PAPER III**).

Regarding the "malting quality" (MQ) parameter of barley, correlation models between the NIR spectra acquired on the samples of barley and the quality parameters of the corresponding malt was searched. The calibration models showed a good degree of predictability, even if these models have been developed using just 40 samples. However, determining these parameters directly on barley as "potential" of malt features can be difficult, perhaps because they are too related to the process. We could therefore develop a calibration that is suitable for a standardized process, but may not be suitable for a different process.

Finally, about NIR control of **beer fermentation**, it was decided to assess the content of **alcohol (% v/v), pH, and the real, apparent and original extract (°P)**. These parameters were then correlated with the spectra of samples of the fermenting wort acquired off-line in parallel with analytical determinations in diffuse reflectance through quartz cuvette and reflective gold. Again,

these correlations between the analytical and spectroscopic data were generated using PLS algorithm and validated through cross-validation, resulting in calibration with a good degree of predictability. Similar results were obtained correlating these analytical determinations performed on **beer samples** and NIR spectra.

The calibration models developed during this PhD thesis research project allows innovative applications of near-infrared spectroscopy in reflectance (NIR) in the production chain of beer.

For example, it is possible to check the quality of the raw materials like barley, maize and barley malt using a rapid, non-destructive and reliable method, with a low error of prediction and with a repeatability comparable with the one of the reference method (ACCREDIA). Then, these new calibration models allow to monitoring the malting process, measuring the moisture content and other quality parameters during germination. Moreover it is possible to obtain an estimate of the "malting quality" (MQ) of barley, to predict whether if its germination will be rapid and uniform and if a certain type of barley is suitable for the production of beer and spirits. Finally, the NIR technique can be applied to monitor the brewing process, using correlations between NIR spectra of beer and analytical parameters. These innovative results are potentially very useful for the actors involved in the beer production chain, especially the calibration models suitable for the control of the malting process and for the assessment of the "malting quality" of barley, which need to be deepened in future studies.

1. NIR SPECTROSCOPY

1.1 Spectroscopy: basic concepts

An ideal method for the determination of chemical composition in a routine food-manufacturing schedule should be non-invasive, non-destructive and rapid to ensure timely processing of the food being analysed.

The development of rapid analytical methods for food products relies mainly upon two approaches: the use of physical properties of substrates as an information supply and the automation of chemical methods. Most rapid analytical methods based on the physical properties of food products are spectroscopic methods. Spectroscopy can be split into two large groups [7]: photonic spectroscopy, which is based on the study of the interaction of an electromagnetic wave with matter, and particle spectroscopy. The first group comprises spectroscopic methods exhibiting an analytical potential for rapid control. The second group is represented by mass spectrometry and derived methods.

All the spectroscopic methods are based on the interaction between electromagnetic radiation and the matter. Electromagnetic radiation, of which visible light forms a tiny part, has a double nature, because it consists of a series of energy packets called photons, which behave as waves that are propagated from a source and move in a straight line if they are not reflected or refracted. The undulatory phenomenon is a magnetic field associated with an electric one, whose vectors are orthogonal and propagate at a rate of $c = 3 \times 10^8$ m/s.

The photons' energies E and the waves' frequencies v are proportional the one to each other and their relationship is defined by the Bohr-Einstein's law:

$$E = h \cdot v \quad \text{(Eq. 1.1)}$$

where h is Planck's constant (6.6×10^{-34} J s).

The frequency, (v) measures the times of repetition of the wave's shape in a second, in cycles per second (s^{-1}, or Hertz, for which the symbol is Hz).

Waves can then be characterized by another value, the wavelength λ, which is the distance covered by light during a full cycle. Considering that the speed of the wave is c meters per second and that there are v cycles per second, the frequency v is related to the wavelength λ and velocity c by law:

$\lambda \cdot v = c$ (Eq. I.2)

In spectroscopy, the wavelengths are expressed using different units, aiming to avoid the manipulation of large number in the considered spectral region. Usually centimeter, millimeter, micrometer (1 µm = 10^{-6} m), nanometer (1 nm = 10^{-9} m), angström (1Å = 10^{-10} m) are used. Another unit is generally used in the mid-infrared spectral region, the wavenumber, "$\bar{v}$", defined as the inverse of the wavelength expressed in centimeters. The conversion relationship is $\bar{v}$ *(cm^{-1}) =* $10^7/\lambda$, with λ expressed in nanometers; and λ *(nm) =* $10^7/\bar{v}$, with $\bar{v}$ expressed in cm^{-1}.

Conventionally, wavelength expressed in nanometers will be used for the near-infrared spectral region and wavenumber for the mid-infrared spectral region.

Spectral regions, several of them being of interest for analytical purposes, can be defined as a function of wavelengths, which are related with the energy of the electromagnetic fields by the Eq. 1.1. All the spectroscopic methods, except mass spectrometry, can be classified according to the energy involved during measurement (see Fig. 1.1) [8]:

- X-ray region (wavelengths between 0.5 and 10 nm) is involved in energy changes of electrons of the internal layers of atoms and molecules. The photon energies of X-rays knock out inner shell electrons causing ionization.

- Far-ultraviolet region (10–200 nm) is the zone corresponding to electronic emission from valence orbitals. In the near-UV region (200–350 nm), electronic transitions of the energetic levels of valence orbitals are observed. This spectral region is characterized by the absorption of peptidic bonds in proteins and of molecules presenting conjugated double bonds such as aromatic amino acids of proteins or vitamins such as vitamins A and E. In this wavelength range, luminescence (fluorescence and phosphorescence) may also be observed.

- The visible region (350–800 nm) is another zone where electronic transitions occur. Molecules exhibiting a large number of conjugated double bonds such as carotenoids, chlorophylls, and porphyrins absorb energy in this region. And their absorption properties may be used to evaluate the color of food products.

- The near-infrared (NIR) region (800–2500 nm or 12500–4000 cm^{-1}) is the first spectral region exhibiting absorption bands related to molecule vibrations. This region is characterized by harmonics and combination bands and is widely used for composition analyses of food products.

- The mid-infrared (MIR) region (2500–25000 nm or 4000–400 cm^{-1}) is the main region of vibrational spectroscopy. This region retains information, allowing organic molecules to be identified and the structure and conformation of molecules such as proteins, polysaccharides, and lipids to be characterized. In general, the absorption of an infrared radiation corresponds to an energy change ranging between 2 and 10 kcal mol^{-1}.

- In the microwave region (100 μm–1 cm), absorbed energy is related to molecule rotation. The radiofrequency region (1 cm–10 m) is the region investigated by nuclear magnetic resonance (NMR) and electron spin resonance.
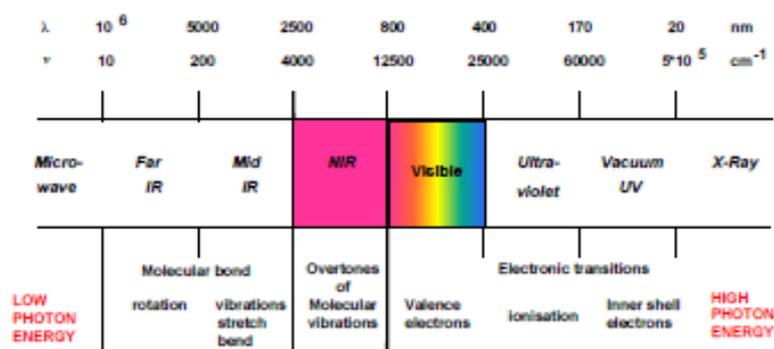
Figure 1.1. - The electromagnetic spectrum [15]

1.2 Vibrational Spectroscopy: basic concepts

Infrared photons have weak energies that correspond to covalent bond stretching and bending vibrations in molecules. There are three different vibrational spectroscopy methods: mid-infrared [MIR], near-infrared [NIR] and Raman. Although the three techniques are very different in several aspects, their basic physical origin is the same: signals in the MIR, NIR, and Raman spectra of chemical compounds can be observed as a consequence of molecular vibrations. However, while Raman spectroscopy is a scattering technique, MIR and NIR spectroscopy are based on the absorption of radiation [9].

1.2.1 The classical mechanical model for a diatomic molecule: the harmonic oscillator (Hooke's law)

The simplest classical model employed to have a didactic insight on the interaction of radiation and matter in the infrared spectral region depicts a diatomic molecule as two spherical masses (*m1 and m2*) connected with a spring with a given force constant (*k*). Hooke´s law states that the energy (*E*) of this system is given by [9]:

$$E = \frac{h}{2\pi} \sqrt{\frac{k}{\mu}} \quad \text{(Eq. 1.3)}$$

where h is Planck's constant and μ is the reduced mass:

$$\mu = \frac{m_1 \cdot m_2}{m_1 + m_2} \quad \text{(Eq. 1.4)}$$

Given d_e as the equilibrium internuclear distance d, and $x = (d - d_e)$ as the displacement coordinate, the potential energy (U) curve of such an oscillator is parabolic in shape and symmetrical about the equilibrium bond length d_e, as showed in Fig. 1.2A and defined by the equation [9]:

$$U = \frac{1}{2} \cdot k \cdot x^2 \quad \text{(Eq. 1.5)}$$
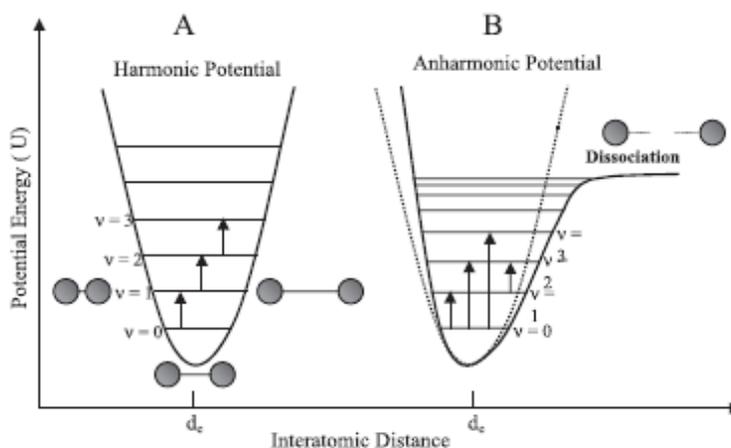
Figure 1.2 - Graph of potential energy as a function of interatomic distance
(A: harmonic model, B: anharmonic model) [15]

Figure 1A shows the behaviour of the potential energy as a function of atom displacement from the equilibrium (minimum energy) position. This first approach is useful to understand the concept of vibrational energy. However, it fails when a microscopic system such as molecules is being considered. The failure arises from the fact that molecular systems can not assume the continuous energy profile predicted by the classical "balls-on-spring" model.

The molecular system can only have certain discrete values of vibrational energy (E_n), defined through a quantum mechanical treatment by the Schrödinger equation [10]:

$$E_n = (n + \frac{1}{2}) \cdot h \cdot v_0 \text{ (Eq. 1.6)}$$

where n is the vibrational quantum number, E_n is the energy associated with the nth quantum level and v_0 is the fundamental vibrational frequency. In the classical model this frequency is defined by:

$$v_0 = \frac{1}{2\pi} \cdot \sqrt{\frac{k}{\mu}} \text{ (Eq. 1.7)}$$

According to the Boltzmann distribution, most molecules at room temperature populate the ground level n = 0, and consequently the allowed, so-called fundamental, transitions between n = 0 and n = 1 dominate the vibrational absorption spectrum. The potential of MIR spectroscopy as a structure elucidation tool is based on the fact that the majority of absorption bands of chemical compounds corresponding to fundamental vibrations occur in this wave number region (4000 to 200 cm−1).

The energy of the electromagnetic radiation that is absorbed in order to promote the molecule to an excited level should match the difference between two adjacent energetic levels. Therefore, the photon energy "E_p" must be [10]:

$$E_p = \Delta E = E_{n2} - E_{n1} = \Delta n h v \text{ (Eq. 1.8)}$$

A disappointing restrictions for NIR spectroscopy of the harmonic model is that the energy levels expressed in Equation 1.6 are equidistant and transitions are only allowed between neighboring energy levels, so it is not possible that Δn is greater than 1 (see Fig. 1.2A):

$$\Delta n = \pm 1 \text{ (Eq. 1.9)}$$

Transitions with Δυ = 2 or greater are forbidden by the harmonic/quantum model and, in this way, most of the observable phenomena in the NIR region, the overtones bands, should not exist.

Also, the vibrations in the harmonic model are independent and their combinations would not exist under the restrictions imposed by the model. Nevertheless, both overtones and combination bands exist.

In conclusion, for the harmonic oscillator $\Delta n = 1$ and $Ep = h\nu$, which matches the predicted equal energy difference between one state and the other of immediately higher energy.

The classical analogue to this behaviour is the concept of resonance. In this concept, the physical characteristics of a "string" stretched between two supporting points, such as its linear density and the force by which it is stretched, will define its natural frequency of vibration (as a guitar string does). The amplitude of this natural vibration (therefore, its energy) can be increased by exposing the string to an acoustic wave propagating in the air, with the same frequency, produced, for example, by a distant stroked string with the same characteristics. The first string undergoes no energy change if the acoustic wave frequencies and the natural frequency do not match each other. Similarly, only radiation of a certain frequency (and wavelength) can excite the vibrational levels of molecules.

However, this model fails in the molecular world because it is not a quantum model. In the "string world" , the energy they can obtain from the exciting mechanical wave can increase continuously while a "quantum string" is able to vibrate at only a given frequency and at only some pre-defined amplitude.

Polyatomic molecules

As a rough estimate, the vibrational movements of two atoms of a diatomic molecule can be considered to be like the compression and extension movements of a spring— the atoms can attract or push away, as described by the harmonic model.

Considering a molecule with N atoms, each atom can be located by three coordinates: x , y , and z . The molecule consequently has 3 N characteristic coordinates or 3 N degrees of freedom or 3 N fundamental vibrations or 3 N vibration modes. If the values of these coordinates were constants, the molecule would be " frozen " and the bond lengths and values for the stretching angles would be constant. However a molecule can move and deform in the space at room temperature. The degrees of freedom are split in three groups corresponding to translation, vibration and rotation. A translation movement requires three degrees of freedom among the 3 N ones, allowing 3 N - 3 degrees. If the molecule is non-linear, three additional degrees of freedom, associated with the three orthogonal axes, are necessary to describe rotation movements, leading to 3 N - 6 degrees or fundamental vibrations [10].

A normal mode of vibration of a polyatomic molecule can be defined as a state of vibration where each atom has a simple harmonic movement around its equilibrium position. Each atom of the molecule exhibits the same oscillation frequency and in general, the oscillations are in phase. Figure 1.3 shows the vibration modes for a nonlinear molecule, the water.
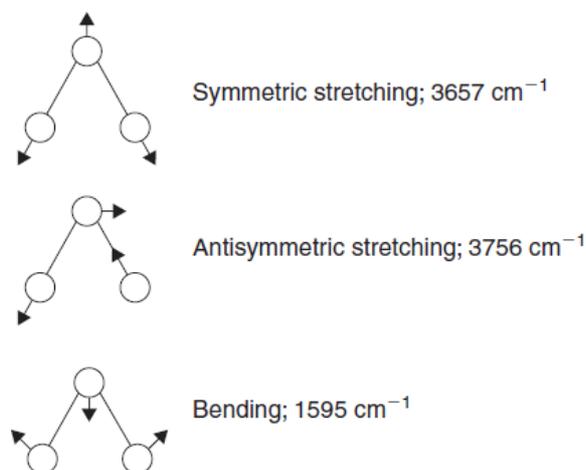
Figure 1.3 - Normal vibration modes for a water molecule [15].

A molecule may exhibit one (or more) plane of symmetry. Water molecules present an axis of symmetry, C_2, and two planes of symmetry (Figure 1.4).
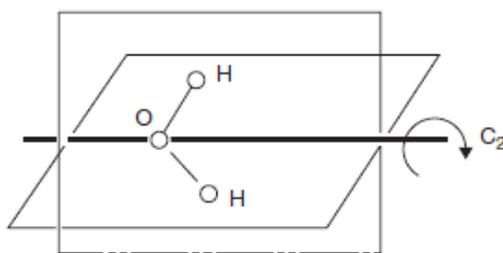
Figure 1.4 - Axes of symmetry and planes for water molecule [15].

A consequence of the plane of symmetry is the existence of symmetric and antisymmetric vibrations (Figures 1.3). By convention, the vibrations are classified according to the wavenumber and as a function of their degrees of symmetry. In that way, the symmetric stretching vibration of water exhibiting the highest frequency ($3652 \ cm^{-1}$) is called ν1 . The symmetric bending vibration observed at $1590 \ cm^{-1}$ is named ν2 , and the antisymmetric bending vibration at $3755 \ cm^{-1}$ is called ν3 . These three frequencies, found in the infrared spectrum of water, are fundamental frequencies.

11

In general, the bonds between light atoms vibrate at higher frequencies than the bonds between heavy atoms. It is observed for carbon atom bound to another atom: when the reduced mass, μ , increases, the frequency decreases. The frequencies of C–H, C–D, C–O, C–Cl, and C–Br bonds are 3000, 2280, 1100, 800, and 550 cm^{-1}, respectively. However the strength constant, k , of the bond also has to be taken into account. For example, due to a higher strength constant, the H–F bond vibrates at a higher frequency than the C–H one. The strength constant also changes as a function of the type of bond: the value of the strength constant for the C=C bond is about twice that of the C–C one. As a consequence, the vibration frequency of C=C is located at 1650 cm^{-1}, compared with 1200 cm^{-1} for C–C. It has also been demonstrated that bending movements are less energetic than stretching ones. In that way, the bending frequency of C–H bond is close to 1340 cm^{-1} , whereas its stretching frequency is observed at about 3000 cm^{-1}.

The intensity of the bands is related to the nature and polarity of the bond. Indeed, the C=O bond, formed by different atoms and highly polarized, strongly absorbs in the MIR region, while C=C bond absorbance in the MIR region is much weaker [11].

1.2.2 The anharmonic model (Morse's curve)

Figure 1.2 B shows a more realistic mechanical model for a diatomic molecule. The molecule is still approximated by two balls connected with a spring. However, the model considers some non-ideal behaviours of the oscillator, taking into account the repulsion between electronic clouds when the atomic nuclei approach (notice how the potential energy rises fasten than in the harmonic model) and a variable behaviour of the bond force when the atoms move apart from one another. In fact, in a real molecule, the over displacement ("strengthening of the spring") of the atomic nuclei will cause molecule bond rupture with consequent dissociation of the atoms. A complex function of the potential energy is assumed to describe the last effect which can be approximated by using higher order terms of displacement, as depicted in the equation [12]:

$$U = k_1 x^2 + k_2 x^3 + k_3 x^4 + .. \quad \text{(Eq. 1.10)}$$

A function that approximates the anharmonic behaviour of a diatomic molecule is the Morse function that describes the potential energy of the molecule using the equation:

$$U = D_e \cdot \left[1 - e^{-a \cdot (d - d_e)} \right]^2 \quad \text{(Eq. 1.11)}$$

where a is "*a*" constant for a given molecule, "D_e" is the spectral dissociation energy, "d_e" is the equilibrium distance between the atoms and "*d*" is the distance between the atoms at any instant.

Applying quantum mechanics to the Morse equation results in the vibrational levels being described by the equation [12]:

$$E_n = h \cdot v \cdot \left(n + \frac{1}{2} \right) - X_m \cdot h \cdot v \cdot \left(n + \frac{1}{2} \right)^2 \text{ (Eq. 1.12)}$$

in which "X_m" is the anharmonicity constant of the vibration, whose value is between 0.005 and 0.05.

Unlike the harmonic oscillator, energy levels are no longer equidistant and the strict selection rule of Equation 1.9 is expanded to transitions over more than one energy level (Eq. 1.13).

Furthermore, the potential energy curve is represented by an asymmetric Morse function, as shown in Figure 1.2B. Generally, a nonlinear molecule containing N atoms will have 3N – 6 vibrational degrees of freedom, while a linear molecule has only 3N − 5. The number of vibrational degrees of freedom represents the number of fundamental vibrational frequencies of the molecule or the number of different "normal modes" of vibration. For a given molecule, a normal mode of vibration corresponds to internal atomic motions in which all atoms move in phase with the same frequency, but with different amplitudes. Additionally to these normal vibrations transitions corresponding to:

$$\Delta n = \pm 2, \pm 3 \text{ (Eq. 1.13)}$$

are now also allowed and are called first, second, and so on, overtones.

The anharmonic/quantum model also predicts that the separation between two adjacent energy levels decreases with *n*, the vibrational quantum number.

The intensities of overtone absorption bands depend on the anharmonicity, and it has been shown that vibrations with low anharmonicity constants also have low overtone intensities.

X─H stretching vibrations, for example, have the largest anharmonicity constants and therefore dominate the spectra in the NIR region. Table 1.1 summarizes the anharmonicity constants of the vibrations of some characteristic functionalities [12].

$\chi \nu(CH)$	$\sim 1.9 \times 10^{-2}$
$\chi \nu(CD)$	$\sim 1.5 \times 10^{-2}$
$\chi \nu(CF)$	$\sim 4 \times 10^{-3}$
$\chi \nu(CCl)$	$\sim 6 \times 10^{-3}$
$\chi \nu(C=O)$	$\sim 6.5 \times 10^{-3}$

Table 1.1 - Anharmonicity Constants χ for Selected Vibrations [15].

Apart from overtones, combinations of different vibrational transitions (sum and difference "tones") may also be observed. In fact, under the assumptions of the anharmonic model, the vibrations are no longer independent of each other and can interact with one another. Therefore, the total vibrational energy (E_n) contains cross-terms from more than one vibration in the molecule [12]:

$$E_n = \sum h \cdot v_r \cdot \left(n_r + \frac{1}{2} \right) - \sum \sum hX_{rs} \left(n_r + \frac{1}{2} \right) \left(n_s + \frac{1}{2} \right) \text{ (Eq. 1.14)}$$

for $r \leq s$; in which v_r and n_r are the fundamental frequency and the quantum number of vibrational mode r, respectively, and X_{rs} is the anharmonicity constant for the interaction of vibrational modes r and s.

These two types of bands, the overtones and the combination bands, are the most common absorption bands in the NIR spectral region.

However, the probability of these transitions decreases significantly with their order, and generally the absorption bands corresponding to overtone or combination vibrations have much lower intensity than their fundamental analogs. Contrary to the MIR, the NIR region contains almost exclusively absorption bands that can be assigned to overtone and combination vibrations.

Unfortunately, the overlap of these overtone and combination bands strongly decreases the specificity of NIR spectroscopy (especially for interpretation purposes) and was one of the main reasons why this technique has been neglected by conservative spectroscopists for such a long time. However, the availability of (a) chemometric evaluation procedures for qualitative discrimination and quantitative determination [25–28] and (b) the perception that the low band intensities can be advantageously exploited in terms of larger sample thicknesses and therefore much easier sample handling has eventually led to the breakthrough of the NIR technique [12].

1.3 Origin and intensity of a NIR absorption band

So far, it is possible to understand from theory that radiation of a given frequency, capable to supply exactly the energy between two vibrational levels or of their overtones or combinations of two or more vibrations, can be absorbed by the molecule and can produce excitation to a higher vibrational energy level. The match of radiation energy with the energy difference between two vibrational levels causes a selective response of the molecular system to the incident radiation. It means that in a given wavelength range, some frequencies will be absorbed, others (that do not match any of the energy differences possible for that molecule) will not be absorbed while some will be partially absorbed. This complex figure of the intensity of absorption versus wavelength constitutes the absorption spectra of a substance or sample.

However, only the energy match between photons and vibrational levels is not sufficient for radiation absorption.

For a vibration to be active, it is necessary that the electrical oscillating field of the electromagnetic wave (light) can interact with the molecule. This can only occur if the displacement of the atoms in a vibrational mode can produce a change in the dipole moment of the molecule or in the local group of vibrating atoms.

Moreover, interaction of infrared radiation with a vibrating molecule, however, is only possible if the electric vector of the radiation oscillates with the same frequency as the molecular dipole moment, μ (selection rule). Thus, a vibration is infrared active only if the molecular dipole moment is modulated by the vibration and [9]:

$$\frac{\partial \mu}{\partial q} \neq 0 \quad \text{(Eq. 1.15)}$$

where q is the vibrational coordinate. The requirement of a dipole moment change during the vibration makes MIR spectroscopy specifically sensitive to polar functionalities.

While this is true considering a fundamental mode, it is worth noting that, for combination bands permitted by anharmonicity, it would be necessary that only one of the combining vibrations be active (causing dipole change). This feature may cause some vibrations, which can not be observed in the middle infrared, to be displayed by a NIR spectrum.

The intensity of a given absorption band is associated with the magnitude of the dipole change during the displacement of atoms in a vibration and with its degree of anharmonicity. Both

phenomena are present in great intensity associated with bonds involving the hydrogen atom and some other heavier element such as carbon, nitrogen and sulphur. The O-H, C-H, N-H and S-H bonds tend to present high anharmonicity and high bond energy with fundamental vibrational transitions in the region of 3000 – 4000 nm. Therefore, it allows to predict the overtones and combinations of the fundamental vibrations of such bonds to occur in the region of energy associated with NIR photons, as shown in Fig. 1.5. Intensities are in between 10, for combinations, up to 1000, for successive overtones, times lower than the absorption resulting from fundamental vibrations [13].
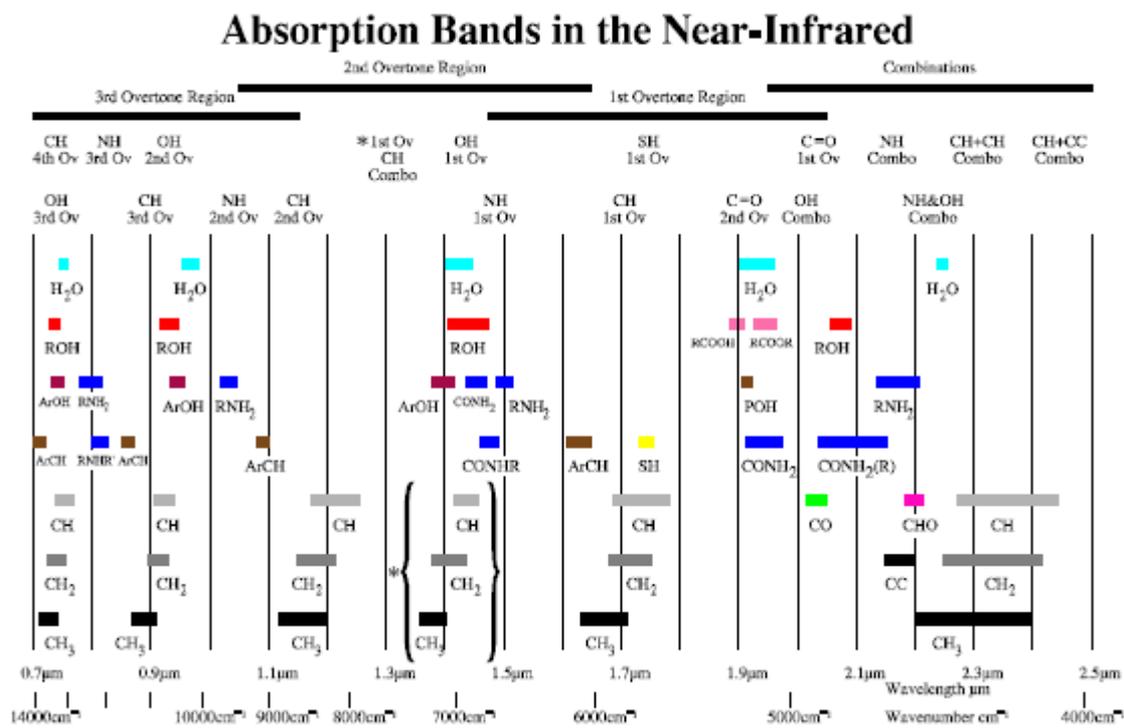
Fig. 1.5 - Absorpition Bands in the Near-Infrared [13]

The spectral occurrences in the NIR region are dominated by overtones and combination absorption bands. However, some other , characteristic effects, associated with the higher order terms in equation 1.10, may be observed and contribute to the complexity of the NIR spectrum. These effects are called resonance. Coupling or resonance between different vibrations of the same functional group can occur as a function of the third and fourth order terms of equation 1.10.

A resonance that leads to a perturbation of the energy levels can occur if two vibrational levels belong to the same symmetry species and have similar energy. Such an accidental degeneracy of, for example, an overtone or a combination band that has the same symmetry and nearly the same

16

frequency as that of a fundamental vibration is called Fermi resonance. This kind of resonance occurs between a fundamental and an overtone when their difference in energy is very low, and it leads to two relatively strong absorption bands that are observed at somewhat higher and lower frequencies than the expected unperturbed frequency positions. When this perturbation takes place, the weaker absorption in the spectrum "steals" intensity from the stronger one, so typically this type of resonance results in a greater separation between the position of the two bands and in the intensification of the overtone or combination band. Typical examples of Fermi resonance have been analyzed for the NIR spectra of CO_2, but this phenomenon has also been reported for numerous other compounds [14].

Darling-Dennison resonance may promote the interaction between two high level overtones of a molecule and a combination band, for example, and is particularly intense in the NIR spectra of water, but it can also occur in other molecules containing symmetrically equivalent X—H bonds. Thus, of the three normal modes of water ($v2$ bending vibration (1595 cm−1), $v3$ antisymmetric stretching (3756 cm−1), and $v1$ symmetric stretching (3657 cm−1)) the two stretching vibrations absorb at similar wave number positions but belong to different symmetry species and therefore cannot interact directly. However, energy levels of these vibrations associated with specific vibrational quantum numbers n1, n2, and n3 can interact if they belong to identical symmetry species and have similar energies. These interactions then lead to several pairs of NIR absorption bands with appreciable intensities.

The complexity of the combination spectral region in the NIR spectrum of hydrocarbons is partly due the possibility of resonance between the combination bands and high order overtone for C-H bonds.

The primary practical consequence of both types of resonance on a NIR spectrum is the possibility of the appearance of two instead of one band in the combination region (1600 – 2500 nm).

Finally, a few comments shall be made on the concept of local modes as compared to normal modes. The main idea of the local mode model is to treat a molecule as if it were made up of a set of equivalent diatomic oscillators, and the reason for the local mode behavior at high energy (>8000 cm−1) may be understood qualitatively as follows. As the stretching vibrations are excited to high energy levels, the anharmonicity term $\chi^- v0$ (Equation (2.9)) tends, in certain cases, to overrule the effect of interbond coupling and the vibrations become uncoupled vibrations and occur as "local modes" [14].

The absorption bands in the spectrum can thus be interpreted as if they originated from an anharmonic diatomic molecule. This is the reason why NIR spectra are often said to become simpler at higher energy. Experimentally, it is found that the inversion from normal to local mode character occurs for high energy transitions corresponding to $n \geq 3$.

1.4 A comparison of the qualitative and quantitative aspects of RAMAN, MIR, and NIR spectroscopy

The different excitation conditions of Raman, MIR, and NIR spectroscopy lead to extremely different signal intensities of these techniques for the same vibration of a specific molecular functionality.

Whereas scanning MIR and NIR spectrometers operate with a polychromatic source for the individual frequency range from which the sample absorbs specific frequencies corresponding to its molecular vibrational transitions (mostly fundamental vibrations for the MIR and overtone or combination vibrations for the NIR), in Raman spectroscopy the sample is irradiated with monochromatic laser light whose frequency may vary from the VIS to the NIR region, and more precisely:

1. Excitation by a VIS-laser (in the range from 400 to 800 nm) combined with monochromatization of the scattered radiation by a holographic grating and simultaneous detection of the dispersed, narrow frequency ranges by a charge-coupled device (CCD) detector.

2. NIR-laser excitation (1064 nm) and measurement in a Fourier-Transform (FT) spectrometer.

Both of these alternatives establish only compromises and the choice of the applied technique depends on the individual problem.

If a molecule is irradiated with visible radiation, it may be excited to an energy level of the next higher electronic state. Return to the ground state or an excited vibrational level of the original electronic state can easily proceed via fluorescence. Thus, for a large proportion of samples, irradiation with visible light causes strong fluorescence by additives or impurities (or by the sample itself), which will superimpose and in many cases inundate the Raman spectrum of the sample.

The use of NIR-laser excitation confers a number of advantages on a Raman system. Both fluorescence and self-absorption are very much reduced in the Raman signal, and, owing to the lower energy of the excitation radiation, thermal degradation is also less of a problem. However, these advantages are partly neutralized by the disadvantages of using a low-frequency laser as the

source. The NIR-Raman technique is obviously less sensitive due to the ν^4-dependence of the scattering efficiency [15]:

$$I_{Raman} \approx \nu^4_{exc} \text{ (Eq. 1.16)}$$

This radiation excites the molecule to a virtual energy state that is far above the vibrational energy levels of this anharmonic oscillator for a VIS-laser and in the range of high overtones for an NIR-laser excitation. From the excited energy level, the molecule may return to the ground state by elastic scattering, thereby emitting the Rayleigh line that has the same frequency as the excitation line and does not contain information in terms of the molecular vibration. If it returns to the first excited vibrational level by inelastic scattering, the emitted Raman line (so-called Stokes line) has a lower frequency (wave number), and the difference to the excitation line corresponds to the energy of the fundamental transition that can also be observed as an MIR absorption band. In the case of the anti-Stokes line, where the starting level is the first excited vibrational state and the molecule returns to the ground state by inelastic scattering, the emitted Raman line is of higher frequency (here too, the frequency difference to the excitation line corresponds to the fundamental transition) but of lower intensity compared to the Stokes line, due to the lower population of the excited state (law of Boltzmann). Commonly, the Stokes lines are used for practical Raman spectroscopy.
One of the limiting factors for the application of the Raman technique, however, becomes evident by comparing the intensity of the laser source and the scattered radiation, because a sensitive detection of the Raman line alongside an efficient elimination of the Rayleigh line are experimental prerequisites for the successful application of Raman spectroscopy[15]:

$$I_{Raman} \approx 10^{-4} I_{Rayleigh} \approx 10^{-8} I_{source} \text{ (Eq. 1.17)}$$

Raman and MIR spectroscopy cover approximately the same wave number region, with the Raman technique extending further into the far-infrared (FIR) region (down to about 50 cm^{-1}), but these techniques complementary because of different selection rules. In fact, a molecular vibration can be observed in the Raman spectrum if there is a modulation of the molecular polarizability "α" along the vibrational coordinate "q" [15].

$$\frac{\partial \alpha}{\partial q} \neq 0 \text{ (Eq. 1.18)}$$

Hence, Raman spectroscopy is primarily sensitive to vibrations of homonuclear functionalities (e.g., C==C, C--C, S--S), whereas the most intense MIR absorptions can be traced back to polar groups (e.g., C--F, Si--O, C==O, and C--O--C). For this reason, the application of these two complementary techniques can be very helpful for the efficient elucidation of a molecular structure.

On the other hand, NIR spectroscopy covers the wave number range adjacent to the MIR and extends up to the VIS region (4,000 to 12,500 cm^{-1}). NIR absorptions are based on overtone and combination vibrations of the investigated molecule, and owing to their lower transition probabilities, the intensities usually decrease by a factor of 10 to 100 for each step from the fundamental to the next overtone. Thus, the intensities of absorption bands successively decrease in the direction from the MIR to the visible region, thereby allowing an adjustment of the sample thickness (from millimeters up to centimeters), depending on the rank of the overtone.
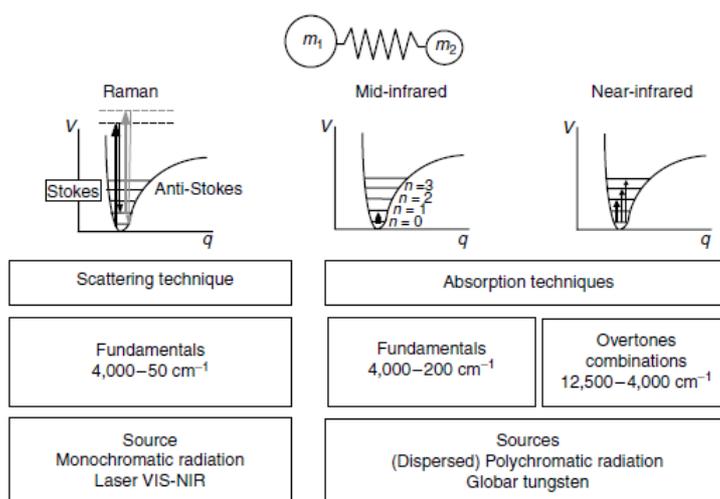
Figure 1.6 - The principles of Raman, MIR, and NIR spectroscopy [15]

This is a characteristic difference to MIR and Raman spectra, where the signal intensities of the fundamental vibrations vary irregularly over the whole frequency range and depend exclusively on the excitation conditions of the individual molecular vibrations, leading to the complementarity of the Raman and MIR technique as structural elucidation tools.

NIR spectroscopy, on the other hand, requires, in addition to the dipole moment change, a large mechanical anharmonicity of the vibrating atoms. This becomes evident from the analysis of the NIR spectra of a large variety of compounds, where the overtone and combination bands of CH,

OH, and NH functionalities dominate the spectrum, whereas the corresponding overtones of the most intense MIR fundamental absorptions are rarely represented.

One reason for this phenomenon is certainly the fact that most of the X−H fundamentals absorb at wave numbers >2000 cm^{-1} so that their first overtones already appear in the NIR frequency range.

The polar groups leading to the most intense fundamental absorptions in the MIR (e.g., ν(C−F), ν(C=O), ν(Si−O)) on the other hand absorb at wave numbers <2000 cm^{-1}, so that their first (and sometimes higher) overtones still occur in the MIR region. Owing to the intensity loss for each step from the fundamental to the next overtone, the absorption intensities of these vibrations have become negligible by the time they should occur in the NIR range. The best example in this respect is the ν(C−F) absorption band at about 1200 cm^{-1} (e.g., of poly[tetrafluorethylene]), which is one of the most intense absorption bands in the MIR owing to the large dipole moment of the C−F bond. However, because of the small anharmonicity constant (see Table 1.1), the first and the second overtones that are expected at about 2400 and 3600 cm^{-1}, respectively, have already strongly reduced intensity, and no further overtone vibrations of this functionality can be observed in the NIR region.

In fact, poly(tetrafluorethylene) is used as a non-absorbing standard material (Spectralon®) for the NIR region.

Anharmonicity plays also an important role in the evaluation of the fundamental and overtone vibration intensities of functionalities with a high hydrogen-bonding tendency such as ν(O−H) and ν(N−H). In fact, owing to its larger anharmonicity, the intensity of the ν(N−H)$_{free}$ overtone absorption is strongly enhanced relative to the corresponding overtone vibration of the associated N−H-groups. Hydrogen bonding is equivalent to increasing the mass of the vibrating H-atom, thereby leading to a reduction of mechanical anharmonicity of the ν(N−H)$_{assoc}$ vibration and a decrease of its absorption intensity. The uncontrolled use of absorption intensities without proper care for their absorptivities (a) in Beer's law would therefore lead to dramatic errors in the estimation of the extent of hydrogen bonding.

The superposition of many different overtone and combination bands in the NIR region causes a very low structural selectivity for NIR spectra compared to the Raman and MIR analogs where many fundamentals can usually be observed in isolated positions. Nevertheless, NIR spectra should also be assigned in as much detail as possible with reference to their molecular origin; this allows a more effective application for research purposes and combination with chemometric evaluation procedures. For the assignment of overtones and combination bands in the NIR to their corresponding fundamentals in the MIR, it is recommended that the wave number notation be used instead of the widespread wavelength (nm or µm) scale. It should be mentioned, however, that the

wave-number positions of the overtones deviate with increasing multiplicity from the exact multiples of their fundamentals owing to the anharmonicity of the vibrations [15].

Raman

$$\frac{\partial \alpha}{\partial q} \neq 0$$

Polarizability

| Homonuclear e.g., C=C | Functionalities | Polar e.g., C=O |

High structural selectivity

$I_{Raman} \sim c$

MIR

$$\frac{\partial \mu}{\partial q} \neq 0$$

Dipole moment

NIR

$$\frac{\partial \mu}{\partial q} \neq 0 / \text{Anharmonicity} \quad m_2 \ll m_1$$

Anharmonicity

CH/OH/NH Functionalities

Low structural selectivity

$$\log \frac{I_0}{I} = A = a \cdot b \cdot c \quad \text{(Beer's law)}$$
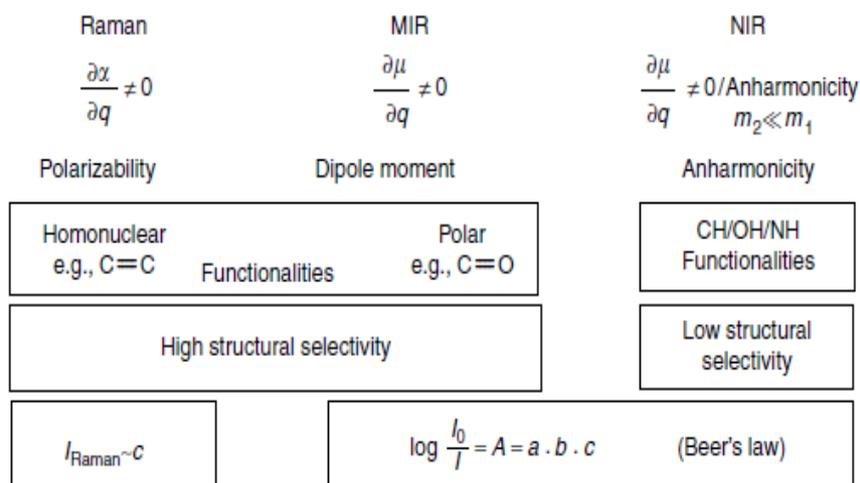
Figure 1.7 - Specific characteristics of Raman, MIR, and NIR spectroscopy [15]

As far as the quantitative evaluation of vibrational spectra is concerned, MIR and NIR spectroscopy follow Beer's law:

$$A = \log \frac{I_0}{I} = a \cdot b \cdot c \quad \text{(Eq. 1.19)}$$

Where I_0 is the incident radiation; I is the transmitted radiation; A is the absorbance; a is the absorptivity; b is the sample thickness and c is the sample concentration

The Raman intensity I_{Raman} is directly proportional to the concentration of the compound c to be determined. To avoid compensation problems, in most cases, quantitative Raman spectroscopy is performed with an internal reference signal in the vicinity of the analytical absorption band being analyzed.

An important issue for the implementation of a technique as an industrial routine tool is the sample preparation required for this technique. In this respect, Raman and NIR spectroscopy have considerable advantages over MIR spectroscopy, which usually requires individual sample preparation steps before data acquisition. Only the technique of attenuated total reflection (ATR) circumvents time-consuming sampling procedures for MIR spectroscopy [15].

1.5 NIR instrumentation

1.5.1 Sources

a) Halogen lamps

NIR spectrometers operate with a polychromatic source, which most commonly are tungsten-halogen lamps. In fact, these lamps remain the overwhelming favorite choice among NIR sources due to theirs good performance characteristics at reasonable cost. Lamps for low voltage operation are particularly rugged and reliable, although the filament may be vibration sensitive.

Tungsten-halogen lamps are filled with a halogen gas to extend the life by recycling evaporated tungsten back to the filament, thereby avoiding premature bulb blackening and failure. The quartz envelope must operate at sufficiently high temperature to maintain this cycle. The life of a lamp is a function of filament design and operating temperature. Increasing the voltage 10% increases the output and shifts the energy peak to shorter wavelengths while reducing the lamp life to approximately 30% of the rated life. Decreasing the voltage 10% may increase the lamp life by a factor of four at the cost of reduced output intensity, particularly in the visible region. Further voltage reduction may cause failure of the halogen cycle and reduce lamp life. In the tungsten filament spectra, which are a function of both the temperature of the filament and the emissivity of tungsten, peak in the very near infrared (VNIR), between 800 and 1000 nm, drops rapidly towards the blue and more slowly towards longer NIR wavelengths. Lamp temperature variation is a potential source of significant baseline drift in the measurement. Temperature stabilization or frequent baseline reference measurements can minimize this drift.

b) Alternative NIR sources

b.1) Light emitting diodes (LEDs)

Light emitting diodes (LEDs) are an attractive source because of their efficiency, small size, and cool operation. These devices can produce NIR radiation with a band width of about 30 - 50 nm, centered in any wavelength of the spectral region. The instruments can employ a set of LEDs as sources of narrow bands of near infrared radiation or use them to produce a polychromatic, highly stable source whose radiation is dispersed by using common monochromator devices such as those based on gratings or filter optics.

More extensive use of LED sources has been limited by the unavailability of longer NIR wavelengths, thus, this technology is primarily useful in the very near infrared (VNIR) region below 1100 nm. In fact, common LEDs can be purchased at low prices for the shorter NIR wavelength range (700 – 1100), although the LEDs operating at higher wavelengths are still expensive [16].

b.2) Tunable lasers

As a source, tunable lasers provide very high intensity, narrow band radiation, particularly useful for in-vivo multi-spectral imaging. The spectral range of a specific laser is limited, which may present problems in some applications. The high cost and complexity of such sources has restricted their use primarily to research applications.

1.5.2 Wavelength selection

From a polychromatic source, contrary to Raman and MIR spectroscopy, scanning NIR spectroscopy offers the largest multiplicity of monochromator/detection principles. In fact, the modern NIR instruments can be classified in terms of the technology employed for wavelength selection, as shown in Table 1.2.

I. Filter Instruments
  Fabri-Perrot (interference)
  Acousto-Optic Tunable Filter (AOTF)

II. LED source self band selection instruments

III. Dispersive
  Grating - Plane or Concave

   Single Beam
   Dual Beam
   Multichannel (detector array)
   Multiplexed (Hadamard)

IV. Interferometric (Fourier Transform)

Table 1.2 - NIR Instrument Classification Based on Wavelength Selection Technology [19].

a) Filter Instruments

a.1) Interference (Fabri-Perrot) filters photometers

The simplest and cheapest NIR instruments are filter photometers. Photometers are distinguished from the other types of spectrometer that will be considered, which are all spectrophotometers, because they do not produce a continuum spectrum. A photometer uses filters mounted in a rotating wheel to select small ranges of wavelengths in the spectrum (Fig. 1.8). The filter wavelengths are chosen depending on the desired analysis, for example protein, moisture, and oil.

Filter photometers lack the flexibility of other instrument types, and can be prone to errors if the temperature changes. They have the advantage of low cost, however, and can be useful for dedicated analysis either in the laboratory or on-line in a production facility [17].
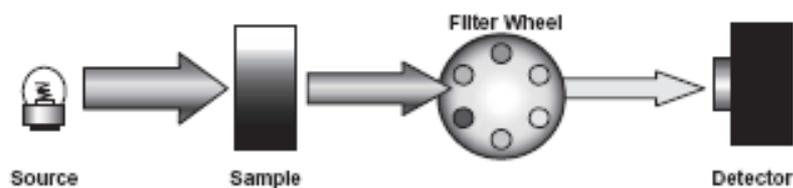
Figure 1.8 - Schematic representation of filter photometer operation [19].

a.2) Acousto-Optical Tunable Filters (AOTF) spectrophotometers

Filters (AOTF) are modern scan spectrophotometers employing a technology that allows constructing instruments with no moving parts, capable of reaching very high scan speeds over a broad range of the NIR spectral region. If necessary, random access to any number of wavelengths necessary to perform a given analytical determination can be easily implemented. Scan speed is fast and up to 2000 wavelengths can be selected per second. The scan speed is usually limited by the detector response time.

The AOTF operating in a non-collinear configuration (acoustic wave and radiation beam propagating at almost perpendicular angles), shown schematically in Figure 1.9, is a device made of a birefringent crystal of TeO_2, cut in a special angle. Other materials can be used but the characteristics of TeO_2 are suitable for the NIR regions and has been chosen by all instrument manufacturers. A piezoelectric material (usually $LiNiO_4$) is attached to one end of the crystal which, under excitation from an external radio frequency signal (rf), produces a mechanical (acoustic) wave which propagates through the crystal. The acoustic wave produces a periodic variation of the refractive index of the crystal in a frequency determined by the rf signal, in the range of 50 to 120 MHz. The interaction of the electromagnetic wave and the acoustic wave causes the crystal to refract selectively a narrow wavelength band.

25

The birefringence of the TeO_2 crystal leads to the production of two monochromatic beams whose angular separation is about 7°. Both or only one diffracted beam can be used by NIR instruments. Dual beam (with a reference beam produced by splitting one of the beams) or single beam instruments can be found. Alternatively, the two monochromatic beams can be employed for the construction of a dual beam instrument with an optimized use of the radiation throughput.

The non-moving parts concept of the AOTF-based NIR spectrophotometers impart to them some unbeatable qualifying characteristics for use in the field or on the factory floor, aiming at in-line monitoring. The wavelength precision is about ±0.05 nm and the resolution is dependent on the wavelength, with typical values in the range 5 to 15 nm for the wavelength in the range 1000 to 2500 nm [18].
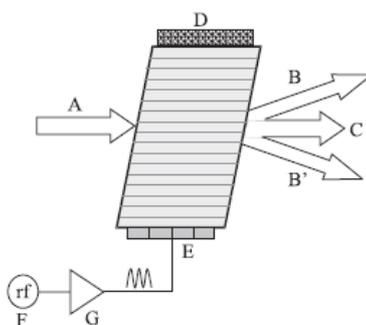
Figure 1.9 - Monochromator based on an Acousto-Optic Tunable Filter.
A, incident polychromatic radiation; B and B', monochromatic beams (same wavelength); C,
remaining polychromatic radiation; D, acousto absorber; E, piezoelectric transducer; F, generator
of radio-frequency signal, and G, radio frequency amplifier [19].

b) Light emitting diodes (LEDs) source self selecting band instruments
See sources.

c) Dispersive spectrophotometers

c.1) Scanning Dispersive Spectrophotometers
Dispersive instruments based on diffraction gratings were employed in the early days of NIR spectroscopy and were responsible for the research initially developed to consolidate NIR spectroscopy as an analytical tool.

In this type of spectrometer broadband light is directed to the sample, and the transmitted or reflected light is then passed through a narrow slit. A diffraction grating then disperses the light into separate wavelengths, which are scanned across an exit slit by moving the grating. The discrete wavelengths that pass through the exit slit are sequentially measured by the detector.

Figure 1.10 shows the principle of operation of a scanning dispersive spectrometer.

Like the other types of spectrophotometer, these instruments are flexible and offer a full range of analytical techniques. Some high-end instruments with double beams are also capable of very high photometric precision. Moreover, the instruments based on grating monochromators present the advantage of a relatively low cost when compared with other scanning instruments employing modern technologies. The main disadvantages of dispersive instruments are the slow scan speed and a lack of wavelength precision, which deteriorates for long term operation due to mechanically driven mechanism fatigue.

This can affect instrument stability, existing calibration models, and make transfer of a calibration model to a new instrument difficult.

Also, the presence of moving parts limits the use of dispersive instruments in the field and in more aggressive environments [19].
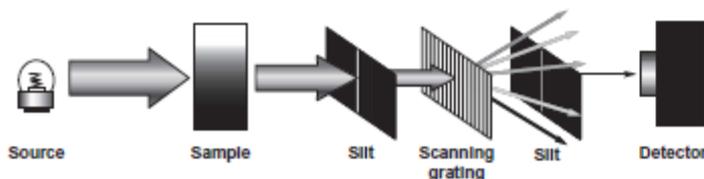
Figure 1.10 - Schematic representation of the principles of a scanning dispersive spectrophotometer [19].

c.2) Detector Array Dispersive Spectrophotometers

On the other hand, recent evolution in sensor production technology gives dispersive optics a longer life. In fact, in a detector array dispersive spectrometer the scanning grating, exit slit, and detector in Figure 1.10 are replaced by a stationary grating and a detector array. Since different wavelengths fall on different detector elements they are measured almost simultaneously, so detector array dispersive spectrometers can be very fast. Today is possible to construct linear arrays of PbS and InGaAs sensors containing up to 256 independent elements. Placed in the focal plane of plane or concave grating optics, the sensor array allows to scan an entire spectra in a few milliseconds, without the use of moving parts. The limited number of elements in the array, however, means that

this type of spectrometer is usually either low resolution, or only covers a limited portion of the spectrum. With no moving parts, these instruments are also very rugged. The dispersive optics can also be improved regarding the signal-to-noise ratio, by making use of a Hadamard multiplexed design spectrophotometer, which permits a theoretical gain in the signal-to-noise ratio of an individual signal after Hadamard transformation[19].

d) Fourier transform (FT) NIR spectrophotometers

A schematic representation of a Fourier transform instrument is shown in Figure 1.11. The broadband light source is directed to a Michelson interferometer. The interferometer consists of a beamsplitter, a fixed mirror, and a mirror that moves back and forth very precisely.

The beamsplitter reflects half of the light to the fixed mirror, and transmits the other half to the moving mirror. Both the fixed and moving mirror will direct the light back to the beamsplitter, where they interfere. This interference changes as the moving mirror is displaced, and therefore the intensity of the light at the detector changes.
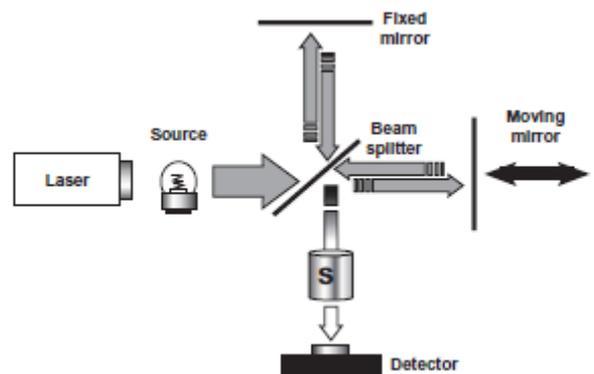
Figure 1.11 - Schematic representation of an interferometer in a FT-NIR system [19].

The intensity as a function of mirror displacement is called an interferogram, which must then be Fourier transformed to obtain the spectrum. $I(t) = \Gamma(v)\cos(4\pi vvt)$. The detector signal intensity for a broadband source is [19]:

$$I(t) = \Gamma(v)\cos(4\pi vvt) \quad \text{(Eq. 1.20)}$$

The mirror velocity v of interferometers is generally chosen so that the modulation frequency (2vv) is in the audio range. For example, a typical mirror velocity for a rapid scanning interferometer is v

= 0.64 cm s^{-1}. If the interferometer covers the spectral range of 10,000 to 4,000 cm^{-1}, then the modulation frequencies the detector must respond to lie in the range of 12.8 to 5.1 kHz. The interferogram of a broadband source is represented by

$$I(t) = \int_{-\infty}^{+\infty} \Gamma(v)\cos(4\pi v v t)dt' \quad \text{(Eq. 1.21)}$$

The spectral response F(v) of the interferometer can be calculated using the cosine FT pair of the preceding equation:

$$\Gamma(v) = \int_{-\infty}^{+\infty} I(\tau)\cos(4\pi v v t)d\tau' \quad \text{(Eq. 1.22)}$$

Fourier transform spectrometers offer high resolution, good speed, and high signal-to-noise ratios. Their biggest advantage, however, stems from the fact that the position of the moving mirror is controlled using a HeNe laser. The inherent wavelength stability of the laser results in very high wavelength accuracy and precision.

This in turn means a calibration model is very stable over time, and permits easy transfer of calibration models between instruments.

Spectrophotometers based on the use of interferometers and Fourier transform to recover the intensities of individual wavelengths in the NIR region are, undoubtedly, the instruments combining most of the best characteristics in terms of wavelength precision and accuracy, high signal-to-noise ratio and scan speed (although slower than AOTF based instruments).

In principle, an interferometer-based spectrometer has several basic advantages over a classical dispersive instrument:

1. Multiplex advantage (Fellgett advantage). In a dispersive instrument, a wavelength is measured, the grating is moved, and another wavelength is measured sequentially. If a scan takes time T, and m spatial elements are examined, the wavelength element is examined for $\Delta t = T/m$.

The more spatial elements (higher resolution), the smaller amount of time that the wavelength is measured. Therefore in a dispersive instrument, assuming the main source of noise is the detector, signal-to-noise is reduced by $\sqrt{m}$. All frequencies in the spectra are measured simultaneously in a FT-NIR spectrometer for the entire time T. This is because an interferometer can modulate at frequencies that are proportional to the wavelength. The time advantage is even larger, since it is

directly proportional to m. A complete spectrum can be collected very rapidly and many scans can be averaged in the time taken for a single scan of a dispersive spectrometer.

2. Throughput advantage (Jacquinot advantage). For the same resolution, the energy throughput in an FT-NIR spectrometer can be higher than in a dispersive spectrometer, where it is restricted by the slits. In combination with the multiplex advantage, this leads to one of the most important features of a FTIR spectrometer: the ability to achieve the same signal-to-noise ratio as a dispersive instrument in a much shorter time. The theoretical throughput of an optical system is dependent on the solid angle of the optical path Ω and the areas A of the detector and sources.

For an FT-NIR spectrophotometer, the solid angle is limited by the aperture (also known as the J-stop) that gives the desired resolution, Δv. The $\Omega_{max} = 2\pi(\Delta v)/(v_{max})$ for an interferometer. For a grating system the max throughput and solid angle is related to a term that relates to the focal length and the characteristics of the grating (α). The other important term is the size of the slit that gives the desired resolution $\Omega_{disp} = 2\pi\alpha(w\Delta v)/(v^2)$ where w is related to the slit width. The ratio of the solid angles gives an expression of the Jacquinot advantage $J = (\Omega_{max})/(\Omega_{disp}) = (v^2)/(\alpha w v_{max})$.

For higher wavenumbers the Jacquinot advantage becomes greater.

The Jaquinot gain arises from the high radiation throughput achieved for a Fourier instrument, due the fact that it does not employ entrance or exit slits to limit the radiation intensity reaching the detector. Typical wavelength accuracy is better than 0.05 nm and the resolution can achieve values below 1 nm in the NIR region, at cost of decreasing the scan speed.

3. Connes advantage. The intrinsic wavelength scale in an FT-NIR spectrometer provides wavelength repeatability better than one part in a million. The wave number scale of an FT-NIR spectrometer is derived from a HeNe laser that acts as an internal reference for each scan. The wave number of this laser is known very accurately and is very stable. As a result, the wave number calibration of interferometers is much more precise. If a calibration standard such as the NIST standard reference material SRM1920 is used, the wave number calibration is more accurate and has much better long-term stability than the calibration of dispersive instruments.

4. Negligible stray light. Because of the way in which the FT-NIR spectrometer modulates each source wavelength, there is no direct equivalent of the stray light effects found in dispersive spectrometers.

5. Constant resolution. In dispersive instruments, throughput is typically optimized by adjusting the slit width during the scan. Thus, signal-to-noise is constant but resolution varies. Instead, in FTNIR spectrophotometers the resolution is defined by the J-stop (Jacquinot stop) aperture size, which does not change during data collection. Increasing the length of the scan for an interferometer increases resolution. Narrowing the slit width in a dispersive spectrometer increases resolution, but this is

limited by how narrow a slit can be reliably closed. Because of this, FT-NIR spectrophotometers typically have a maximum resolution value much higher than even research-grade dispersive spectrometers.

6. Continuous spectra. Because there are no grating or filter changes, there are no discontinuities in the spectrum.

These advantages turn the NIR spectrophotometer based on interferometric measurement and Fourier transform into an unbeatable research instrument. On the other hand, the spectrophotometer is not as robust as an AOTF-based instrument which is assembled without any moving parts.

The price of a Fourier based instrument is comparable with the AOTF-based spectrophotometer and, therefore, both are considered expensive relative to other options.

Currently available systems also offer a full range of analytical techniques (i.e. solids, pastes, powders, liquids, probes, vial holders, integrating spheres) on a single instrument, with easy computer controlled switching between different modes.

1.5.3 NIR detectors

An infrared detector is a photodetector that reacts to infrared (IR) radiation. The two main types of detectors are thermal and photonic. The detectors used in NIR instruments are photonic, based on semiconductor materials with narrow band gaps that generate carriers (holes and/or electrons) by absorbing photons of the incident radiation, which causes electronic excitations.

The photonic detectors have high response time and sensitivity, but usually these have to be cooled to cut thermal noise. There are two types of photonic detectors, the photoconductive and photovoltaic detectors. In photoconductive detectors, the resistivity of the detector element is monitored. In instruments with response extending to 2500 nm the most commonly used detectors are PbS (Lead Sulphide photoconductive) detectors. Compared with other detectors in the same wavelength region, the PbS detector has superior features, such as higher detection capability, and faster response. It also operates at room temperatures, but the dark resistance, photo sensitivity and response characteristics change depending on the ambient temperature.

In a photovoltaic detector the voltage changes in response to incident radiation, generating an electrical photocurrent in response to the absorbed photons. In a simple p–n semiconductor junction, incident radiation leads to a flow of current over the junction, and the device acts as a photoconductive detector. But if the diode is in series with a very high resistance, the voltage across that resistance changes with the intensity of the incoming radiation, so that the diode then acts as a photovoltaic detector. Various semiconductor materials such as silicon, indium antimonide, and

gallium arsenide can be used for the detectors. They can operate from visual wavelengths to 10 µm or longer. The most commonly used photovoltaic detectors in NIR spectroscopy are Si (Silicon) and InGaAs (Indium Gallium Arsenide) [19].

1.6 Measurement modes

NIR spectrometry started as an unique technique when Karl Norris proposed that the spectral measurement could be obtained by analysing the information content of that portion of radiation diffusely reflected by solid samples instead of the weaker signal of transmitance. Today, diffuse reflectance is one of the various possibilities for employing the NIR spectral region. Figure 1.12 depicts the most common measurement modes employed by NIR spectroscopy [19].
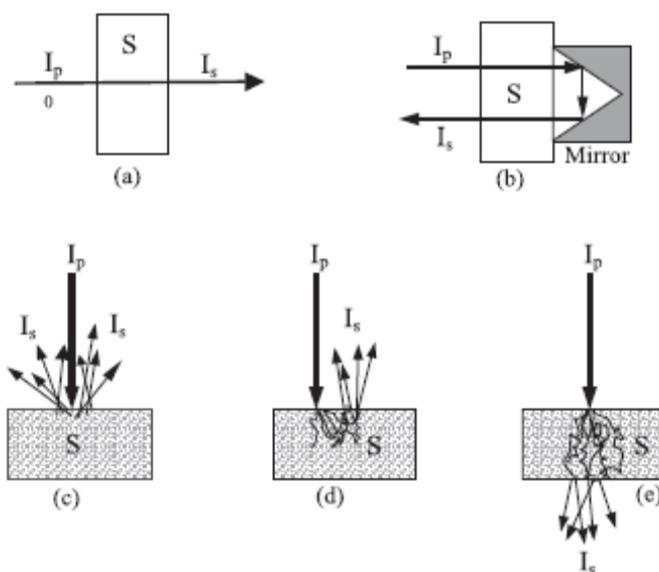
Figure 1.12 - Modes of measurements employed in NIR spectroscopy: (a) transmittance; (b) transflectance; (c) diffuse reflectance; (d) interactance, and (e) transmittance through scattering medium [19].

Transmittance (Figure 1.12a) is obtained as in conventional UV-VIS spectroscopy. In transmittance measurements light is directed at a sample, where some of the light is absorbed and some is transmitted to the detector. This type of measurement is called Near-infrared transmittance (NIT) used for samples that are liquids or transparent foils.

The main accessories used for transmission measurements are a vial holder or a liquid probe.

The benefits of vial holders include a temperature-regulated block, and custom-sized disposable glass vials. Transparent samples are measured in glass/quartz cuvettes with typical optical paths varying from 1 to 50 mm. The optical path is, in principle, defined by the spectral region being probed. It goes from a larger to a shorter path as the wavelength goes from the higher order overtones to the combination region around 2200 nm.

The fiber optic-based liquid probe can be used for either off-line, at-line, or in-line measurements. Light from the spectrophotometer is directed through fiber optic cable to a pair of mirrors that direct the light through a cavity in the probe head. The cavity has a fixed pathlength that enables a liquid sample to enter.

In transmittance measurements the entire pathlength of a sample is integrated into the spectral measurement, thereby reducing errors due to nonhomogeneity of samples. Transmittance techniques are most useful for measuring large particles. For fine particles, the front surface scatter brings about a loss of energy transmitted through a sample with the net effect being a decrease in the signal-to-noise of the instrument. In transmittance, higher frequency energy is most commonly used due to its greater depth of penetration into the sample. The higher frequency energy (800 to 1400 nm) is more susceptible to front surface scattering than lower frequency energy. Transmittance measurements must therefore be optimized, taking into consideration the relationships between the frequency used for measurement, front surface scatter, and the pathlength of the sample. In transmittance measurements, particle size can be small enough to begin to scatter most of the energy striking the sample. If the particle size is sufficiently small, the instrument will not transmit enough energy through the sample for the detectors to record a signal.

Figure 1.12b shows a special way to obtain a transmittance measurement which is referred to as transflectance. When a mirror is placed behind the sample, the light transmitted through the sample is reflected back through the sample. Transflection thus measures a combination of transmission and reflection. This technique is useful for emulsions, gels, and liquids. This measurement mode is frequent when optical bundle probes are employed. The difference in relation to a simple transmitance measurement is in doubling the optical path as the radiation beam passes twice through the sample.

Many substances in solution follow Beer´s law, showing a linear relationship between concentration and absorbance, but it is important to be aware that the same limiting factors found in other spectral regions restrict the real systems from following Beer´s law over a wide concentration range. These limiting factors originate from the instrument and/or are characteristics of the sample constituents as, for example, the non-linearity of the detection system and the changes in hydrogen bond patterns as the concentration of the various species undergo relative concentration changes.

The best reference substance for transmittance and transflectance measurements should be carbon tetrachloride (CCl_4), which shows no absorption bands in the whole NIR region. However, this toxic substance must be avoided unless it can be safely conditioned in a sealed flask. Empty cells and an internal reference beam have also been employed to calculate the transmittance (and absorbance) of transparent samples.

Diffuse reflectance measurement of solid samples (Figure 1.12c), is a distinguishing measurement mode employed in NIR spectroscopy. When light is reflected from rough surfaces or powders, it is referred to as diffuse reflectance. In diffuse reflectance, scattering and absorbance by solid granules contribute to change the signal intensity.

Depending on the sample, light may penetrate beyond the surface a significant distance. There is therefore the potential of quantifying the components within the sample.

A rigorous treatment of the signal obtained in this type of measurement was established by Kubelka and Munk. The mathematical treatment results in the following equation that should replace Beer´s law, which is valid only for transparent homogeneous materials, and establishes a linear relationship between the absorbance A, which is a function of the concentration (C), and the diffuse reflectance (R) [19]:

$$A = f(C) = \frac{(1-R)^2}{2R} \quad \text{(Eq. 1.23)}$$

where R is the reflectance $= I_R/I_{R0}$, where I_R is the intensity of radiation reflected by the sample and I_{R0} the same quantity reflected by an non-absorbing material over the whole spectral range of measurement. The measurement of I_R and I_{R0} requires the collection of the scattered radiation by a perfectly reflecting reference (100% in all wavelengths) and, therefore, is seldom employed. In fact, the Kubelka-Munk equation is rarely employed, being substituted by more practical, although certainly non-linear, equation much used for developing analytical methods based on reflectance measurements, such as [19]:

A = f(C)= log1/R (Eq. 1.24)

The relationship does not depart much from the Kubelka-Munk prediction and, for small changes in the reflectance (R) (which are common for many applications) can be assumed to present a linear behaviour with the concentration of the analyte. In the same way, raw transmittance is converted to absorbance using the expression log 1/T.

There are two types of accessories that measure by diffuse reflectance: the diffuse reflectance probe, or the integrating sphere.

The diffuse reflectance probe uses a fiber-optic cable with multiple fibers. Half of the fibers in the bundle are typically used to transmit the light to the sample, and half to return the reflected light to the spectrometer. A schematic diagram of a diffuse reflectance probe is shown in Figure 1.13.
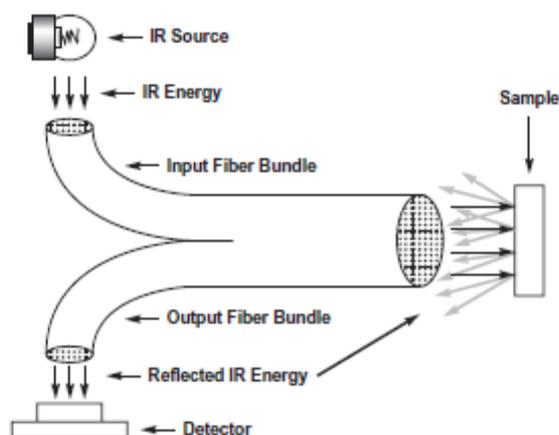
Figure 1.14 - Schematic diagram of a diffuse reflectance probe [19].

A second type of diffuse reflection accessory is an integrating sphere. In an integrating sphere light is directed onto a sample, as shown in Figure 1.14. The reflected light is measured by a detector, which is mounted in the wall of the gold-plated sphere. An integrating sphere is well suited to inhomogeneous sample because of the large sampling area. If the sample is very inhomogeneous a rotating cup can be placed on the integrating sphere to provide further averaging.
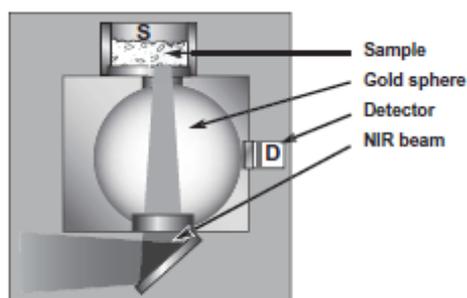
Figure 1.14 - Schematic representation of an integrating sphere [19]e.

Figure 1.12 shows two more measurement modes employed in NIR spectroscopy. In the interactance mode (Figure 1.12d) a higher probability is given to the incident beam to interact with the sample. Consequently, the emerging beam (collected at a place somewhat distant from the location of incidence) contains more information on the sample constituents and reflects better the actual composition of the sample. Figure 1.12e shows the transmitance measurement of dense solid samples.

1.7 Analytical information from NIR spectral data

Despite its complexity and the presence of broad and superimposed absorption bands, NIR spectra have high information contents. This information content has been intensively exploited for qualitative and quantitative chemical and physical analytical purposes.

1.7.1 Qualitative applications

Sometimes the interest is in the use of the NIR spectrum as a source of information for qualitative identification of samples. The complexity of the spectrum forbids its direct use, as is done with the middle infrared region, where clear absorption bands can be easily distinguished and attributed, for example, to the presence of certain functional groups. Nevertheless, correlation charts showing where the absorption bands of O-H, C-H, N-H and S-H bonds of distinct compounds are located in the NIR spectral region can be found and used as a first approach for qualitative analysis. NIR spectroscopy is not suitable for structure elucidation. However, it has been widely employed for fast and direct access to identify starting products used, for example, by the pharmaceutical industry.

Interpretative NIR spectroscopy, or the use of such band characteristics as intensity and wavelength position for attribution of the origin of the spectral features, has been recommended as a support to guide and understand the results obtained for modern chemometric mathematical and statistical classification methods. In fact, the development of any analytical method based on NIR should be preceded by an exhaustive exercise aimed at correlating chemical knowledge about the sample, usually available beforehand, and spectroscopic features or vice-versa. The results of this exercise could anticipate spectral occurrences, their intensities, and the possible effect of parameters, such as temperature, on the NIR spectrum and the best wavelength regions to look for qualitatively and/or quantitatively relevant information.

1.7.2 Quantitative applications

Table 1.3 summarises the sample properties, about which a NIR spectrum, in principle, is capable to give information, and some application examples. The information present in a NIR spectrum can be employed in the usual way to estimate the concentration of a given substance in a sample or to estimate a bulk or physical property when these can be, in any instance, reflected in significant changes in the intensity and/or wavelength of the spectral features produced by the sample. These features may be indirectly associated with the analyte.

I.   Chemical Composition
     I.1. Protein
     I.2. Humidity/water
     I.3. Hydrocarbons
     I.4. Carboxylic acids
     I.5. Amines
     I.6. Oil/fat
     I.7. Sucrose/glycose
     I.8. Additives in fuels

II. Bulk Properties
     II.1. Density
     II.2. Digestibility
     II.3. Viscosity
     II.4. Motor fuel octane number
     II.5. Reid pressure
     II.6. Seed germination
     II.7. Distillation parameters
     II.8. Fruit ripeness
     II.9. Total dissolved solids

III. Physical Properties
     III.1. Particle size/fiber diameter
     III.2. Temperature
     III.3. Mechanical properties
     III.4. Thermal and mechanical pre-treatment
     III.5. Molar masses of polymers

Table 1.3 - NIR application examples [15].

For quantitative applications, NIR spectroscopy is not very sensitive. Most of the quantitative applications are targeted to determine major constituents in the sample. In general, the detection limit is about 0.1% (m/m), although, for some specific applications and under favourable characteristics of the sample matrix and analyte, NIR can reach lower values.

Anyway, this technique presents some positive properties that make it widely acceptable as one of most used on/in line detection methods in food and other areas.

For example, compared to other non-destructive techniques, NIR spectroscopy does not need any sample preparation. Hence the analysis is very simple and rapid, which is a requirement for on-line application [20]. Furthermore, NIR technique allows several constituents to be measured simultaneously until 0.1% concentration [21]. Finally, the relatively weak absorption due to water enables high-moisture foods to be analyzed.

Industries involved with foods and beverages have traditionally used NIR measurements for quality control, blending, and process control. Developments in computer science and chemometrics have prompted parallel developments in the on/in-line NIR techniques, and have attracted considerable attention from food researchers.

With respect to cereals, in many countries the price of grain is determined by its protein content, starch content, and/or hardness, often with substantial price increments between grades. Several studies show grain quality parameters to be significantly variable, even when harvested in the same field and at the same time [22, 23]. NIRS technology has made it possible to directly measure different constituents in the grain products [24, 25, 26, 27, 28]. Furthermore, its ability to be installed on the harvesting machine itself is advantageous for on-line determination and grading. Engel et al. (1997) [29] described an approach for inspecting grain protein on-line by the use of NIR analysis. On-line measurement of grain quality with respect to moisture and protein content by a NIR measurement device [30] that was installed in a bypass unit of the clean grain elevator in a conventional combine harvester has been possible.

NIR spectroscopy is applied extensively to the analysis of barley for both quality assessment and control purposes and it has been used successfully to monitor barley protein and moisture [31].

The Technical Committee of the American Society of Brewing Chemists (ASBC) reported collaborative trials to assess use of NIR spectroscopy for determination of protein and moisture in whole grain barley [32].

Further NIRS calibrations were developed for the accurate and fast prediction of the total contents of methionine, cystine, lysine, threonine, tryptophan, and other essential amino acids, protein, and moisture in barley [33].

Allison et al. [34] have used a NIR photometer with six filters at 1680, 1940, 2100, 2150, 2230, and 2310 nm to determine the percentage soluble β-glucan content of barleys of different origins. The correlation coefficient between predicted and actual values was 0.85 over the lower soluble β-glucan range (0.4–2%), which is that usually found in commercial barleys.

R. de Sa and G. Palmer in 2006 carried out initial experiments and evaluate the use of Near Infrared Spectroscopy analysis for rapid measurement of β-glucan in single grains of malt and barley [3].

The accumulation of mixed linkage barley (1/3) (1/4)- β-D-glucan (BG) during grain filling at eight stages was studied using standard reference methods and infrared spectroscopy, because fast and non-destructive spectroscopic fingerprinting is obviously advantageous for studying physiological processes such as grain filling, as the techniques are non-destructive, fast and sensitive and it is possible to do real-time analysis [35, 36].

Malt extract is another parameter used in assessment of the malting quality of barley. The conventional methods used in this assessment are tedious and poorly adapted to rapid screening of early-generation breeding lines.

In 1977, Morgan [37] reported the use of the a model NIR photometer to provide rapid analysis of malt hot water extract (HWE).

McGuire [38] calibrated a NIR filter photometer with 54 barley genotypes, then estimated the malt extract of 332 selected lines. The NIR values were highly correlated with the laboratory malt extract values. Coefficients of correlation of 0.95 and 0.98 were respectively obtained for the two series of selected lines.

Ratcliffe and Panozzo [39] have investigated the application of NIR spectroscopy to develop calibrations that can be used in a barley breeding program for identification of experimental lines suitable for the malting and brewing industry in Australia.

The physiological and physical-chemical basis of barley germination was also studied. Vigour was defined as germination percentage after 24 h, and NIT calibrations can be used to predict vigour in malting grade barley [40, 41].

To verify that NIR spectroscopy data represents a physio–chemical fingerprint of the barley seed, physical and chemical spectral components were partially separated by Multiple Scatter Correction and their genetic classification ability verified. Wavelength bands with known water binding and (1/3, 1/4)-b-glucan assignments were successfully predicted by partial least squares regression giving insight into how NIR-data works in classification [42].

Dietary fiber is an important quality parameter of barley (Hordeum vulgare L.) but is extremely laborious to measure. Near-infrared (NIR) transmission and reflectance spectroscopy were investigated as rapid screening tools to evaluate the total dietary fiber content of barley cultivars. [43].

In barley, malting varieties generally were classified as soft grain whereas non-malting or feed varieties were classified as hard. Hardness has also been associated with the level of modification of malt which would imply that grain components within the endosperm (such as starch granules, starch protein matrix and cell wall material) directly impact on modification. Hardness calibrations for the three hardness methods were developed using the NIR[42]. The establishment of fast and non-

destructive methods for the evaluation of quality and safety of raw grains is being demanded nowadays to avoid toxic substance presence. Alfatoxin B1 (AFB1) has been recognised by the International Agency of Research on Cancer as a group 1 carcinogen for animals and humans and the EU Official Journal has established action levels for AFB1 presence in all feed materials between 5 and 20 ppb [45].

In malting, the factor used for assessing barley quality is the HWE. The most successful NIR prediction of HWE was obtained when the malt was produced without additives or addition of water during germination [46].

Lager malt with a maximum S-methylmethionine (SMM) content of 19 ppm is often requested by the breweries. However, average concentrations range from 10 ppm SMM in kilned lager malt to more than 50 ppm in green malt. Assays of SMM in germinating barley samples were carried out [47] using a grating (1100–2500 nm) NIR Systems spectrophotometer with reference data obtained from high-performance liquid chromatography (HPLC).

An increasing number of quality criteria are involved in the evaluation of the final malt. This evaluation is normally based on experience and prior knowledge by the maltster/brewer/breeder, in which each quality parameter is evaluated according to a target or target range. The purpose of this investigation is to study the use of fuzzy logic for the translation of a complex malt quality profile into a simple univariate overall quality index (OQI). NIT spectra were recorded on the 50 malt samples, and the spectra were used in a partial least squares regression (PLSR) model for the prediction of OQI. [48].

Near-infrared calibrations were developed for the prediction of moisture, wort protein, and diastatic power in germinating (green) malt. Spectra of green malt samples were collected over a wavelength range of 400-2,500 nm; however, only energy in a wavelength range of 1,100-2,500 nm was used during calibration development. Wort protein calibration demonstrated a high degree of predictive accuracy, but the performance of the diastatic power calibration was less than satisfactory [49, 50].

Concerning to beer, an investigation about the use of NIR spectroscopy for monitoring beer fermentation [51] has been proposed, even if the data presented here are near-line measurements of the raw liquor. The fermentation parameters studied were ethanol concentration, specific gravity (SG), optical density (OD) and dry cell weight (DCW), and the aim was to establish the performance in terms of predicting biomass and composition that could ultimately be obtained from an on-line probe.

Another recent application of NIR absorption is the evaluation of the ethanol content [52]. The measurements can be performed without sample preparation and the results are in excellent

agreement with the standard analytic procedures, and this fact demonstrates the potential of NIR interpretive spectroscopy for determining ethanol directly in a broad range of different beers.

2 BEER PRODUCTION

The principal phases in beer production are shown in Fig. 2.1.
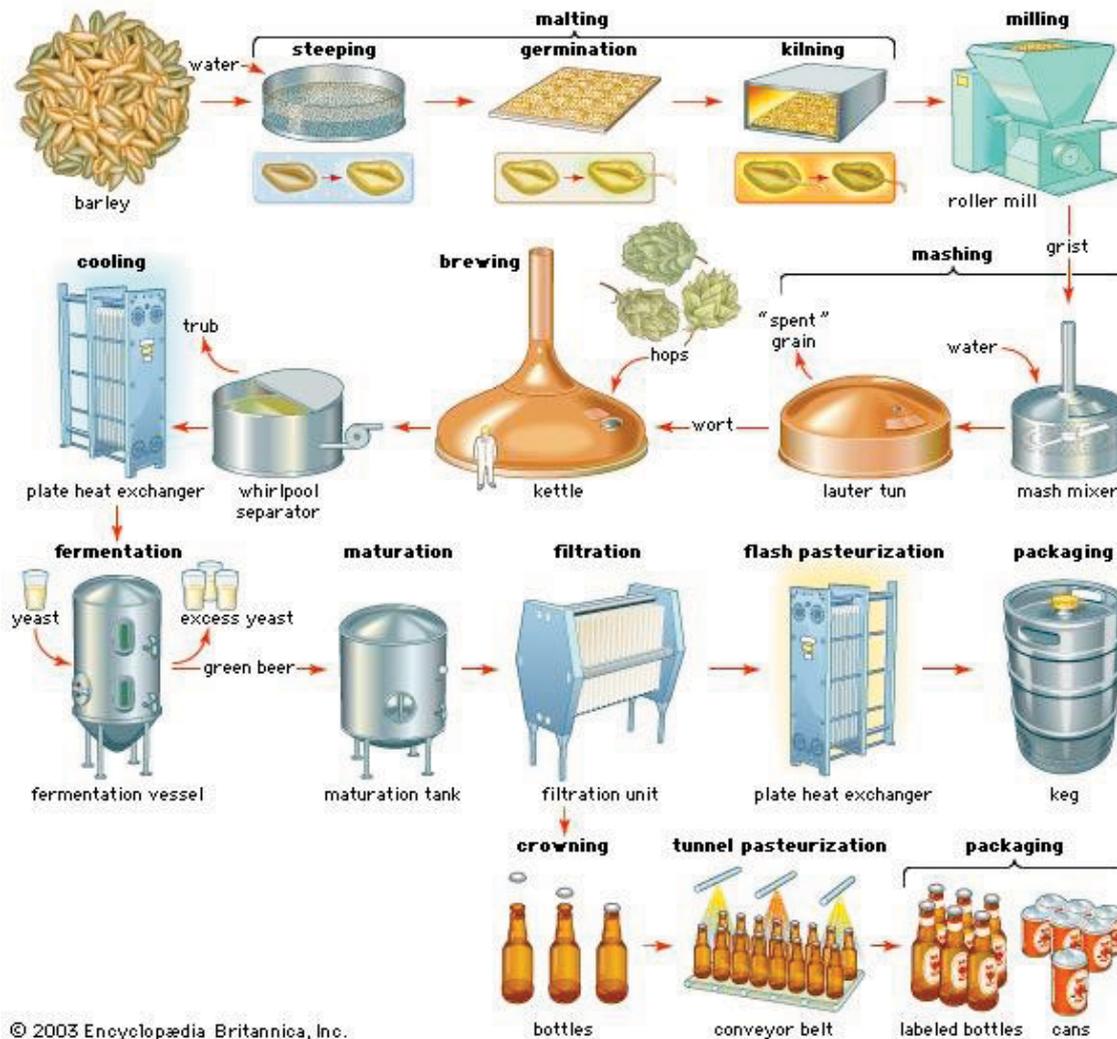
Fig. 2.1 . The principal phases in beer production

2.1 The raw materials for beer production

Four raw materials are required for beer production: barley, hops, water and yeast. The quality of these raw materials has a decisive influence on the quality of the final product.

2.1.1 Barley

Barley (*Hordeum vulgare*) supplies the starch required for beer production. This starch is converted to fermentable extract in the brewhouse. It is necessary to produce, by cultivation of suitable varieties, barleys which provide extract-rich malts.

After wheat, maize and rice, barley is the most important cereal grown in the world with an annual production of 170 million tonnes, of which around the 20% is used for the production of malt, the starchy essential source in the beer production [53].

The reasons for its wide distribution are due to its high productivity associated with low costs of cultivation and early harvesting.

Barley can be divided into the winter type, the seeds of which are sown in about the middle of September, and the spring type, which is sown in March and April. Both types are subdivided into varieties which, depending on the arrangement of the corns on the ear axis (rachis), are classified as two-row or multirow. In the case of multirow barleys there are three fertilizable florets at each node on the rachis. Each of these, after pollination, develops into a barley corn of grain. When seen from the above, the groups of three grains appear alternately on the right and on the left forming six-row barley. When the rachis internode segments are relatively long, often only four rows can be made out because the lateral florets of any one node overlap those of the adjacent nodes. Consequently, in four-row barley these florets seem to be in four, rather than in six rows. In the case of two-row barleys only one grains develops at each node because the lateral florets are sterile. From above only one grain can be seen on the right and one on the left. Two row barleys produce large, plump grains usually with thinner, finely wrinkled husks. Consequently such barleys have relatively large amounts of useful contents and less husk, and so contain less polyphenolic and bitter substances. The grain are all very uniform and their extract content is comparatively high. Two-row barleys are preferably grown as spring barleys and combine all the desirable features for malt and beer production. Six-row barleys produce grains of uneven size. Because the grains do not have sufficient room to grow fully, those in the rows from lateral florets are thinner and these grains are curved at the distal end where the awn is attached (twisted grains) [54]. The yield of winter barley is about 6-7 tons/ha which is substantially higher than that of spring barley (about 4.5 t/ha) and it is of course due to the shorter growing season for the latter (150 day as opposed to 300 day for winter barleys). For malting and brewing purposes the two-row spring barleys are by far the most suitable. However increasing numbers of two-row winter barleys are being developed whose quality is

almost as good of as that of the two-row spring barleys, with higher yield. It has been demonstrated that it is possible to use either winter or spring malting barley varieties for beer production. Traditionally, spring malting barley is produced in regions with moderate temperatures and adequate rainfall throughout the growing season. Winter malting barley, on the other hand, is mostly grown in the milder and semi-arid parts of Europe.

Barley is the grain most suitable for the production of beer for several reasons: during germination produces an amount of enzymes (amylase) higher than other cereals and it is protected by the husks (consisting mostly in cellulose) from damages that can lead to losses of its important features. Moreover, these husks still adhere to the grain, even after threshing and processing to malt. Consequently it is able to form wort filtration layer required in a later production stage. Before use in the brewery the barley must first be converted into malt.

Together with these physiological features, the chemical composition of barley, with its high starch content, is the most suitable of all the other cereals for the production of beer [53].
The chemical composition of malting is represented by of total carbohydrates for the 70-85%, proteins for the 10.5 to 11.5% (1.68 to 1.84% if the content is expressed as total nitrogen), inorganic substances for the 2-4%, lipids for the 1.5 to 2.0% and other substances for the 2.1%.

The most important parameters for malting barley are resistance to plant diseases, low in protein, rapid absorption of water, high energy of germination, high yield potential and high enzyme content [54].

Barley Evaluation

The quality of the barley offered for purchase or delivered has a decisive effect on the quality of the malt and the beer produced from it. Barley evaluation is therefore very important for a maltster.

a) Hand Evaluation
Barleys for malting are selected mainly on the basis of the variety and the grow site. In addition to the rapid methods commonly used today when barley is delivered, the evaluation of the barley from its external appearance, is important. There are not reference methods for this kind of evaluation. The examined features are [55]:

a.1) Colour and brightness: the barley should have a light yellow colour and a bright, uniform appearance. Greenish corns indicate premature harvesting. Barley that has suffered rain damage appears grey and dull. Brown tips may be a varietal characteristic, but in most cases are caused by wet harvesting and this leads to water-sensitive corns. Red corns (red coloured endosperm) indicate a massive infestation of Fusarium, which could lead the formation of gushing in the beer. Barley with red corns is not suitable for malting.

a.2) Amount of impurities (purity)

a.3) Damaged corns (not intact)

a.4) Corn shape: the corns should be large, well-filled and rounded. Such barley corns usually produce more extract and contain less protein than thin long corns. However, the corn shape depends primarily on the variety.

a.5) Uniformity

a.6) Presence of pests: the most common grain pest is the grain weevil. Corns that have been attacked by a grain weevil clearly show the holes eaten away and float on the top during steeping. Such damages barley cannot be used to make malt.

a.7) Appearance of the seedling (sprouting): with very wet harvest the barley batch may contain already germinated corns. Such batches are unusable for malt production because the barley does not then germinate uniformly.

b) Physical Properties Examinations (grading by size %)

Grading by size is the most important physical examination of barley.

The barley is sorted by 2.8 mm, 2.5 mm and 2.2 mm vibrating sieves into four components. Everything which remains on sieve 1 (2.8 mm) and sieve 2 (2.5 mm) is Grade I (well-filled barley). Everything which passes thought sieves I and II but is retained on sieve III is Grade II. Everything that passes thought sieve III is screenings and is sold feed barley. Because 100 g of barley is always examined, the weight in g is equal to the percentage of each fraction.

Normal values for the well-filled fraction are more than the 85% for average malting barley, more than the 90% for fine malting barley and more than the 95% for premium quality malting barley[56].

c) Chemical properties examinations

c.1) Water Content (%)

The moisture content of barley is 14-15% on average. The moisture content can vary between 12% in very dry harvesting conditions and over 20% in wet conditions. More precisely, it is less than

13% in the South region of the European Brewery Convention (EBC) barley and malt committee, and it is more than the 16% in the North region, where consequently the barley should be dried before a long term storage. In fact, barley must have moisture content below 15% for long term storage. Moreover, the determination of the moisture content is important because the amounts of the other components are related to the dry weight[55, 56].

c.2) The protein content (% and %dm)

The protein content of barley has an important role in malt and beer production. Protein rich barleys are more difficult to process and produce a higher malting loss. Every percent of additional protein results in approximately one percent less extract. The protein content therefore is a particularly important item in the barley supply contract. The nitrogen content of barley can vary from 8 to 11 to 16%. The normal commercial requirement is therefore a maximum of 11.5% protein in the dry matter [55].

c.3) Carbohydrate (% and %dm)

Carbohydrates are quantitatively the most important class of compounds, but they differ considerably from one another with regard to their importance in processing and the quality of the end product [55]:

- Starch is the most important constituent and forms 50-65% of the barley. It is formed in the slowly ripening barley grain by assimilation and subsequent condensation of glucose to form an energy reserve which is metabolised by the seedling in its initial growth phase. The starch is deposited in granules in the endosperm cells. The starch granules contain up to 5% lipids and 0,5% protein substances and consist of two different structures, amylose and amylopectin, which are built of glucose residues. Amylose consists of 200 to 400 α-glucose units linked in an unbranched helical chain by oxygen bridges at the 1,4-positions. Amylopectin is a chain of α-glucose units linked by oxygen bridges at the 1,4-positions too, but there are also 1,6 linkage spaced 15 to 30 glucose units apart and so the amylopectin molecules look like branched trees and they may contain up to 6000 glucose residues.

- Hemicellulose are the main constituents of the endosperm cell walls. They consist of β-glucans (80 to 90%) and pentosans (10 to 20%), which together form the rigid framework of the endosperm cell walls. β-glucans consist of long chains of glucose molecules bound together by 1,3 and more often by 1,4-bonds. The β-glucan is contained at 4 to 7% in the barley and in the cell walls of the endosperm tightly linked with higher molecular protein substances and pentosans. When β-glucan goes into solution, the molecules become associated with one another

as the result of the formation of hydrogen bonds and form so-called fringed micelles. The breakdown of β-glucan can have an adverse effect on the finished beer, like an improvement of viscosity and haze.

d) Physiological examinations

Germinative energy (%)

The germinative energy is the percentage of corns which, at the time of the test, germinate under normal malting conditions. The germinative energy test shows whether the corns have started to germinate after 3 and 5 days. A high germinative energy indicates a healthy barley condition and that malting will be successful. The germinative energy after 3 days should be as close as possible to that after 5 days. For a brewing barley, at least the 95% of the corns should be able to germinate [55].

2.1.2 Adjuncts

The enzyme potential of malt is sufficient to catabolize additional starch. Consequently, in many countries it is allowed the use of unmalted cereals, called adjuncts, as alternative starchy sources in beer production. These adjuncts replace part (usually the 15-20%) of the malt. The reasons that make preferable the use of these alternative raw materials are economic, because usually these unmalted cereals are cheaper than the malt, and qualitative, because they can contribute to the final organoleptic quality of beer with their compositional characteristics. The unmalted cereals used as adjuncts around the world depend on their availability in each country (eg. sorghum in Africa, rice in Asia, maize in Europe and the U.S.A.). In Italy the maize is the most widely used adjunct, and it can be used up to a maximum of 40% of the wort extract (% dm) [55, 57, 58].

Maize

Maize (corn) is harvested with a water content of 25 to 30% and brought to a water content of 10 to 14% by drying. The dry matter of maize consists of 75-80% of carbohydrates, 9-12% of protein, 4-5% of oil and small amount of crude fiber and inorganic substances. The oil is located in the germ of the corn. Because of concern about the foam damaging effect of the oil, maize is degermed before processing and thereby practically freed from oil. The degermed maize then has an oil

content of about 1%, but oil contents of up to 1.5% are also tolerated. Maize is degermed dry before processing, the germ and husk being removed by a plan-sifter and aspirator. Maize can be processed in the following products: grits, (0.3 to 1.5 mm) refined corn grits (0.5 mm, prepared by steeping the grits for 30-40 hours in hot water at 50°C), flakes (the starch is pregelatinized) and syrup (the starch is hydrolyzed to simple sugars with acid hydrolysis and/or enzymes). During processing to grits or flakes the protein content decreases to about 7-9%. This protein remains largely undissolved during mashing so a smaller protein content, corresponding the maize fraction, must be expected and this can affect the supply of low molecular weight protein to the yeast. Maize starch is similar, even in its external shape, to barley starch. Moreover, the gelatinization temperature of corn starch is 60 to 70°C and so no problems should be expected during processing. The extract content of degermed maize is 88 – 90% dry weight and consequently the same as for malt [55, 57, 58].

Maize evaluation:

a) *Moisture content %,* which is expected to be between 10 and 15%.

b) *Extract content %dm*, which is expected to be between 88 and 90%.

c) *Content of lipids %dm*, which is expected to be between 0.5 and 1.5%.

2.2 Malting and barley malt

Beer can not be made without malt, even if there are recent studies concerning the use of 100 % of un-malted barley for brewing, with a combination of microbial enzymes and endogenous enzymes of barley [59]. Anyway, these studies are pioneering, and the traditional brewing to date involves the use of barley malt as a source of all the enzymes necessary to the process.

Consequently malt production from barley is the first step in beer production. In fact, it is not possible to use directly barley for the beer production, but it has to be transformed into malt, to allow the production of many enzymes that are present in dormant barley seeds, which play a key role during the mashing, degrading the starch in the seed. It is of course possible to make malt from other cereals, for example wheat, rye or sorghum, but barley malt has proved to be the most suitable malt for beer production. The purpose of malting is to produce enzymes in the germinating barley kernel and to cause certain changes in its chemical constituents. For this purpose the barley is made to germinate trough the absorption of water and this germination process is interrupted at the appropriate time by a drying procedure, known as kilning. In addition, malt is characterized by

aromatic features which depend not only on its composition but also by the kilning program, which can not lead to the same results drying the barley instead of the malt. Finally, the greater friability of the malt makes it more suitable for beer production [57, 58, 60].

So the purposes of the malting process are:

1) To induce the synthesis of enzymes (especially hydrolases) that serve to degrade the large molecules of reserve substances present in the endosperm of the seed and to allow their solubilization.

2) To degrade proteins, in order to produce essential nutrients for yeast.

3) To form important substances in the color and flavor.

4) To remove undesirable substances, such that the dimethyl-sulphide is removed during drying.

2.2.1 The malting process

The malting process consists of four main steps are: cleaning (and grading), steeping, germination and drying.

a) Cleaning, or purification, and grading.

During this process attention is paid to removal of unmaltable impurities (foreign cereals, sand, small stones, string, straw, earth, metal fragments, half-corns, etc.). and removal of other contaminants such as weed seeds which reduce malt quality and increase the moisture content. Of courses it is not possible to remove all this foreign material with a single machine. Barley cleaning therefore involves several machines and devices connected in series. After cleaning the barley kernels are separated into different homogeneous fractions according to their size through a mechanical sieve. this stage of

Size is important for the production of beer, because large and thick-bellied kernels contain more starch than small and thin kernels. Small kernels take up water more quickly than large ones during steeping and would result in even malt quality if they were not separated. The barley is therefore sorted into fractions of even kernel size by using sieves with 2.2 and 2.5 mm slit widths in order to obtain homogenous malt [57, 58, 60].

b) Steeping

Once the barley is cleaned and calibrated it is immersed in water in large flat-bottomed tanks, to stimulate its germination. The purpose of the steeping process is to supply water to the interior of the kernel. As a result the enzymes become active and the life process known as germination

begins. During the steeping the seed absorbs water and its initial moisture of 14-15% increases up to 42-44% in the pale malt and 44-47% in the dark malt. Once the humidity is around 30%, the embryo of the kernel starts the process of germination, synthesizing growth hormones (gibberellins), which stimulate the formation of hydrolytic enzymes. These enzymes begin to degrade various reserve substances such as starch, proteins and β-glucans. During the germination, the respiration of barley results in oxygen consumption and CO_2 and heat production, which must be removed to prevent the asphyxiation of seeds. This explains the need for alternate periods of immersion of the seeds in water and periods of exposure to air. The steeping process is an about 24 hours series of repeated cycles of immersion in water at a 12-14 ° C, followed by air breaks at the same temperature. The water absorption rate varies with temperature and the duration of immersion in water, but also with the size of the grains, the barley variety and the climatic season of sowing. Consequently, the steeping programs (temperatures, numbers and duration of immersions and breaks) are different for diverse barleys and malting plants [57, 58, 60].

c) Germination

During germination a new barley plant is produced from the kernel. To form the new plant the barley needs a large amount of energy and building materials which must be produced by respiration and other metabolic processes. Before the new plan can react with the environment and make starch itself by assimilation, it must draw on the reserve materials present in the endosperm. At the start of the malting process the endosperm contents are in a stable high molecular weight form. These substances must be degraded to products consisting of smaller molecules before they can be transported by water. This degradation is performed by enzymes which are formed during germination. The production of enzymes is the main purpose of malting. These enzymes are absolutely essential for breaking down of large molecules during mashing. To avoid loss of material the enzymatic degradation processes are restricted during malting. The processes occurring during germination are: growth processes, enzyme formation and metabolic changes. The growth processes concern the development of rootlets and acrospires. Towards the end of steeping the rootlets break through the base of the grain. They are rubbed off after kilning during malt cleaning and form part of the malting loss. To keep the rootlet loss low, germination is performed at the lowest possible temperature for the shorter possible time. Loss due to rootlet is about 4% if the malt dry weight. The acrospires break through the head but not the husk and grow under the husk on the dorsal side of the kernel towards its tip. Because the acrospires do not grow out the husk, unlike the rootlets, they are not rubbed off malt during cleaning, and so they do not constitute a loss. The length of acrospires is related to the progress of the metabolic changes inside the kernel. Because modification should only

proceed to a certain extent, since otherwise changes to the storage materials and therefore losses would be too large, the acrospires should only reach a length of about 2/4 to 3/4 of the grain length in the case of Pilsner malt and about 3/4 to 1/1 for dark malt. Concerning the enzymes formation, the ones interesting for maltsters are: starch degrading enzymes (α-amylase, β-amylase and limit residual dextrinase), cytolitic enzymes (endo-β-glucanase, exo-β-glucanase, β-glucan solubilase and endo-xylanase), proteolytic enzymes (proteinases and peptidates), fat degrading enzymes (lipases, in particular the lipoxygenases) and phosphoric acid splitting enzymes (phosphatases). During germination, enzymes are not only formed and increased in amount, they are also used to a limited extent, so that nutrients can be supplied to the seedling. The enzymes therefore cause changes which all lead to the production of low molecular weight compounds. Because the degradation products are, however, subsequently respired or transported to build new cell material in the seedling, they are no longer available during later processing. Maltsters therefore have an interest in allowing respiration and formation of new cell tissues to occur only to a limited extent. The changes to storage materials of particular interest to maltsters are the solubilization and degradation of β-glucans, and the starch, protein and fatty matter (lipids) breakdown. Germination is performed in pneumatically operated plants and only seldom on the floor, or in large horizontal tanks (Saladin), in the dark. During germination is important that the germinating barley has a water content greater than 40%. While part of the water evaporates, new water (sweat) in continuously produced by respiration. If insufficient sweat is formed, water must be added. During the five or six days of the germination, the steeped barley must be periodically moved and reshuffled with air to remove the carbon dioxide and to provide sufficient oxygen. With too much aeration, too much of the grain is lost by respiration. If aeration is insufficient, intramolecular respiration begins which may lead to the death of the seedling. Respiration is greatest at the beginning of germination and can be later reduced. It causes the increase of the temperature. This increases the losses caused by respiration and rootlet growth, and the amount of enzymes formed are reduced. From the third day of germination the respiration can be reduced by avoiding the temperature to increase over the maximum temperatures, which are 17-18°C for Pilsner malt and 23-25°C for dark malt. During the germination barley is called "green malt" [57, 58, 60].

d) Kilning

The kilning of green malt is performed in special rooms where air at a controlled temperature is introduce. The purpose of the kilning process is to interrupt the germination to prevent further transformation and losses. For this reason, the water content is lowered from over 40% to less than 5% to make the malt more storable and to increase its preservability. With the lowering of the water

content, all life processes in malt such as germination and modification as well as further enzymatic activity are stopped. In contrast, the enzymatic potential formed should be completely retained. In addition to removal water from the kernel, the temperatures reached during the drying promote the formation of compounds of the Maillard reaction, a series of reactions that occur from condensation of a carbonyl group of a sugar, an aldehyde or a ketone, with an amino group of an amino acid or a simple peptide. The obtained products characterize the aroma and the color of malt and vary their intensity depending on the time and temperature of drying.

In fact, the temperature during the initial stages of drying should not be too high to avoid inactivation of the enzyme complexes formed during germination. When heating the moist green malt during kilning, care must be taken not to destroy the enzymes, because they are needed to break down substrates in the brewhouse. It is important to protect the enzymes to a large extent, and for this reason the malt must first be pre-dried before it is subjected to high temperatures. The moist starch in the green malt gelatinizes at the high temperatures and after cooling the malt is no longer suitable for use. Its inside has a glass-like appearance (vitreous malt). On heating while the starch is wet, unusable vitreous glassy malt is formed. The temperature must only be raised above 50°C when the water content has been decreased to 10-12%. The slow lowering of the water content at temperatures of 40-50°C is known as withering or initial drying. Long initial drying times at low temperatures have a favorable effect on the flavor stability of the beer.

When the humidity is below 10%, the water still present in the malt is tied, so to be removed it needs that the temperature raises up to 80-85 ° C.

The drying process thus enters its final phase, called "curing", which finishes when the humidity drops below 5%. The temperatures used during the this last stage determine the extent of the Maillard reaction and the tone of the final color of malt. A curing temperature of 80-85°C, which is typical of pale malts (Pils or Lager, with a color of 2.5 to 3.5 EBC), does not affect the enzymatic activity seed, while a curing temperature of 100 to 105°C, as in the case of dark malt (Munich type), reduced of about one third the enzymatic activity of the seed. To produce dark malt all the conditions which lead to the formation of aroma-producing Maillard products (melanoidins) are favored. A Munich pale malt, with a color of 13-15 EBC, provides the basis for the dark beer character and is used for up to 85% of the grist. A Munich dark type, with a color of 20-25 EBC, when used as 25 to 40% of the grist, helps to intensify the aroma. For the roasted maltsthe temperatures reaches 185°C, while the very dark malts (chocolate, Carafa, etc.). the roasting temperatures exceeding 200°C and then all the enzymes are denatured. For this reason they have to be used in amounts of 0.5 to 2% of the grist to obtain a dark beer color and the typical aroma. Colored malts are produced with coloring values of 800 to 1600 ECB (chocolate). Drying times

vary from 24 to 48 hours depending on the type of malt to obtain. For example, the caramel malts are obtained heating the seed at about 60-70°C for 60-90 minutes and the dark caramel malt is heated in about 60 min with rapid removal of stream to 150-180°C for 1 to 2 hours to caramelize the corn contents. As a result of this, brown colored compounds with a typical caramel aroma are formed earlier. Depending on temperature and exposure time, this process can either be intensified and reduced. Caramel malt is used to give the beer an increased depth of color and improved body and to emphasize the malty character.

So, through our use of different times and temperatures throughout the malting process, may be obtained for different needs, different types of malt such as:

- Light Malt (for beers like Pilsner and Lager)

- Dark malt (dark and pale Munich)

- Vienna Malt type (used to correct over-pale malts, to produce "golden" beer and to improve palate fulness).

- Caramel malt (Light and Dark)

- Chocolate Malt (roasted very dark)

- Acid malt (acidifying the malt-water mixture)

- Malt Short - Checked (for the improvement of the foam)

On average, one cycle takes about seven days of malting.

The malt is thus certain quality requirements are determined through chemical analysis and physical properties [57, 58, 60].

2.2.2 Malt

Malt evaluation

Malt can be examined by hand evaluation, physical and physiological examinations and chemical-technological methods [55, 58, 60].

a) Hand evaluation

The hand evaluation of malt involves examination of the colour, smell, taste and aroma, brightness and the amount of impurities. Hand evaluation only gives rough indications.

b) Physical Properties Examinations (grading by size)

Grading is performed as for barley. At least 85% of grains should be grade I (bigger than 2.5 mm).

c) Physiological examination (acrospire length)

Acrospire development gives an indication of the uniformity of germination. The acrospires should reach a length of about 2/4 to 3/4 of the grain length in the case of Pilsner malt and about 3/4 to 1/1 for dark malt. Lower values lead to under-modified malt and higher values lead to over-modified malt. The acrospire length provides indications about the barley germination and the malt homogeneity. It can be also useful to estimate the DMS content and the Lypoxigenase activity. This value depends on the barley variety and dormancy, the husks integrity and thickness and the protein content. A high value of average acrospire length is correlated with high values of friability, Kolbach Index, Diastatic Power and wort colour, with high contents of dimethysulphide (DMS), soluble nitrogen, Free-Amino Nitrogen (FAN) and β-glucan and with low values of viscosity and extract. On the other hand, a low value of average acrospire length can provides useful information about the barley, like low germinative energy and respiration problems during the germination.

d) Chemical properties examinations

d.1) Water content (%)

The water content is a very important parameter to be determined on malt, for commercial and qualitative reasons. The water content depends on the type of malt. Normal values are from 3.0 to 3.5% for pale malt and between 1.0 and 4.5% for dark malt.

The commercially acceptable limit is usually 5%. Dry malt is a hygroscopic product and it is necessary to avoid moisture absorption, in order to ensure its preservation. Moreover, this parameter allows obtaining the amount of dry matter, which is relevant for brewers.

d.2) The nitrogen content (% and %dm)

Normally, nitrogen content in malt s up to 0.5% lower in malt than in barley. It is usually expressed as protein content % dry matter (calculated as N x 6.25). The protein content in malt should be between 10 and 11% dm. In fact, every percent of additional protein results in approximately one percent less extract. On the other hand, too low nitrogen content can lead problems of yeast nutrition, insufficient beer foam stability and low enzymatic activity.

d.3) Total β-glucan content (% and %dm)

β-glucans are the degradative products of the cell wall of the malt. If the cell wall is not sufficiently degraded, the lautering of the mash is not smooth. Beer with high β-glucan content may cause haze after freezing. Normal values are between 0.5 and 1.5% dry matter.

d.4) Diastatic Power (WK)

It is expressed in Windisch-Kolbach units (WK). It is the determination of the activity of α-and β-amylase, and then it expresses the enzymatic power of the malt. It is usually more than 200 WK for pale malt, while in a malt dried at higher temperatures it tends to decrease; the higher the temperature the lower the enzymatic activity.

d.5) Dimethysulphide (DMS, ppm)

It is a compound which can produce an undesirable vegetable-like or cabbage flavour and smell in the beer. Since the formation of DMS starts during malting, it is necessary to know how it originates. There are three ways in which DMS is formed:

1) An inactive precursor of DMS, S-methyl methionine (SMM) is formed during the germination phase. It is heat-labile and is split into an active precursor (DMS-P) and free DMS on heating.

2) The active precursor (DMS-P) breaks down on heating to volatile DMS. It is therefore necessary to dispel this DMS by means of long and intensive heating (kilning, wort boiling). This splits the DMS-P and completely removes the volatile DMS which is produced.

3) Under the conditions of the kilning process, a small part of the S-methyl methionine (SMM) can be converted to sulphoxide (DMSO), which is difficultly volatile. This DMSO can later be reduced by the yeast (or also contamination organisms) to DMS. The amount of DMSO and its influence is small, however.

The influence of the barley and the malting on the DMS-P content is very great. The barley variety has a considerable influence, as does growing region, year and climate. About the malting process, the higher the germination moisture level, the higher the DMS-P content and also the thiobarbituric acid. Greater protein breakdown also releases more DMS-P. Higher germination temperatures also lead to the production of DMS-P. Increasing the duration of germination brings about a large increase in DMS-P. An increase of the initial drying temperature reduces the DMS content, but increases the thiobarbituric acid. The curing temperature has a great influence on the DMS-P content. The higher the curing temperature, the more DMS-P is converted into DMS and expelled. The DMS-P content is on average 400 µg/100 g (4 ppm) in dry weight for pale malts and it should not exceed 5 ppm.

<u>d.5) Friability (%)</u>

It is a physical method to evaluate the modification of the malt. The higher is the percentage of friable grains, the greater is the endosperm modification, and then it is easier to extract soluble substances from the seed. Its value should be greater than 80.

Glassy corns (%). They are not-modified grains, so their presence, which indicates bad-malting, should be less than 2.5%. These malts are difficult to grind and have a high content of β-glucans and protein complexes.

Congress mash

The most important feature of malt is of course its behaviour in the mashing process. For this reason, a standard mashing procedure, called Congress mash, is performed in the laboratory. The wort obtained is called Congress wort and immediately analysed for various quality parameters.

<u>d.6) Fine extract (% and %dm)</u>

The fine extract is amount of soluble substance obtained from 100 g malt finely ground by mean of a DLFU disc mill, with a distance of 0.2 mm between the discs. It is the most important quality parameter of malt and it is obtained from the specific gravity of the Congress wort by means of the sugar table (Plato table) for 20°C. The extract yield is reported as a percentage both on an "as is" basis and also related to dry weight. Normal values for extract yields are between 79 and 82% dry weight for pale malt, and from 75 to 78% dry weight for dark malt. The malt is considered better the higher the extract yield.

<u>d.7) The wort colour (EBC)</u>

Although the wort colour provides no reliable information about the beer colour, it is always measured because it gives an indication of the type of malt used. The beer colour, which is an important feature of each style, is determined by the type of malts used even if the process (especially boiling) has a great influence on it. A sample of malt is mashed following the Congress mash procedure. The mash is filtered and the wort is collected. The wort is clarified through a 0.45 micron membrane filter until bright. The colour of the wort is determined by measuring the absorbance at 430 nm and multiplying by the appropriate factor. The value is obtained in EBC (European Brewery Convention) units, which can range from a minimum of 3 EBC units for a malt type Pils, up to 1200-1400 EBC units for a chocolate or black malt type.

Normal values are:

For pale malt   up to 4 EBC units

For medium coloured malt   5 to 8 EBC units

For dark malt   9.5 to 20 EBC units

It is positively correlated with the temperature (Maillard reaction), with the moisture during the kilning process and with the protein content. It also depends on the barley variety. A barley with a high value of colour has also high values of acrospire length and β-glucan and total nitrogen content and low values of moisture, soluble nitrogen and DMS content and low values of Kolbach Index, Diastatic Power and friability.

d.8) The wort viscosity (cP or mPA • s)

From the viscosity of the Congress wort conclusions can be drawn about the future behaviour of the wort during lautering and about the filtration of the beer. This value can also provide information about the endosperm modification, in particular the degree of degradation of stored starch and cell wall. The viscosity of the Congress wort at 20°C is determined using a calibrated viscometer of an appropriate type (glass capillary viscometer, rotary viscometer, falling ball viscometer). Congress wort has a viscosity of 1.5 to 1.6 mPA • s, calculated at 12%, has 1.6 to 2.0 mPA • s.

d.9) The soluble nitrogen content (%dm)

The soluble nitrogen content refers to the nitrogen compounds which have dissolved in the Congress machine procedure. Normally there is 0.55 to 0.75% soluble nitrogen in the dry weight extract of the Congress wort.

The Soluble Nitrogen is used to calculate the Kolbach index (N_K) parameter, which is defined as the ratio between the soluble and total and soluble nitrogen:

$$N_K = \frac{N_S \cdot 100}{N} \quad \text{(Eq. 2.1)}$$

Where N_S is soluble nitrogen and N is total nitrogen, both expressed as percentage of dry matter in the flour. The Kolbach Index shows what percentage of the total nitrogen in the malt is dissolved in the Congress mashing procedure. Consequently NK is an indicator of the proteolytic modification of the malt.

The malt modification is judged by its Kolbach number:

Below 35   under-modified

From 35 to 41   well modified

Over 41   very highly modified

d.10) The free amino nitrogen (FAN) content (mg/L)

The amino nitrogen analysis measures low molecular weight nitrogen compounds in the Congress wort like amino acids, ammonia and, in addition, the terminal α-amino nitrogen groups of peptides and proteins. Proline is partially estimated. Normal values are from 120 to 160 mg/L.

d.11) pH of the wort

During the brewing, many important processes proceed better or more quickly at lower pH, and for this reason, the pH of the Congress wort is supposed to be lower than 5.90. On the other hand, the pH value should not be less than 5.80, otherwise it can indicate an over-modified malt or a sulfuration during the kilning process.

d.12) Fermentability (%)

The fermentation of the Congress wort after boiling provides a measure of the attenuation fro brewing. It should be higher than 79%.

d.13) Difference Fine/Coarse Extract (%)

It is the difference in yield between the extract obtained by applying the Congress mash to finely ground (F, 0.2 mm) and coarsely ground (C, 0.7 mm) malt. It is a measure of the degree of modification of the seed. If its value is below 2%, the seed is very well modified and then it is easy to extract the soluble substances in the mashing process; a value around 3-4%, means that the malt is not very well modified.

d.14) Hartong Index Vz 45 (%)

It is a measure of the percentage yield of extract obtained by mashing the malt flour at 45 °C for 1 hour. It is an is an indicator of the degree of modification of malt. Its value should be higher than 36%.

2.3 Brewing and beer

The stored malt is delivered to the brewery where, before using it for brewing, it is analyzed to assess its physical and chemical characteristics, performing the same analysis carried out in the malting plant. Then, the malt is used for beer production.

2.3.1 The brewing process

The most important process in beer production is the fermentation of sugars contained in the wort to form alcohol and carbon dioxide. To provide the necessary conditions for the realization of this process, the initially insoluble components in the malt must be converted into soluble products, and in particular soluble fermentable sugars must be produced. The formation and dissolving of these compounds is the purpose of wort production.

a) Malt milling

In order to give the malt enzymes the opportunity, during mashing, to act on the malt contents and to degrade them, the malt must be broken into small fragments. The greater the extent of comminution the larger the surface area available for enzymatic attack and the better breakdown of the malt material. This mechanical process of breaking malt into smaller pieces is called milling. Milling must be finer the less well modified the malt and the higher is the water content. The malt is fragmented in a grist mill. The malt used for a brew is called the grist. Depending on the process used a distinction is made between:

- Dry milling,
- Wet milling (conditioning),
- Hammer milling

The most commonly used mills in breweries are dry grist mills. In them the malt is crushed in a dry state between rollers arranged in pairs. Depending on the number of rollers, the mills are classified as 2, 4, 5 or 6 roller mills. Anyway, a whole series of considerations must be taken into account when fragmenting the malt. In fact, after mashing the wort must be run off and in this filtration process, depending on the mash separation equipment used, the husks are needed as a filter material. In the "lauter-tun" separation, the husks are required for mash separation, so they must be disintegrated as little as possible during milling. A dry husk fragments easily and filterability is greatly reduced by the small fragments produced by disintegration of the husks. On the other hand the husk is more elastic the wetter it is, and it is easier to protect. Consequently lautering is more rapid. The process of wetting the husks is known as conditioning. When a modern mash filter is used the filtration is performed through a very small pore polypropylene cloth. Therefore when

using such a mash filter the malt can be   very finely ground by a hammer mill and very good yields are consequently obtained [57, 58, 61].

b) *Mashing*

Mashing is the most important process in wort production. During mashing the grist and water are mixed together (mashed) and the contents of the malt are thereby brought into solution and with the help of enzymes, the extract is obtained.

The substances in the malt grist are only partly soluble. Only soluble substances can pass into beer. It is therefore necessary to convert the insoluble materials in the grist into soluble materials during mashing. All of the substances which go into solution are referred as to extract. Examples of soluble substances are sugars, dextrins, inorganic substances and proteins. Insoluble substances include starch, cellulose, part of the high molecular weight protein and other compounds which remain as spent grains at the end of the filtration process. The purpose of mashing is to completely degrade the starch to sugar and soluble dextrins. As a results of this other extract substances are also produced. Most of the extract is reduced during mashing by the action of the enzymes which are then allowed to act at their optimum temperatures and pH [58, 59].

The most important enzymes involved during mashing are:

Enzyme	*Optimum T °C*	*Optimum pH*	*Function*
Phytase	30 - 52	4.4 – 5.5	Wort pH reduction by hydrolysis of phytic acid
β-glucanase	36 - 45	4.5 – 5.0	β-glucan breakdown
protease	45 - 55	4.6 – 5.2	Protein breakdown
β-amilase	58 - 63	5.2 -5.6	Starch breakdown (maltose)
α-amilase	68 - 73	5.4 – 5.8	Starch breakdown

Table 2.1 - *The most important enzymes involved during mashing.*

The most important property of enzymes is their action in breaking chemical bonds in their substrates. This activity depends on various factors:

- Temperature: mashing involves increasing the temperature of the mash to the optimum temperatures for the enzymes which one wants to activate, and maintaining a rest at that temperature. The rest occurs at the temperature optima of the enzymes:

    - 45 to 50°C protein, β-glucanase and lowering pH rest
    - 62 to 65°C maltose production rest
    - 70 to 75°C saccharification rest
    - 75 to 78°C final mash temperature

    By mashing at 62 to 63°C the highest possible maltose content and highest attenuation limit is achieved. Maltose-rich worts ferment more quickly and hold the yeast more in suspension. By exceeding these temperatures and mashing for a long time at 72 to 75°C, a dextrin-rich beer with a low attenuation limit is obtained.

- Time: the enzymes certainly do not work uniformly throughout mashing. The maximum enzyme activity is reached after 10 to 20 min. After 40 to 60 min enzyme activity at first decreases rapidly, but the reduction in activity continuously decreases. With increasing mashing time the concentration of the extract solution increases. But the rate of increase becomes slower and slower, and form an economic point of view it does not make sense to continue the mashing for a time considerably longer than 60 min.

- pH: the enzymatic activity is very dependent on the pH. By mashing within a pH range of 5.5 to 5.6, which can be regarded as the optimum pH range for both amylases, the extract content can be increased, more fermentable sugar is produced and the attenuation is higher. However, the "normal" mash pH, depending on the composition of the brewing water and the malt, is higher, between 5.6 to 5.9. it is therefore advantageous to lower the pH value when mashing, for example by using acid malt or lactic or citric acid.

Depending on the way in which the temperature is raised, mashing process are classified into two types:

- Infusion, when the entire mash is heated up, with appropriate rests, to the final mashing temperature and no part of the mash is removed to be boiled separately.

- Decotion, when the temperature is increased by removing part of the mash and boiling it. By pumping back to the remainder of the mash the temperature of the total mash is increased to the next higher rest temperature. This process can be repeated several times (one, two or three mash procedure) [58, 59].

c) Lautering

At the end of the mashing process the mash consists of a watery mixture of dissolved and un-dissolved substances. The aqueous solution of the extract is called wort, the insoluble part is referred to as spent grains. The spent grains consist essentially of the husks, the seedling and other materials which do not go into solution on mashing or have again been precipitated during wort boiling. Only the wort is used for beer production and for this purpose it must be separated as completely as possible from the spent grains. This separation process is called lautering. During lautering as much as possible of the extract should be recovered. Lautering is a filtration process in which the spent grains play the role of the filter material. It occurs in two stages:

- running off of the first wort,
- sparging (washing out) of the spent grains (second wort).

When the first wort has been drained from the spent grains, the latter still contain extract. For economical operation this extract must be recovered. The extract retained by the spent grains is washed out by hot water. This process is called sparging. The thinner wort running off is called second wort. Its extract content decreases rapidly at first and then more and more slowly since the last extract is washed out of the spent grains only with difficulty. As the temperature increases the viscosity of the liquid decreases, but because α-amylase is destroyed at 80°C it is necessary to keep below this temperature during lautering. Sparging gradually dilutes the wort. In order to obtain the desired wort concentration at the end of lautering, the first wort must contain 4 to 6% more extract than the beer to be produced.

Lautering can be performed by using two systems [58, 59]:

- lauter tun: lauter tun are the oldest and by far the most widely used mash separation equipment. A lauter tun consists of a cylindrical vessel, on the slotted double bottom of which the spent grains are retained and filter the wort.
- mash filter: mash filters providing a competing system to the lauter tun, but they are not as widely used. Instead of the thick spent grains layer in the lauter tun, thought which filtration occurs, in a mash filter the spent grain layer is only about 4 to 6 cm thick and the filtration occurs here primarily through close meshed filter cloths. This enables better recovery of the extract absorbed in the spent grains.

d) Boiling

The wort obtained after lautering and sparging is boiled for 50 (to 60) min. During this time the hops are added. The end product of wort boiling is the cast wort. During boiling a number of important processes occur [57, 58]:

- Extraction and transformation of hops components. During wort boiling bitter and aromatic hop components are transferred into the wort. The hop resins or bitter substances are the most important component of hops for beer production because they give the beer its bitterness taste. The α-acids are completely insoluble in cold wort. In boiling wort, changes in the structures of α-acid occur which are referred to as isomerization. The iso-compounds produced are much more soluble than the α-acids from which they are formed.

- Formation and precipitation of protein-polyphenol-compounds. Compounds consisting of proteins and poliphenols of hops and compounds consisting of proteins and oxidised polyphenols are insoluble in the hot wort and precipitate during wort boiling as break. By break is meant the flocculent particles fromed during wort boiling. It is desirable to eliminate these compounds as completely as possible. Some compounds formed from protein degradation products and polyphenols remain in solution during wort boiling and do not precipitate until the wort is cooled. Precipitation is encouraged by a low pH and a longer wort boiling, but for economic reasons is preferable to boil no longer than necessary.

- Evaporation of water and consequent concentration of the wort. Today a rate of evaporation of 4% of the wort content is aimed for with good evaporation efficiency. In fact, evaporation of water uses energy and energy is expensive. Is therefore preferable to boil no longer than necessary and to do not evaporate unnecessary large amount of water.

- Wort sterilization. During boiling all micro-organisms contained in the wort are killed and the wort is sterilized. From on now, extreme biological caution is necessary.

- Destruction of all enzymes.

- Formation of reducing substances and increase of the wort color. During wort boiling substances are formed which can react with the oxygen in the wort and thereby exert a reducing effect. These substances are called reductones. These include, for example, melanoidins from the Maillard reaction, Stracker aldehydes and tannins oxidized. Then, during boiling the wort color increases and only becomes pale again during fermentation.

- Decrease of pH. The wort becomes slightly more acid since the melanoidins formed on boiling are acid and the hops also contribute some acid. The pH of unboiled wort without mash acidification is about 5.5 to 5.6, while the pH of the cast wort is about 5.4 to 5.5. Many processes proceed better or more quickly at a lower pH. Is therefore preferable to control the pH during boiling and, if necessary, to acidify it to a pH value of 5.2 – 5.4. In fact, at these pH values there is a better precipitation of protein-polyphenols complexes, a less increase in wort color, a better clean tasting hop bitterness and a more intense

sterilization. A disadvantage of using a lower pH is the worse hop bitter substances utilization, as a result of which more hops are needed.

- Evaporation of undesirable aroma substances. The wort contains a range of more or less volatile aroma substances which partly have a negative effect on the beer aroma. In order to establish an optimal aroma profile, it is necessary to remove these undesirable aroma substances from the wort. As well as DMS, these undesirable substances also include fat degradation products such as hexanal, several Strecker aldehydes such as 2 methyl-butanal and Maillard products such as furfural.

e) Clarification, or removal of the coarse break (whirpool)

The break from the cast wort is now called coarse break, as well as boiled or hot break (trub). It consists of large particles, 30 to 80 μm in size, which are slightly heavier than the wort and in general settle down well to form a compact mass if they are given sufficient time. The coarse break must be removed since it is not only no value in further beer production, but also actually detrimental to quality. In fact, it hinders wort clarification, it coasts the yeast, it increases the amount of break-rich sediments and thereby it increases the loss, it contains the fatty acids of the malt, and it makes beer filtration more difficult. The amount of coarse break is about 6000 to 8000 mg/L following casting-out and it should, after its removal, have been decreased to 100 mg/L. the aim, however, is the total removal. Whirpool is the most elegant method for hot break removal and it is least costly alternative of all trub removal methods.

The whirlpool is a cylindrical vessel in which the wort is tangentially pumped, and this produces a whirling circular motion of the wort inside the vessel. The bottom of whirlpool vessel may have variable geometry (flat, sloping or tapered). With the combination of three forces (centrifugal force, hydrostatic pressure and friction forces), the hot trub, being heavier than the liquid, tends to collect on the centre of the vessel bottom. The way in which the wort is pumped into the whirlpool is particulary important. The wort inflow velocity should not exceed 3.5 m/s. Often much smaller velocities are sufficient to cause the wort to rotate and produce the whirlpool effect. The rest period in the whirlpool is 20 to 30 min. It is important to keep the thermal exposure of the wort after boiling as low as possible and therefore the rest in the whirlpool should be kept as short as possible. A shorter rest produces less coloring and better flavor stability in the beer. This system of separation, does not allow to remove the cold trub formed by the breakdown products of proteins and polyphenols, that remains in solution and does not precipitate until the wort is cooled [57, 58].

f) Wort cooling and aereation.

64

Because yeast can only live and ferment at low temperatures, the hot wort must me cooled as quickly as possible to 7 to 10°C for low fermentation and to 15 to 20°C for high fermentation. During this process the initially clear wort becomes turbid because of the formation of the cold break. For rapid fermentation the yeast must be supplied with the optimal amount of oxygen. The aeration of cold wort for yeast nourishment is the only time during the entire beer production process that oxygen is deliberately added. The oxygen is taken up by the yeast within a few hours and does not damage the wort quality [57, 58].

g) Fermentation

Once cooled the wort, it is sent to fermentation tanks where the yeast is inoculated. To transform the wort into beer, the sugars must be fermented by enzymes in the yeast to ethanol and carbon dioxide by the yeast. The reactions occurring during fermentation can be differentiated into those which occur in the main fermentation and those occurring in maturation, but the processes overlap. It is therefore necessary to consider the changes which occur during fermentation and maturation as continuous process. It is particularly important to consider the fermentation by-products which have a significant effect on the taste, aroma and other characteristic properties of the beer. The formation and the partial degradation of these products is closely related to the metabolism of yeast.

The amount of yeast to inoculate varies depending on the situation, but a practical rule suggests inoculums of one million cells per milliliter of wort for each °P. Numerous different strains can be distinguished among yeast strains predominantly used in breweries. In brewing practice these are divided into two major groups, the top fermenting yeasts (Saccharomyces cerevisiae) and bottom fermenting yeasts (Saccharomyces carlsbengensis). The names top fermenting and bottom fermenting yeast strains are derived from their characteristic appearance during fermentation; bottom fermenting yeasts settle to the bottom towards the end of fermentation. Top fermenting yeasts also settle to the bottom at the end of fermentation, but much later than the bottom fermenting strains. Top and bottom fermenting yeasts also differ with regard to fermentation temperature. Fermentation with bottom fermenting yeasts are performed between 4°C and 12°C. In the case of top fermenting yeasts 14°C to 25°C is used. The temperature control is determined by the brewer. As soon as the yeast is inoculated, it consumes the available oxygen in order to multiply itself by respiration. In absence of oxygen, yeast is the only living organism which can change from respiration to fermentation. In this case, glucose is converted to alcohol (ethanol) and CO_2 as described by Gay-Lussac equation [57, 58, 62, 63]:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \text{ (Eq. 2.2)}$$

The alcohol which is produced still contains a lot of energy so that the energy acquired by the yeast cell during fermentation is around the 4% smaller than it is in respiration, and the multiplicative capacity is 30 times lower. Nevertheless at the end of fermentation the amount of yeasts is 5-7 times the initial inoculums for top fermenting beers and 3-5 times the initial inoculums for bottom fermenting beer. The yeast usually reach the stationary phase in 90'-120' min from the beginning of fermentation. Then, the cellular multiplication stops because of the reduced availability of sugars and the high number of cells and the high concentration of alcohol. The main fermentation lasts for 6-8 days for bottom fermenting beers and for 2-4 days for top fermenting beers. Apart from the alcohol and the CO_2 produced during the fermentation, a number of metabolic by-products from the yeast are passed into the beer, some of which either react with each other or change their amount or composition. A distinction is made between green beer aroma substances (diacetyl, aldehydes, and sulphur compounds) which are biochemically removed from the beer during fermentation and maturation, and mature beer aroma substances (higher alcohols and esters) which are formed during fermentation and not removed during maturation [57, 58, 62, 63]:

- Diacetyl: is the most important immature beer aroma. Above its threshold (0.15 mg/L) it gives beer an unclean, sweetish to revolting taste, which in high concentration is responsible for the aroma of butter. Because pentandione also acts in a similar way, although with a higher taste threshold, these substances are considered together and referred to vicinal diketones. Yeast form only the precursor of vicinal diketones by its metabolism. These precursors give rise to the vicinal diketones by oxidative decarboxylation outside, and independent of, the yeast cells. The diacetyl and pentandione formed can only be removed again by the yeast cells.

- Acetaldehyde: is the most important aldehyde, which occurs as a normal intermediated product in alcoholic fermentation. It is excreted into the green beer by yeast during the first three days of fermentation. It is responsible for the "green" young beer flavor. In the further course of fermentation the concentration of acetaldehyde decreases because it is removed. Consequently the immature beer flavor constantly decreases. In the young beer phase the aldehyde content is about 20 to 40 mg/L and it decreases to a value of less than 8 to 10 mg/L in finished beer.

- Sulphur compounds: yeast metabolism results in the formation of volatile sulphur compounds, such as H_2S (hydrogen sulphide), mercaptans and other compounds, which even in small concentrations have a very strong smell and taste. H_2S is produced during fermentation from sulphur-containing amino-acids, and it is very volatile, so it is partially

desorbed during fermentation and maturation by the ascending carbon dioxide bubbles. The same process leads to the removal of DMS. Mercaptans are oxidized by oxygen to disulfides, which are less dangerous for the beer taste.

- Higher alcohols: they are finished beer aroma compounds, which give to the beer a fruity taste. They are formed by the yeasts from amino acids and sugars. About the 80% of the higher alcohol is formed during the primary fermentation. Concentrations of higher alcohols above 100 mg/L damage the flavor of the beer. The content of higher alcohols is 50 to 60 mg/L for bottom fermenting beers and 60 to 90 mg/L for top fermenting beers.

- Esters: are the most important aroma compounds in beer and to a large extent determine its aroma. Higher ester concentrations can, however, give a beer with an unpleasant bitter, fruity taste. Esters are formed during fermentation by esterification of fatty acids and also by the esterification of higher alcohols. Beer contains about 60 different esters of which, however, ionky about six are of much importance for the flavor properties of beer: ethyl-acetate, isoamylacetate, isobutylacetate, β-phenylacetate, ethylcaproate and ethylcaprylate. Their concentration increases mainly in the vigorous phase of fermentation. Their concentration in the maturation stage is dependent on the secondary fermentation and the amount of esters may double with a long secondary fermentation. The ester content depends on the beer type and the original wort gravity. Top fermenting beers contain up to 80 mg/L of ester and bottom fermenting beers contain up to 60 mg/L of ester.

Besides the formation of by-products a number of other reactions and changes occur during fermentation which are very important [57, 58, 61, 63]:

- Lowering of the pH value. At the end of fermentation, the pH of beer is 4.2 to 4.6, while in the wort at the time of inoculation the pH is about 5.2 to 5.6. This decrease in the pH value depends on many factors, including the formation of organic acids as a result of deamination of amino acids, the use of primary phosphate ions by the yeasts, the uptake of ammonium and potassium, nitrogen ions by the yeasts and the release of hydrogen ions. A value of pH below 4.4 has a positive effect because it accelerates the precipitation of bitter substances and polyphenols and leads to a finer beer in the flavoring and taste. An increase in pH may instead be an indication of yeast autolysis.

- Changes in the composition of nitrogen compounds.

- Lightening of about 3 EBC units in the beer color.

- Clarification of the beer, because of the precipitation of bitter substances and polyohenols.

- Dissolving of CO_2 in the beer.

At the end of the primary fermentation the yeast is collected (about 2-3 liters/hl of wort) from the bottom of the fermentation vessel where it settled. At this point the secondary fermentation or maturation takes place. It is performed at a temperature from -1 to 2 °C for a period ranging from several weeks for top fermenting beers to several months for bottom fermenting beers. During this phase three fundamental phenomena that turn beer green beer in mature beer take place [57, 58, 61, 63]:

- saturation of beer with carbon dioxide. The production of carbon dioxide which allows the saturation of beer in tanks under pressure arises from the metabolism of the yeast not removed at the end of primary fermentation. In packaged beer the CO_2 content is about 0.45 - 0.5%. Only about the 15% of the CO_2 produced during the fermentation remains dissolved in the beer. The solubility of CO_2 in beer decreases with increasing temperature and increases proportionally with the pressure.

- clarification of beer, which is caused by flocculation of yeasts and the precipitation of protein-polyphenol-compounds.

- refinement of the beer taste. The yeast not removed at the end of primary fermentation remove the green beer aroma substances (diacetyl, aldehydes, and sulphur compounds) and produce the mature beer aroma substances (higher alcohols and esters).

After maturation, beer can become spoilt and unpleasant very quickly. There are several reasons for this [57, 58, 62, 63]:

- any micro-organism (contaminants) in the beer can multiply, make the beer hazy and as a result of excreting metabolic product, make it unpleasant.

- in time colloids in the beer increase in size, for various reasons, and make the beer hazy.

- with time the beer flavor becomes spoilt, because of further undesirable transformation preformed by residual yeasts and enzymes.

Therefore, everything must be done to ensure stability. There are two approaches to this:

- making the beer colloidally stable (filtration)

- making the beer microbiologically stable (pasteurization and sterile filtration)

h) Filtration

At the end of the maturation process the beer is oxygen-free, but up to 1 million yeast cells and other turbidity-causing particles are still contained in suspension which have to be removed without harmful oxygen having access to the beer. Filtration is a separation process in which the yeast cells and other turbidity-causing materials still present in the beer are removed. At the same time substances are removed which would, in the course of the next few weeks or months, themselves

precipitate and make the beer turbid. The purpose of filtration is to make beer so stable that no visible changes occur for a long time [62, 64].

i) Pasteurization

The purpose of pasteurization is to increase the shelf life of beer by killing all the microorganisms still present and by the inactivation of all enzymes responsible for undesirable chemical changes. The heat treatment must be as small as possible, because the heat can have negative effects on the organoleptic properties of beer [62].

The beer can be pasteurized in two different ways:

• Flash pasteurization: it is done directly on beer before packing. The beer is heated by a plate heat exchanger to at least 68 to 72°C and held at this temperature for about 50 seconds [65].

• Tunnel pasteurization: it is carried on the bottles or cans and not directly on the beer. The bottles or cans pass through a tunnel where they are warmed by hot water (up to about 60 °C) for 10 to 20 min [62].

The sterile filtration is designed to remove all micro-organisms without subjecting the beer to heat treatment, considering its negative effects on the quality of beer. However, sterile filtration, in contrast to the pasteurization, does not denature the enzymes that are only partially removed [62, 64].

l) Packaging

The packaging is the final stage of the process of beer production. Beer can be packaged in cans, bottles or kegs and for each type of packaging are used different filling technological processes. In any case, the beer should be scrupulously kept away from the air (oxygen absorbed should not exceed 0.02 to 0.04 mg/L) and always under pressure as any loss of carbonic dioxide are irreversible. Hygiene is essential in all the sectors but particularly for beer packaging plants, because any microbial contamination could waste all the work done before that stage [62].

2.3.2 Beer

Beer evaluation

Quality examination of the finished beer has three different aspects [56, 58, 59]:

    a) beer tasting

    b) microbiological monitoring

    c) chemical and physical properties examination

c) Chemical and physical properties examination

c.1) Determination of the alcohol content and of the original (°P), real (% w/w) and apparent extract (% w/w) of beer

The determination of the original gravity is supposed to provide information about the constituents of the beer. Only a part of the original constituents are still present because a considerable portion are lost during fermentation. The extract content of the wort before fermentation is the original gravity or original wort extract. The unfermentable extract still present in the beer can be measured using for example a simple hydrometer, or a densitometer, and this determination leads to the calculation of the apparent extract of the beer. To obtain the true or real extract content the alcohol must be removed, e. g. by distillation. The determination of alcohol content is also an important parameter to consider in beer quality evaluation.

c.2) Measurement of beer color (EBC)

The color measurement is usually performed visually or spectrophotometrically and given in EBC units. This value is important for the beer quality and it changes according to the type of beer considered. For example, pilsner beers have a color between 6 to 11 EBC, pale beers between 7 to 15 EBC, dark beers from 30 to 100 EBC and bock beers from 8 to 25 EBC.

c.3) Measurement of pH

The pH is always measured in beer analysis. The measurement of pH is very important because all enzymatic processes are dependent on it as is the behavior of the microorganisms. In beer the pH value should be between 4.2 and 4.6.

c.4) Measurement of the oxygen content of the beer (mg/L)

The monitoring of the oxygen content is one of the most important controls in the production of beer. High oxygen values can have a very detrimental effect on the quality of the beer and its flavor stability. This value should not exceed the 0.15 mg/L.

c.5) Measurement of the diacetyl content of beer (mg/L)

Diacetyl is the most important off-flavor of beer, and its value should not exceed 0.10 mg/L.

c.6) Determination of the carbon dioxide content (%)

The dissolved CO2 content of the beer is an important quality parameter, particularly for the production of good tanginess in beer. Normal CO2 contents can be considered to be between 0.45 and 0.60 % by weight in the case of bottom fermenting beers and from 0.40 to 1.00 % by weight in the case of top fermenting beers.

c.7) Measurement of bitterness units (EBU)

Measurement of the bitterness is an important control since the bitterness of the taste greatly affects the beer. Determination of the EBC bitterness units (EBU) is performed spectrophotometrically. Values for different beer types are, for example, from 20 to 40 EBU for a pilsner beer and from 20 to 30 EBU for a pale beer.

3 MATERIALS AND METHODS

3.1 What is a calibration

Generally speaking, every quantitative method aims to determine a system property Y quantitatively from a measured system parameter X. For this reason, to construct a calibration is necessary to find a correlation between a measured system parameter X, ie a spectrum, and the analytical property of interest Y. This determination normally requires two steps: calibration and analysis (prediction) [66, 67, 68].

1) Calibration: During this process, a correlation between the measured quantity X (spectrum) and the properties of interest Y, is searched for. The property of interest Y value is known for the samples used for the calibration (training set), as shown in Figure 3.1 . This correlation is expressed by the calibration function b, otherwise known as "regression coefficient"or "b-factor" [66, 67, 68]:

$$Y = X \cdot b \text{ (Eq. 3.1)}$$

Calibration:

| X-Data (Spectra) | + | Y-Data (analytical values) | = | Calibration Function b |
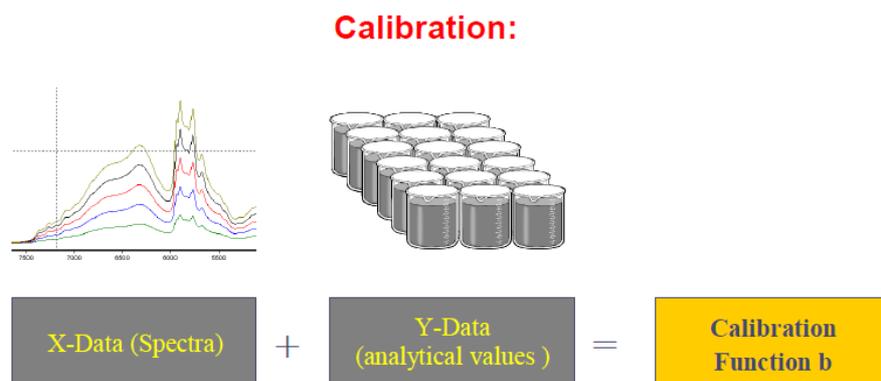
Figure 3.1 - graphic representation of a calibration [66]

2) Analysis:

After calibration, the analysis is performed. By relating the calibration function b with the measured parameter X, the system property of interest Y of an unknown sample is determined, as shown in Figure 3.2 [66, 67, 68]:

Analysis:
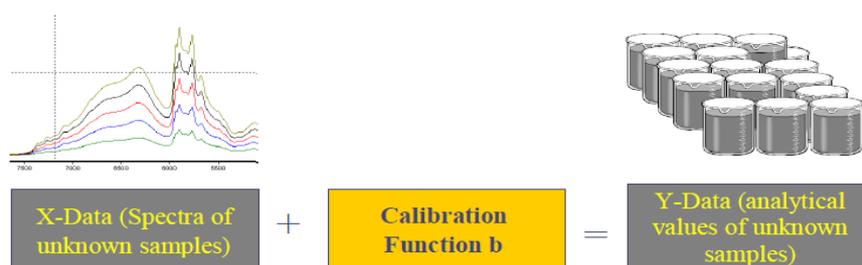
Figure 3.2 - graphic representation quantitative determination of substances using a calibration [66]

Then it quickly becomes evident that the development of a calibration function is critical to the success of the analysis.

3.1.1 Types of Calibration

There are two types of calibration, the monovariate and the multivariate analysis. Calibration monovariate, because of its many limitations, makes it impossible to draw information from complex NIR spectra, so the calibration discussed in this thesis is obviously multivariate, and makes use of modern chemometric methods of data processing [66, 69, 70].

Monovariate calibration

In the case of monovariate calibration, only one spectral information, e. g. peak height or peak area, is correlated to the reference value. These calibration for individual components, based on the Beer-Lambert law for a single wavelength, calculate a linear correlation, concentration/absorbance for each component (2 components = 2 wavelengths), as shown in Figure 3.3 [66, 69, 70].
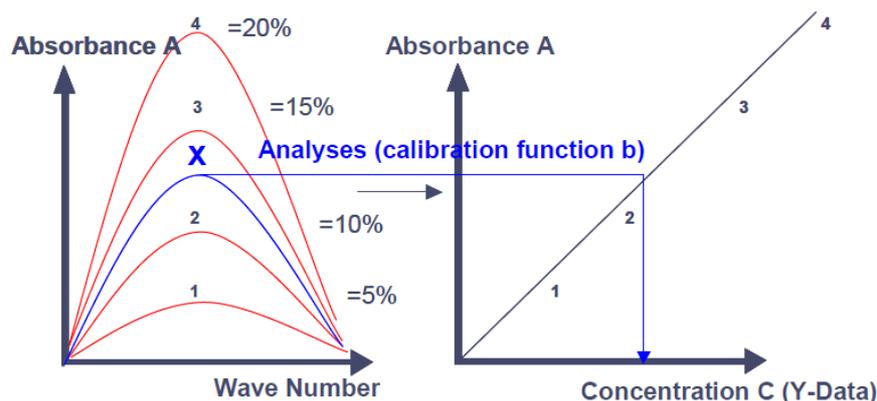
Figure 3.3 - Schematic representation of a monovariate calibration [66]

In most cases, the calibration monovariate shows a poor predictive ability, because of its limitations [66, 69, 70]:

- The concentration of analyte is only related to a single point of the spectrum, and therefore neither outliers nor the presence of interfering components can be determined.
- The statistical variation of the signal, such as detector noise, is directly incorporated into the spectral data.
- A satisfactory univariate calibration of multi-component systems, requires a sufficient separation of peaks. This, in the case of NIR spectroscopy, it is simply impossible.
- In the analysis of multi-compounent systems, a linear relationship between the absorbance values and the concentrations of the various analytes at the measured wavelength is assumed. In many cases, this is not true for real systems because intermolecular forces or temperature effects can lead to distortion of the respective analytes bands.

Multivariate Calibration

The multivariate calibration generally combines a large amount of spectral information with the corresponding reference sample. This leads to higher precision and error stability. It can be used for single or multiple component systems. Compared to classical monovariate calibration, this technique does not use a spectral data evaluated at a single wavelength, but the full spectrum, measured over the entire frequency range. The advantage of this type of calibration is the largest amount of spectral information used, so even the smallest differences between the spectra can be identified. This allows to identify outliers and to recognize the spectral structures in noisy areas (higher signal to noise ratio) [66, 69, 70].

Moreover, as seen previously, because of the overlapping of bands in the NIR region it is not possible to use simple linear regressions for the calibration (as in the case of UV-Vis for example). For this reason, over the time many mathematical models have been developed to solve the problem of overlapping spectral in multivariate calibrations [66, 69, 70].

Currently, NIR spectroscopy is the analytical technique which most applies chemometrics. Chemometrics is the use of mathematical and statistical techniques for extracting relevant information from analytical data, in the present case, the NIR spectral data. Both Chemometrics and NIR technology have evolved in a symbiosis where NIR spectroscopy achieves more robust identification and quantitation models and extends its applicability, while posing new challenges to chemometrics that motivate the improvement of many of its techniques [66, 69, 70].

There is an arsenal of chemometric tools dedicated to make use of NIR spectroscopic information. The most common are Classical Least Square (CLS), Inverse Least Square (ILS), Multiple Linear Regression (MLR), Principal Component Regression (PCR) and Partial Least Square Regression (PLSR) [71, 72].

The Classical Least Squares (CLS) model assumes that measurements are the weighted sum of linearly independent signals. In spectroscopy, for example, the CLS model assumes that measured spectra are the sum of pure component spectra weighted by the concentration of the analytes. The main disadvantage of CLS is that the pure responses S of all spectrally-active components must either be known a priori or estimated using known concentrations of all spectrally-active components. These estimates must include any minor components that may not be of interest themselves but may contribute to the measured signal. It is possible to get around the problem of having to know or estimate S by using an inverse least squares (ILS) model. ILS assumes that a regression vector b can be used to determine a property of the system y from the measured variables x (a row vector, such as a spectrum). In the classical model, responses are expressed as a function of pure component concentrations (p), whereas the inverse model expresses a single concentration (y) as a function of the responses. Note that the inverse model formulation is more widely applicable than the classical (CLS) case, for at least two reasons: 1) only the value of the property of interest (y) needs to be known, and 2) this property is not restricted to being a component concentration property. Unfortunately, this approach often fails in practice because of collinearity of some or all of the response variables in X (e.g., some columns of X (variables) are linear combinations of other columns), or because X contains fewer samples than variables (fewer rows than columns). For example, typical spectroscopy calibration problems are extremely ill-conditioned due to a high degree of correlation between absorbances at adjacent wavelengths. Also, the number of

wavelength channels offered by the spectrometer is typically greater than the number of calibration samples that are available [71, 72].

For this reason, it is necessary to use calibration methods based on variable reduction. These methods combine the advantages of the two above-described least-squares methods. Because they are inverse, indirect methods, they allow individual analytes in mixtures to be quantified without the need to know the other components. Also, they use the information contained in the whole spectrum, and " compress " it into a small number of variables. This avoids the need to select variables and facilitates detection of interferences and outliers. The procedure used to select wavelengths depends on the number of variables available. The aim is to select the calibration equation providing the closest fitting [71, 72].

In multilinear regression (MLR), a specific number of wavelengths (or frequencies), k, are chosen such that k << n. The choice of specific wavelengths (or frequencies) to include in a multilinear regression model is a critical factor in the model development. Several mathematical algorithms have been suggested for making this selection. Alternatively, selection may be based on prior knowledge of a relationship between the absorptions measured and the property or component being modeled. Anyway, regression by MLR suffers from two different problems: 1) the relative abundance of response variables relative to the number of available calibration samples (for the typical spectral calibration problem), which leads to an underdetermined situation, and 2) the possibility of collinearity of the response variables in X, which leads to unstable matrix inversions and unstable regression results. Principal Components Regression (PCR) is one way to deal with both of these problems. Instead of regressing the properties of interest (e.g., concentrations) onto a set of the original response variables (e.g., spectral response variables), the properties are regressed onto the principal component scores of the measured variables, (which, by definition, are orthogonal, and therefore well-conditioned). Like PCR, Partial Least Square Regression (PLSR) involves the decomposition of the spectral data matrix, X, into the product of matrices. Unlike PCR where X is first decomposed, and then regressed versus the reference values, in PLS, the y vector is used in obtaining the decomposition of X. PCR and PLS can be considered standard calibration techniques for NIR spectroscopy. The main advantage of these techniques is to avoid co-linearity problems permitting to work with a number of variables that is greater than the number of samples. A comparison between these two techniques reveals similar results in terms of prediction performance, with no significant difference being reported when both employ the optimised number of principal components (PCs). PLS usually produces good models using a lower number of PCs than its counterpart, PCR [71, 72].

3.2 Constructing multivariate calibration models

Constructing a multivariate calibration model is a complex, time-consuming process that requires careful selection of variables in order to ensure accurate prediction of unknown samples. This requires knowledge not only of the target samples, but also of chemometric techniques in order to obtain a model retaining its predictive ability over time and amenable to easy updating.

The process of obtaining a robust model involves the following steps: choosing the samples for inclusion in the calibration set, determining the property to be predicted by using an appropriate method to measure such samples, obtaining the analytical spectral signal, constructing the model, validating it and, finally, using it to predict unknown samples.

Below is described in detail each step involved in the modeling of analytical data [71, 72].

3.2.1.Selection of calibration samples

This is one of the most important steps in constructing a calibration model. Usually the set of known samples used to develop calibration is called training set. It should be representative of the whole population of all samples that will be analyzed [66, 67, 73]. The training set ideally should encompass all possible sources of physical and chemical variability in the samples to be subsequently predicted. Variability in the samples used to construct the model is due to the body of factors affecting some property of the samples in such a way as to reflect in their spectra. Variability sources can be of diverse nature and origin. In any case, the samples included in the training set should be representative of the whole population and exhibit values of the target parameter uniformly spanning its potential range of variation. New samples will predict by interpolation within the model limits as no accurate prediction can be ensured by extrapolation.

For the development of a multivariate model, an ideal training set will [71, 72]:

- Contain independent samples
- Contain samples which provide examples of all chemical components which are expected to be present in the samples which are to be analyzed using the model, thereby ensuring that analyses involve interpolation of the model;
- Contain samples for which the range of variation in the concentrations of the chemical components exceeds the range of variation expected for samples which are to be analyzed using the model, thereby ensuring that analyses involve interpolation of the model;
- Contain samples for which the concentrations of chemical components are uniformly distributed over their total range of variation;

- Contain a sufficient number of samples to statistically define the relationships between the spectral variables and the component concentrations or properties to be modeled. The number of samples that are required to calibrate an infrared multivariate model depends on the complexity of the samples being analyzed. In general, the number of samples required to build a valid calibration range from 20 to 200, depending on the complexity of the composition of the sample. Obviously, the greater the number of components, most samples will be needed to construct the calibration. If the samples to be analyzed contain only a few components that vary in concentration, then there will be a small number of spectral variables, and a relatively small calibration set is adequate to define the relationship between the variables and the concentrations or properties. If a larger number of components vary in the samples to be analyzed, then a larger number of calibration samples is required for the model development. Determining whether or not a set of calibration samples is adequate can only be done after a model is developed and an estimate of the number of spectral variables required for the model is made. If a multivariate model is developed using three or fewer variables, then the calibration set should contain a minimum of 24 samples after elimination of outliers. If a multivariate model is developed using k (>3) variables, then the calibration set should contain a minimum of 6k spectra after elimination of outliers. If the model is mean centered, a minimum of 6(k + 1) spectra should remain.

- Each sample is associated with two types of variables: independent (spectra) and dependent (the target parameter). The samples included in the calibration set should span the whole variability in both; thus, the selected samples should be uniformly distributed throughout the calibration range in the multidimensional space defined by spectral variability. One simple method for selecting samples based on spectral variability uses a scatter plot obtained from a PCA applied to the whole set of available spectra. Inspecting the most salient PCs in the graph allows one to clearly envisage the distribution of the sample spectra; those to be included in the calibration set are chosen from both the extremes and the middle of the score maps obtained and simultaneously checked to uniformly encompass the range spanned by the quantity to be determined. This method is effective when the first two or three PCs contain a high proportion of the total variance.

The number and type of samples used in this thesis project are better explained in the "3.4 Samples" section.

3.2.2.Reference methods

Reference values are generated by mixing the samples in the laboratory, or by quantitative determination using a different analytical technique. Apart from an even distribution of the concentration values over the entire concentration range, in the case of multi-components mixtures it is important to avoid the collinearity between the values of the parameters to be determined. This means that the individual concentration of the respective components must not increase or decrease equally in the different samples. Models established from collinear data are scarcely robust and can produce mathematical artifacts leading to spurious predictions. In fact, in the case of a collinear data set, some algorithms like PLS can not assign the individual spectral bands to the respective component values. A method set up with collinear data set is useless for the analysis of non-collinear data-set [66, 72].

On the other hand, in the case of material samples where the reference values are determined with an independent analytical method, the reference method used should provide accurate and precise values if the multivariate model finally developed is to be accurate as well. In fact, the presence of inaccurate data, leads to dispersion of the statistical analysis values around the true value. If the quality of the reference data is difficult or impossible to improve, it is advisable to make several repeat measurements of the same samples and to construct an average. Because NIR spectroscopy is a relative technique the accuracy of the multivariate model depends on the reliability of the reference data. However, the precision of the model may be better than that of the reference values since regression averages random errors [66, 72].

The reference methods used in this thesis project are better explained in the "3.5 Reference Methods" section.

3.2.3. Obtaining the analytical signal (spectra acquisition)

The analytical signal (namely the spectra of the samples used to construct the model) should be obtained with the same instrument and under identical conditions as those subsequently used for routine analysis in order to ensure that all spectral will contain the same sources of instrumental variability. The essential condition for spectra to be useful for constructing calibration models is that they should contain the information to be modeled, which is not always the case. One should bear in mind that chemometrics can extract information present in a data set, but not create it from scratch. Also, the amount of information contained in the set should be large enough to allow the development of models with an adequate predictive ability for the target parameters. In fact, during the measurements of spectra of calibration samples must be taken into account all external disturbances that may occur and the measures that can not be avoided by careful preparation of the

sample. Some algorithms like PLS can distinguish between analytically relevant and useless spectral structures. Disturbances are detected during calibration and eliminated in further analysis. The task of the method developer is to measure the samples in realistic conditions. Consequently, all potential disturbance that interfere with the system should be taken into account in order to make the algorithm "learn" to recognize and eliminate them. An example of these disorders are environmental influences (changes in temperature, diffusion of CO_2, or water vapor) that can be varied both the characteristics of the sample and those of the measuring instrument. NIR measurements are not so much sensitive to these environmental disturbance and they are affected noticeably only by the atmospherically dissolved water. Its disturbance is generally compensated by a regular reference (background) measurement. The reference substance should be as inert as possible and have not bands absorption in the spectral range relevant to the analysis. For NIR analysis, especially those on solids, the most used back ground materials are teflon or rough surfaces covered metals able to reflect the light [66, 67, 68, 73]. The stability of the NIR instrument is also very important.

The instrument used during this thesis is a Vector 22/N FT-NIR spectrometer system, equipped with tungsten source, RocksolidTM interferometer, fiber-optic module equipped with Ge-Diode detector and an integrating sphere module equipped with PbS detector for spectra acquisition in diffuse reflectance mode (Bruker Optics, Milan, Italy). All log1/R spectra were recorded on a quartz-bottomed cup (4 cm inner diameter) placed on the integrating sphere optics and, to compensate for the lack of homogeneity, the sample was spinning during the measurement (10 rpm). Absorption spectra were collected at room temperature against a gold-coated background by means of the OPUS software (version 5.5 or 6.5, Bruker Optics) in the spectral range of 11,500–4,000 cm^{-1} (900-2500 nm) with a resolution of 8 cm^{-1} using 64 scans for averaging (the same number of scans was used for the background). In order to correct the long term drifts affecting the Reflectance/Absorbance spectrum, due to changes in the water and CO_2 content in the optical path of the instrument, one reference background was collected for each sample, to calculate the sample absorbance spectrum, by using the ratio between the sample and reference signals . During the four years of this study the spectrometer was of course subject to different instrument checks to test the instrumental stability. On day-by-day basis a "Performance Qualification (PQ) test protocol" has been applied to ensure that the instrument was working properly. The "PQ test protocol" consists of the several long-term stability single tests, which compare the measured data to a set of reference data which have been recorded after installation, major repair or exchange of optical components. The single tests performed during a "PQ test protocol" are: Deviation from 100% Line; Interferogram Peak Amplitude, Energy Distribution (Single-Channel Spectrum), X-Axis

Frequency Calibration Test (Wavenumber accuracy) and Y-Axis Reproducibility Test (Photometric Accuracy). Through these tests, it has been possible to detect any changes in the source power, especially using the "Energy Distribution" test. In fact, if the power of the spectrometer source decreases, the distance between the test single-channel spectrum and the reference single-channel spectrum will increase and pass the maximum allowed value. On the basis of the "PQ test protocol" results, it was necessary to change the lamp every year. In this way, it was possible to avoid fluctuation in the spectra due to the natural change in the light source intensity. As soon as the source has been changed, a new reference spectrum was measured, to perform a new "PQ test protocol". Moreover, a "Operational Qualification (OQ) test protocol" was performed to check the instrument performance for several parameters (resolution, sensitivity, scan time, alignment and linearity) comparing them to the instrument specifications.

3.2.4. Calculation of the calibration model

Calculating a calibration model involves processing the analytical signal in order to establish its most simple possible relationship with the target parameter (whether an analyte concentration or some physical property of the sample) [69, 70].

The aim of calibrating is to calculate the parameters in an equation allowing a property in future, unknown samples to be accurately determined (i.e. with as small as possible a departure from the actual values). The quality of calibration models can be assessed via some statistical parameters,

In this thesis the PLS (Partial Least Squares, on Latent Structures or Projections) algorithm has been used for the calculation of multivariate calibrations. This algorithm provides a continuous relationship in space between two variables related to each other.

Calling X (K x N) the matrix of spectral data (with K = wavelengths and N = samples) and Y (P x N) the matrix of concentration data (P = analytes), the PLS regression (PLSR) assumes that the information contained in these two matrixes can be concentrated into a smaller number of variables in order to reduce signal to noise without losing relevant information.

This method regresses the new variables rather than the measured responses, and hence simplify construction of the calibration model and interpretation of the results. Their importance lies in the ability to solve—at least partly—typical problems such as the following:

- *Poor selectivity: measurements of the variables X can be influenced by the presence of interferents accompanying the analyte. The PLS algorithm leads to the elimination of noise and the highlighted new relevant features in the X data, which are correlated with the Y matrix.*

- *Redundancy and collinearity: the information contained in X can be redundant or even correlated. The PLS algorithm leads to data compression. Moreover, PLSR uses an orthogonal space for the regression, thereby avoiding the problems derived from collinearity between variables*

- *The lack of an accurate knowledge of the influence of Y on X: One may not know all sample components influencing X or their mutual interactions.*

Briefly, PLS involves a principal component analysis (PCA) of the data matrix and the reference values of the parameter to be modeled, contained in the y vector (PLS1) or matrix (PLS2) are used in obtaining the decomposition of X by a least squares regression.

The aim of the PLS algorithm is to build a calibration model using the minimum number of factors (or latent variables).

The eigenvectors derived from the spectral data matrix X containing all information relevant to the investigation system and are used to predict concentrations instead of the original spectra.

In the case of PLS regression, the factors are extracted using a "downward" order, ie the first factor characterizes the biggest change in the spectrum observed correlated with the analytical properties of interest and so on. The greater the number of factors, most changes are characterized.

Principal component analysis

Principal component analysis is a variable compression method that reduces the data set of matrix X (K x N) to a much smaller number of A variables called principal components (PCs). The corresponding mathematical model is constructed from the expression [69, 70, 71, 72]:

$$X = TP^T + E \text{ (Eq. 3.2)}$$

where T (N x A) is a matrix containing the A scores for the PCs, P (K x A) that containing the A loadings for the PCs and E (K x N) the residuals matrix of the model.

The scores are the intensities of the new A variables for the samples and the loadings the new variables obtained from the original ones. The PCs are orthogonal to each another, so both vectors are completely uncorrelated. One major consequence of the orthogonality in the PC vectors is that correlation is completely eliminated by using the new variables instead of the original X .

The aim of PCA is to identify the directions, allowing the original data matrix to be reduced to a simpler one while deleting useless information. The mathematical algorithm used simply calculates the eigenvectors and eigenvalues of a matrix; as can be easily demonstrated, if the variables X are centered, then the vectors of the loadings p a (with a 1, 2, … , A PCs) are the eigenvectors of the

matrix (X^TX) and those of the scores t a the eigenvalues of the matrix (XX^T). The most common among the computational algorithms available for this purpose calculate PCs in a sequential manner via an iterative least-squares process followed by subtraction of the contribution of each component. This means that the first PC extracted explained the maximum variation and so on.

Partial Least Squares Regression (PLSR)

The PLSR algorithm uses the information contained in both the spectroscopic data matrix, X , and the concentration matrix, Y , during calibration and compresses data in such a way that the most variance in both X and Y is explained. In this way, PLSR reduces the potential impact of large, though irrelevant, variations in X during calibration.

As in PCA, matrices X and Y are centered or autoscaled prior to resolution into factors. Matrix X is used to extract a few latent variables (a = 1, 2,…,A). Thus, each matrix is resolved into a combination of A factors ($A \leq K$), which allows the simultaneous calculation of [69, 70, 71, 72, 74, 75]:

$$X = TP^T + E = \sum_{a=1}^{A} t_a p_a^T + E \text{ (Eq. 3.3)}$$

$$Y = UQ^T + F = \sum_{a=1}^{A} u_a q_a^T + F \text{ (Eq. 3.4)}$$

With M samples, A factors, K variables and P analytes, matrices T (M x A) and U (M x A) will be the scores matrices for blocks X and Y , respectively; matrices P^T (A x K) and Q^T (A x P) the loadings matrices for blocks X and Y , respectively; and E and F the residuals matrices for blocks X and Y , respectively.

The process is started by calculating a small, though adequate, number of latent variables W(X) (loading weights), which are extracted from the variables in matrix X; the desired number of latent variables are stored in a scores matrix T which is used to iteratively model the variables in X and Y until convergence is reached.

Similarly, the variables in Y can be modeled from those in X via the matrix of regression coefficients B . The coefficients in B can be estimated as a function of the loadings of X and Y, namely P and Q respectively, in addition to W(X) [69, 70, 71, 72, 74, 75]:

$$\hat{B} = W(P^T W)^{-1} Q^T \text{ (Eq. 3.5)}$$

Unlike PCA, the loadings do not coincide fully with the direction of maximum variation since they have been corrected in order to maximize the predictive ability of matrix Y .

If only the concentration of one of the components in Y is to be determined, even if all others are known, then the algorithm, PLS1, is a simplified version of the complete algorithm and is designated as PLS2.

For calibration, the regressors matrix $\hat{B}$ allowing a sample to be predicted without the need to resolve it into scores and loadings matrices is calculated. Thus, if the spectrum for a given sample is defined by vector x_i , the concentrations of the analytes y can be calculated from [66, 72]:

$$\hat{y}_i = x_i^T \hat{B} \quad \text{(Eq. 3.6)}$$

Evaluation of the model

The quality of calibration models can be assessed via some statistical parameters, of which those allowing the mean error for the whole population rather than a single sample are to be preferred. The statistics typically used to assess the quality of calibration models calculate the error of prediction as the summation of the squares of the residuals, which is usually designated as the Predicted Residual Error Sum of Squares (PRESS) [66]:

$$PRESS = \sum_{i=1}^{N}(y_i - \hat{y}_i)^2 \quad \text{(Eq 3.7)}$$

Where N is the number of samples in calibration, y_i is the true value (determined with the reference method) of the analyte for the i-sample, and $\hat{y}_i$ is its estimate calculated by the model.

From this parameter, it is possible to calculate the Root Mean Square Error of Calibration (RMSEC), which is also used for the evaluation of the calibration, and which should be as lower as possible [66]:

$$RMSEC = \sqrt{\frac{PRESS}{N}} = \sqrt{\frac{\sum_{i=1}^{N}(y_i - \hat{y}_i)^2}{N}} \quad \text{(Eq. 3.8)}$$

3.2.5. Validation of the calibration model

To assess the quality and robustness of a multivariate calibration is necessary to perform a validation of the method. Currently there are no theoretical models that allow to directly estimate

the error related with a calibration function, so it is necessary to use a set of test samples with known concentrations (see Figure III.15) [66, 76].

To validate a multivariate calibration method it is possible to use two methods: internal validation (or cross-validation) and external validation (or test-set validation):

a) Internal validation (cross validation)

In the case of an internal validation, individual samples (defined by the user) are taken from the calibration set. It is possible to exclude 1 sample (cross-validation leave-one-out) or n samples (cross-validation leave-n-out). Using the remaining samples, a model is established and used to analyze the previously extracted samples. A comparison of the result with the real concentration determined with the reference method shows how precisely the model predict the concentration in the samples. By extracting the samples beforehand, it is guarantee that they are not known to the calibration model and thus they are independent. In fact, only if the data set used for the validation is independent from the one used for the calibration the actual preciseness in prediction can be assessed realistically. To assess the complete data set, the samples analyzed previously are returned to the calibration data set, and a second set of test samples is removed for the analysis. This procedure of removing samples, analyzing them and returning them to the calibration data set is continued successively until all samples have been analyzed once. A comparison of the resulting analyses values (predicted) with the original raw data (true) allows the calculation of the predictive error of the complete data system, the Root Mean Square Error of Cross-Validation (RMSECV) [66]:

$$RMSECV = \sqrt{\frac{\sum_{i=1}^{N}(y_{i-true} - y_{i-predicted})^2}{N}} \quad \text{(Eq. 3.9)}$$

The main advantage of cross-validation is that the same samples used for the validation are used also for the calibration. So it is possible to obtain a model sufficiently robust and validate it even having few samples. The main disadvantage is that leave-one-sample-out cross validation can be too optimistic and lead to an overestimation of the model predictability. Another disadvantage is that the time required for validation are greater than the external validation.

b) External validation (test-set-validation)

The external validation requires two completely independent data set of standard samples. One set is used for the construction of the model (training set) and it does not change during the validation, while the other is used for the validation (test set) in order to calculate the difference between the

reference value and that predicted by the method. This difference is usually expressed using the bias and the Root Mean Square Error of Prediction (RMSEP) [66, 76]:

$$RMSEP = \sqrt{\frac{\sum_{i=1}^{M}\left(y_{i-true}-y_{i-predicted}\right)^2}{M}} \quad \text{(Eq. 3.10)}$$

Where M is the number of samples included in the test set.

For the validation of a multivariate model, an ideal validation sample set will:

- Contain samples that provide examples of all chemical components which are expected to be present in the samples which are to be analyzed using the model;

- Contain samples for which the range of variation in the concentrations of the chemical components is comparable to the range of variation expected for samples that are to be analyzed using the model;

- Contain samples for which the concentrations of chemical components are uniformly distributed over their total range of variation;

- Contain a sufficient number of samples to statistically test the relationships between the spectral variables and the component concentrations or properties that were modeled. The number of samples needed to validate an infrared multivariate model depends on the complexity of the model. Only samples whose analyses are found to be interpolations of the model should be used in the validation procedure. If five or fewer spectral variables are used in the model, then a minimum of 20 interpolation samples is recommended. If $k > 5$ spectral variables are used in the model, then a minimum of 4k interpolation samples should be used in the validation.

The main problem of external validation is to have enough samples to perform it. In fact, very often the number of samples available to construct the calibration function is limited so it is not possible to have an independent dataset of samples for the validation of the method. In this case, the solution is to use the internal validation or cross-validation [66, 76]. The other problem is that the error of prediction value changes according to the type and the number of validation samples.

Anyway, it is always important to remember that the error of prediction should be not considered as a "single value" but it is a statistic that can change according to the type of validation used to estimate it and it has to be considered together with its uncertainty.

The results of the validation (both external and internal) are usually displayed in a graph that represents the predicted values versus the true values. The model has of course a better

predictability if the values are arranged along a line 45° across the chart (bisector). It must be remembered that this is not the straight calibration line but only the PRESS graphical representation [66, 76].
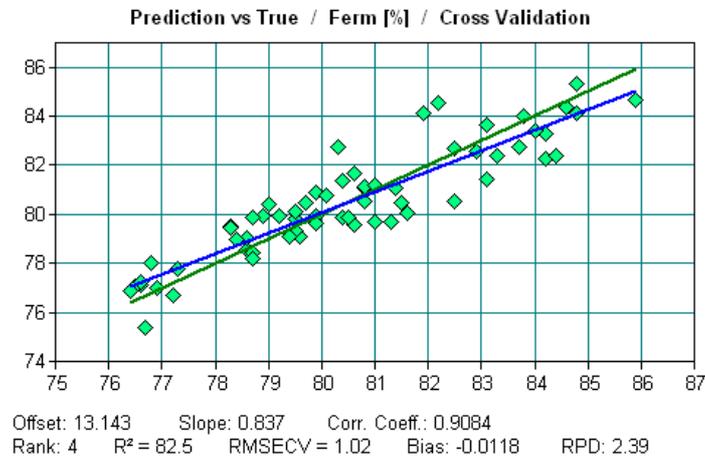
Figure 3.4 – PRESS graphical representation

Where the green line is the bisector and the blue line is the PRESS graphical representation, namely the correlation line between predicted and true values. From the equation of this line:

$y=ax+b$ (Eq. 3.11)

it is possible to obtain the Slope a and the Offset b, and these two parameters can be used for the evaluation of the calibration model. Another statistic typically used to assess the quality of calibration models is a measure of the correlation (linear dependence) between two variables x (true values) and y (predicted values) , giving a value between +1 and −1 inclusive [66, 76]:

(Eq. 3.12)

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

Where x_i is the true value of the i-sample, y_i is the predicted value of the i-sample, $\bar{y}$ and $\bar{x}$ are the mean values, and N is the number of samples used for the validation.

Alternatively, the coefficient of determination R^2 can be used. In the case of a linear regression, R^2 is simply the square of the sample correlation coefficient between the true values and the predicted

87

values and its values vary from 0 to 1. This statistic allows the determination of the amount of variation in the data that is adequately modeled by the calibration equation as a total fraction of 1.0. Thus $R^2 = 1.00$ indicates the calibration equation models 100% of the variation within the data. An $R^2 = 0.50$ indicates that 50% of the variation in the difference between the true values for the data points and the predicted or estimated values for these points are explained by the calibration equation (mathematical model), and 50% is not explained. R^2 values approaching 1.0 are attempted when developing calibration. It is also possible to use the R^2 multiplied for 100.

Another parameter that can be used in order to evaluate the calibration is the Bias, which is a systematic deviation of the measured (predicted) values from the true value [66, 76]:

$$Bias = \frac{\sum_{i=1}^{N}\left(y_{i-true} - y_{i-predicted}\right)}{M} \quad \text{(Eq. 3.12)}$$

A quantitative measure for the preciseness of a validation is the SEP (Standard Error of Prediction): This value indicates the standard deviation of all bias-corrected measured values from the true value and it is calculated as follows [66, 76]:

$$SEP = \sqrt{\frac{\sum_{i=1}^{M}\left(\left(y_{i-true} - y_{i-predicted}\right) - Bias\right)^2}{M-1}} \quad \text{(Eq. 3.13)}$$

From the SEP value ti is possible to obtain the RPD (Residual Prediction Deviation) value, which is the ratio of standard deviation to standard error of prediction [66, 76]:

$$RPD = \frac{SD_{true}}{SEP} \quad \text{(Eq. 3.14)}$$

Where SD_{true} (Standard Deviation) is the standard deviation calculated for the true values.

In this thesis, different kinds of calibration have been used, and they will be better explained in the further parameters.

3.2.6. Optimization of the model

The goal of a calibration model definition is to choose the most accurate and precise calibration model possible and to estimate how well it will perform in future samples.

In principle, there are endless possibilities for the construction of a calibration model. Once that the calibration (and eventually validation) samples have been chosen, and also the algorithm used for the calculation for the calibration has been established, and also the kind of validation, it is still possible to improve the model performances by optimizing certain parameters, among which the most important are:

- Methods of preprocessing

- Spectral range

- Number of calibration factors

All these factors depend on a multitude of system parameters, and they can not be calculated from theoretical considerations. Therefore they must be empirically determined (using the "trials and errors" procedure) by using some models with the possible combinations of these three parameters, validating them and repeating this procedure until the model that achieves the highest value of R^2 and the lowest value of RMSECV (or RMSEP) has been found [66]. The recognition and elimination of outliers are also fundamental to the success of the calibration:

a) Methods of preprocessing

It 's the most important choice together with that of the spectral range.

Pre-treating the analytical signal is intended to suppress the effect on contributions not associated with the information of interest in order to increase the accuracy and precision of the results and to obtain simple, robust models with an acceptable predictive ability. The aim of spectral pretreatments is to model the spectra in order to allows the PLS algorithm to find the best correlation between spectral data and those of concentration.

Although spectral signal pre-treatments reduce the contribution of noise, their efficiency depends on the nature of the noise and the specific treatment used. The selection of the type of pre-processing of data depends mainly on the measurement technique, the type of sample, analysis of the purpose and type of data. The best choice in each situation must be chosen in an empirical manner, using a trial-and-error approach, which is a major disadvantage.

The main goal of pre-processing is to transform data in such a way that the (multivariate) signals will better adhere to Beer's law, which states that absorbance and concentration are linearly correlated [77, 78]:

$A = \varepsilon \, lc$ (Eq. 3.15)

Where ε is the molar absorptivity, l is the (effective) path length, and c is the concentration of the constituent of interest. The estimation of ε or the correct value of l is not important; what is aimed for is that the collective term ε x l is constant for the data set, thus making the relationship between A and c linear.

Many physical and chemical phenomena can cause a deviation from this linear relationship, including scatter from particulates, interferents, molecular interactions, changes in refractive index at high concentrations, shifts in chemical equilibrium as a function of concentration, stray light, changes in sample size/path length, etc, as shown in Fig. 3.5 [77, 78].
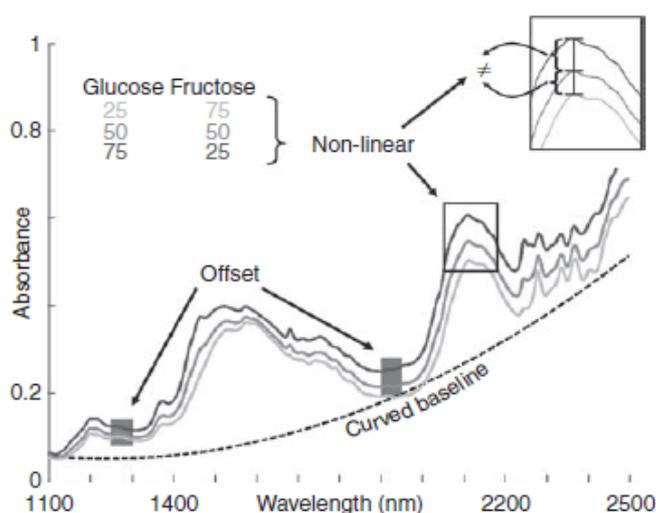
Figure 3.5 - The non-linearity in the spectra is in general caused by two scatter effects: offset and curved baseline [77].

Pre-processing techniques are designed to compensate for these deviations from linear relationships and thus to improve the linear relationship between the spectral signals and analyte concentrations. Pre-processing techniques can be divided into two major groups: those which directly use available reference values for the pre-processing operation, and those that do not. The latter group is thus a reference-independent pre-processing group, and as such provides more general tools suitable for studies such as exploratory studies, for example, where often no reference value is available. The reference-independent techniques can further be divided into two subgroups: scatter correction methods and derivation methods.

1.Scatter correction methods

1.a Constant Offset Elimination

The spectra are shifted linearly such as the minimum value of Y (ie absorbance) is equal to 0. In this way the shift of the baseline is eliminated [66].

1.b Straight Line Substraction

In each selected frequency range, a straight line is adapted to the spectrum using the method of least partial squares. Then, this line is subtracted to the spectrum, in order to eliminate the shift of the baseline [77, 78].

1.c Multiplicative Scatter Correction (MSC)

Multiplicative Scatter (or, in general, Signal) Correction (MSC) is probably the most widely used pre-processing technique for NIR (closely followed by SNV and derivation).

The concept behind MSC is that artifacts or imperfections (e.g., undesirable scatter effect) will be removed from the data matrix prior to data modeling. MSC comprises two steps [77, 78]:

1. Estimation of the correction coefficients (additive and multiplicative contributions).

$$x_{org} = b_0 + b_1 \cdot x_{ref} + e \quad \text{(Eq. 3.16)}$$

2. Correcting the recorded spectrum.

$$x_{corr} = \frac{x_{org} - b_0}{b_1} \quad \text{(Eq. 3.17)}$$

where x_{org} is one original sample spectra measured by the NIR instrument, x_{ref} is a reference spectrum used for preprocessing of the entire dataset, e is the un-modeled part of x_{org}, x_{corr} is the corrected spectra, and b_0 and b_1 are scalar parameters, which differ for each sample. In most applications, the average spectrum of the calibration set is used as the reference spectrum. Then, plotting the sample spectrum against the selected reference spectrum it is possible to find the least-squares regression fitting line through all points. The scalar correction terms are found as the intercept (b_0, additive contribution) and the slope (b_1, multiplicative contribution) of this line as showed in Fig. 3.6, so that the differences between the reference spectrum and the sample spectrum are minimum.
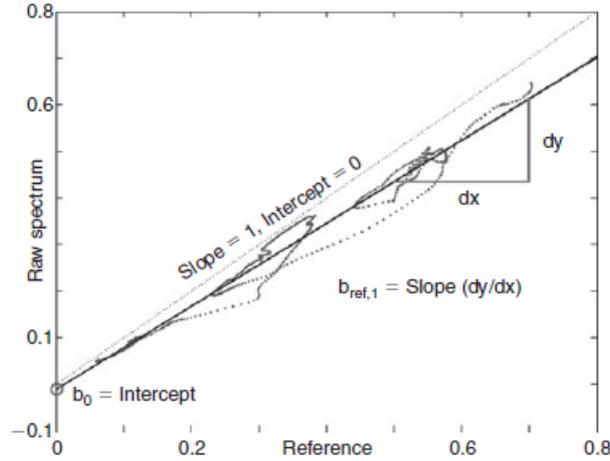
Figure 3.6 - The estimation of the correction coefficients for multiplicative signal correction [77].

It is also possible to apply an expansion of the MSC pretreatment including wavelength corrections. This expansion, inclusion of wavelength dependency, can be seen as a merging of the de-trending technique with the MSC and it is called Extended Multiple Scatter Correction (EMSC). The EMSC algorithm minimizes the signal variability caused by scatter from particulates in the samples (following the basic idea of MSC) with the inclusion of the wavelength dependency.

This expansion includes both second-order polynomial fitting to the reference spectrum and fitting of a baseline on the wavelength axis:

$x_{org} = [1\ x_{ref}\ x_{ref}^2\ \lambda\ \lambda^2\ ]\cdot b + e$ (Eq. 3.18)

where λ is the correction vector for the wavelength-axis dependency and b is a set of scalars (correction coefficients) given by:

$b = [b_0\ b_{ref,1}\ b_{ref,2}\ b_{\lambda,1}\ b_{\lambda,2}]$ (Eq. 3.19)

where b_0 is the offset correction, $b_{ref,i}$ is the correction according the ith order of the reference and $b_{\lambda,i}$ is the correction of the ith order wavelength-axis dependency. From equation ..it can be seen that by removing everything but the two first terms EMSC turns into the original MSC, while removing the reference x_{ref} the equation turns into the standard spectral dependent de-trending equation. Wavelength-axis dependency is most often included as a second-order polynomial fitting on the wavelength axis to the spectra. When no reference correction is included, this simple wavelength

fitting also goes under the name of spectral de-trending and it can be viewed as a baseline correction.

1.d Standard Vector Normalization:

Standard Vector Normalization belongs to the same family of the Standard Normal Variate (SNV) pre-processing and both of them are probably the second most applied methods for scatter correction of NIR data. The basic format for SNV and Normalization correction is the same as that for the traditional MSC [77, 78]:

$$x_{corr} = \frac{x_{org} - a_0}{a_1}$$ (Eq. 3.20)

For SNV, a_0 is the average value of the samples spectra to be corrected, while, for vector normalization, a_0 is set equal to zero. a_1 is the standard deviation of the sample-spectrum.

There is no least squares step in the SNV and normalization solutions, so these operations are all more prone to noise than the more robust MSC.

In principle, a spectrum contains two parts of information: the height of the bands as well as the structure. After normalization, the height information is lost and only the structural information remains. In this way, in measurements in diffuse reflections, the interfering influences of different materials densities or particle sizes can often be minimized.

1.e Min-Max Normalization

The spectra are shifted linearly, so that the minimum Y-value equals to 0. Then, the spectra are expanded, so that the maximum Y-value equals to 2 absorbance units. This pretreatments are effects similar to vector normalization [66].

2.Derivation methods

Savtizky and Golay (SG) popularized a method for numerical derivation of a vector that includes a smoothing step. In order to find the derivative at centre point i, a polynomial is fitted in a symmetric window on the raw data. When the parameters for this polynomial are calculated, the derivative of any order of this function can easily be found analytically, and this value is subsequently used as the derivative estimate for this centre point. This operation is applied to all points in the spectra sequentially. The number of points used to calculate the polynomial (window size) and the degree of the fitted polynomial are both decisions that need to be made[77, 78].

2.a First derivative

By calculating the first derivative, the signals with steep edges get more emphasis that relatively flat bands. This method is used to emphasize pronounced but small features compared to huge broad-banded structures. By calculating the derivative, these structures get a steeper shape which can be evaluated more easily. When using the derivative as a data preprocessing method, it has to be taken into account that the spectral noise is enhanced as well. This superimposes the spectrum as an additional disturbance and can deteriorate the analyte signal [77, 78].

2.b Second derivative

Compared to the first derivative, even extremely flat structures can be evaluated. The disturbing influence of the spectral noise is generally so strong that spectra can be evaluated ina avery restricted spectral range [77, 78].

In this thesis, all these kinds of spectral preprocessing have been used, and also combination of them, and the best ones were chosen using the trial-and-error procedure. In principle, any combination of pre-processing is possible, but the following simple rules have been used as initial guidelines:

- *Scatter correction (with the exception of normalization) should always be performed prior to differentiation. These techniques are all designed for correction of raw spectrum, and have never been thought of as corrections to a differentiated or baseline-corrected spectrum.*
- *Normalization can be used at both ends of the correction, although it is easier to assess the effect of Normalization if it is done prior to any other operation.*
- *The basic difference between SNV with subsequent de-trending and MSC with reference and baseline correction is that, in MSC, both corrections are applied simultaneously, not consecutively. Thus, MSC will generally give a smaller baseline correction than SNV plus de-trending.*

b) Selection of spectral data and the range of frequencies

NIR spectroscopy is called "full-spectrum method", because more experimental data are present, more information is available, the better the model you get. However, the contribution of the noise spectral absorption bands or contaminants can degrade the quality of the model.

It is therefore advisable to perform the first calibration over the entire spectral range, then delete the noisiest areas (such as those at the limit of the range) and consider the best bands with absorbance

values, ie between 0.7 and 1 for spectrophotometers traditional instruments and less than 2.5 in the Fourier transform. In fact, the bands with absorbance values outside these ranges are characterized by very low light intensity, and then a resulting signal is too noisy to be used [66].

Choosing the most suitable spectral range for developing a calibration model is not an easy task and frequently it involves an endless sequence of trial-and-error runs until an adequate predictive ability is achieved. When the spectra for the target analyte and its potential interferents in pure form are available, one can choose the range where the analyte exhibits substantial bands and exclude those where the interferents absorb. However, this approach is useless in the NIR region, where bands are typically narrow and strongly overlapped, and the analyte signal is therefore easily concealed by the signal for the sample matrix. In this situation, one can simply calculate the correlation between the absorbance at different intervals of wavelength and the target quantity in order to plot the resulting vector against the independent variable. Those intervals exhibiting the strongest correlation can be of help with a view to selecting an appropriate range to develop the model [79].

Some algorithms allow the most suitable spectral variables for modeling the independent variable to be identified. The interval partial least-squares regression (iPLS) is one of the more commonly used method. The spectra are divided into a number of intervals of equal width and local PLS models are developed on each of these spectral subintervals. The prediction performance of these local models and the global (full-spectrum) model is compared. The comparison is mainly based on the validation parameter RMSECV (root mean squared error of cross-validation), but other parameters such as r^2 (squared correlation coefficient), slope, and offset are also evaluated to ensure a comprehensive model overview [80].

Sample and/or measurement abnormalities (outliers) as detected by PLS inner relation plots should generally be removed prior to the application of iPLS. Models based upon the various intervals usually need a different number of PLS components than do full-spectrum models to catch the relevant variation in y. This condition is caused by the varying amount of y-correlated information carried by the interval variables (the larger the spectral interval, the greater the number of substances that are likely to absorb/interfere) and is also related to the noise/interference carried by the variables. However, the selected model dimension has to be common to all the local models in order to make a comparison possible. In order to favor the ``best'' spectral region, it is natural to let the simplest interval model (i.e., the one with the smallest number of PLS components) guide the selection of the model dimension. A fair comparison of the global and local models requires that the global and local model dimensions be selected separately. There is a minimal probability for hitting the optimal interval with the equidistant subdivisions. A more optimal interval might be found by carrying out small adjustments in the interval limits, like interval shift and changes in interval

width. Each step is initiated with the optimal interval limits from the previous step. The interval limits are changed one variable at a time and evaluated by the RMSECV provided by application of PLS regression to the interval.

The purpose of iPLS is to optimize the predictive power of PLS regression models by finding one or a few intervals which give better prediction that the one obtained using the full spectrum, and to provide an overview to useful for the spectroscopic interpretation. Its main force is to provide an overall picture of the relevant information in different spectral subdivisions, thereby focusing on important spectral regions and removing interferences from other regions. The sensitivity of the PLS algorithm to noisy variables is highlighted by the informative iPLS plots [80].

In this thesis, the noising parts of the spectra have been deleted. Then, if the calibration is performed using OPUS software, a set of five frequency regions is used (11501.7-7498.1 7501.9-6098 6101.8-5450 5453.8-4597.6 4601.4-4246.6 cm^{-1}). The frequency regions are typical for NIR applications. The five frequency regions are tested on their own and in all possible combinations., and the best combination is chosen for the calibration. If the calibration is performed using the PLS toolbox in MATLAB, the best spectral range have been chosen by iPLS. The number and the width of the spectral intervals are different for each calibration and they will be better explained in the further chapters.

c) Number of calibration factors

The determination of the number of principal components is a crucial point for the quality of the calibration model. The factors (equivalent to principal components) explaining the spectral matrix are sorted in decreasing order according to their contribution to the spectral features. The first factor characterizes the biggest change in the spectrum observed correlated with the analytical properties, then are extracted factors with smaller contribution and finally factors which mainly reflect spectral noise and fluctuations. Thus not all factors are needed to explain the spectral features of the components (the contributions representing noise can be omitted).

The quality of the chemometric model now depends on the choice of the correct number of factors needed; this is also called the rank of the model.

Using an insufficient number of principal components leads to a poor reproduction of the spectral data and therefore the model will not be able to recognize changes in the spectral features. Namely, not all features can be explained by the model and this is called "underfitting".

On the other hand, including too many principal components just adds spectral noise to the regression and does not increase the amount of valuable information ("overfitting").

As a consequence there is an optimum number of factors for every system, i.e. an optimum rank.

A criteria for determining the optimum rank is to look at the root mean square error of prediction (RMSEP) resulting from an analysis of the test set (or the cross validation) and to choose the rank for which these two values reach for the optimum (lowest value of RMSEP and highest value of R^2) [66].

In this thesis, the ranks selected as optimal are the ones for which the RMSEP parameter reaches the lowest value. If two or more ranks gave good results, an F-test has been calculated to verify that these results are not statistically different and then the lowest rank were chosen, in order to prevent overfitting.

3.2.7. Identification and elimination of outliers

An outlier can be defined as any observation not fitting the model. Outlier prediction is important during the calibration modeling and monitoring phases. Outliers are not considered to be part of the group that is designed to be used as a calibration set. In a practical sense, outliers are those samples that have unique character so as to make them recognizably (statistically) different from a designated sample population. It should be noted that the word " outlier " need not be synonymous with "incorrect"; however, one should always ascertain whether an outlier is the result of an actual phenomenon or an artifact arising from some error while constructing the calibration model. In fact, identifying and suppressing outliers is of utmost importance since their presence can adversely affect the robustness and predictive ability of the resulting model. In fact, at any rate it is necessary to examine critically whether the outliers result from a faulty measurement. The elimination of typical but "unpleasant" samples is not permitted. The calibration model would then lack sufficient stability to handle the natural variety of different samples. Frequently, outliers are marked as also samples that lie out of the calibration range. In this case, the identification of such outliers is not due to faulty measurements, but with a lack of robustness of the model, then it may be appropriate to include these samples into the calibration set.

One may encounter three different types of outliers in developing a multivariate calibration model, namely: (a) X -sample outliers (viz. samples, for which the spectra depart markedly from those for the others); (b) Y -sample outliers (viz. samples, for which the model provides a target value considerably different from the actual value); and (c) X –variable outliers (viz. spectral variables that behave markedly differently from the others).

Evaluation criteria for selecting outliers are often subjective; therefore there is a requirement that some expertise in multivariate methods by employed prior to discarding any samples from a calibration set [66, 72, 81].

In this thesis, outliers have been detected by evaluating three parameters:
- *Mahalanobis distance*
- *Spectral residuum and Differ*

a) Mahalanobis distance

The Mahalanobis distance is used to check how well each spectrum "fits" the spectra of the calibration data set. It is defined as the difference between a measured spectrum and the mean value of all spectra in the calibration data set, which was used when reconstructing the spectral data matrux for the given number of samples. The larger this difference, the larger the value of the Mahalanobis distance. There are various possible reasons. External disturbances, such as the contamination of the samples or disturbing temperature drifts, can leads to spectrum distortion and increase the Mahalanobis distance. In the same way, this parameter can grow also if the sample lies outside the concentration range. Mahalanobis distance is closely related to the leverage statistic, h, but has a different scale, to obtain a statistic that is independent of the number of calibration samples (N):

$$\text{Mahalanobis Distance} = (N - 1)\left(h_i - \frac{1}{N}\right) \quad \text{(Eq. 3.21)}$$

The leverage statistic, h, is a scalar measure of where the spectral vector x lies within the multivariate parameter space used in the model.

If x_i is a spectral vector (dimension f by 1) and X is the matrix of calibration spectra (of dimension n by f), then the leverage statistic h_i of the i-sample is defined as:

$$h_i = \text{diag}(x^T(XX^T)^{-1}x) \quad \text{(Eq. 3.22)}$$

Where "diag" indicates the diagonal of the matrix, and the calculation is performed for R factors. The leverage, h_i, is a measure explaining the influence of the i-object on its own prediction. In different words, h_i is a measure of the influence of the i-spectrum on the PLS model. Theoretically, the average leverage statistic for all of the calibration sample spectra has a value of R/N where R is

the number of PLS latent variables, and N is the number of calibration samples. On average, each sample contributes k/n of the spectral variables. For samples that have $h_i > 5R/N$, the sample spectrum is contributing a significant fraction to the definition of one of the spectral variables and to the regression coefficient associated with this variable. Samples with $h_i > 5R/N$ are considered outliers and eliminated from the calibration set in the development of the model [66].

b) Spectral Residuum and Differ

A factorization can never explain the total variance of the spectral (X) or the concentration data matrix (Y). The rest that is not explained by the factorization is called residuum. The residuum is the total difference between the real data and the data reconstructed by factorization. This applies to spectral data as well as to concentration data. Looking at equations 3.3 and 3.4, the residuum matrix are E for the spectral matrix X and F for the concentration matrix Y. The calculation of the residual values is useful to detect outliers. The spectral residuum of the i-sample can be calculated by:

$$SpecRes_i = \sqrt{\sum_{j=1}^{M} \left(x_{i,j} - s_{i,j} \right)^2}$$ (Eq. 3.23)

Where x_i is the measured spectrum and s_i is the spectrum reconstructed by the factorization. The sum is calculated over all the frequency values j.

In the same way, the residual value of the i-sample can be calculated by using the Differ parameter:

$$Differ_i = y_{i\text{-true}} - y_{i\text{-predicted}}$$ (Eq. 3.24)

The better the reconstruction of a factorization is, the smaller are the residual values.

To recognize outliers, the squared residual values are compared with the mean value of all others by calculating the FValue:

$$FValue_i = \frac{(N-1)\left(SpecRes_i \right)^2}{\sum_{j \neq i}^{N-1} \left(SpecRes_j \right)^2}$$ (Eq. 3.25)

$$FValue_i = \frac{(N-1)\left(Differ_i \right)^2}{\sum_{j \neq i}^{N-1} \left(Differ_j \right)^2}$$ (Eq. 3.26)

Samples poorly represented by the PLS vectors have a high FValue. In order to judge whether an FValue might indicate an outlier, it must be compared with the FValue of the other samples of the data set. Therefore, from the FValue and the degrees of freedom it is possible to calculate the FProb value, which indicates the probability that the i-sample is an outlier in the distribution of the Fvalues:

$$FProb_i = \frac{\int_0^{FValue} f(FValue)d(FValue)}{\int_0^{\infty} f(FValue)d(FValue)}$$

(Eq. 3.27)

The limit for the automatic outlier detection is 0.99. If the $FProb_i$ value of the i-sample lies above the limit, the corresponding sample is considered an outliers [66].

3.2.8. Validation of Agreement Between Model and Reference Method

A method for evaluate the agreement between the model and the reference method involves the reproducibility at 95% (R_{95}) of the reference method. It is necessary to calculate the percentage of reference values (true values) that fall in the interval definite by:

$$y_{predicted} - R_{95} < y_{true} < y_{predicted} + R_{95}$$

(Eq. 3.28)

If 95% or more of the reference values fall within this interval, then estimates produced with the multivariate NIR model agree with those produced by the reference method as well as a second laboratory repeating the reference measurement would agree [72].

3.2.9. Precision of Near Infrared Estimated Values

The precision of values estimated from a near infrared multivariate model is calculated from repeated spectral measurements [72]. The number of samples for which repeat measurements is made should be at least never less than three. Calibration curve should be tested by recording more than 10 independent spectra under repeatability conditions and evaluating the predicted values. The normality test of the distributions on each data set was performed by means of the Shapiro-Wilk test with a probability level of p = 95%. Moreover, anomalous data were identified by means of the Huber test, which is based on the evaluation of the median.

If both conditions are satisfied, that is, the distribution is normal and there are no anomalous data, the statistical parameters, such as the average value, the standard deviation (s_r), and the repeatability (95% confidence, r_{95}), can be calculated, and a comparison with the repeatability of the standardized methods adopted as primary methods can be carried out.

The repeatability of the NIR methods was compared with those of the standard methods σ_r which are calculated from the collaborative trial determined repeatabilities of the standard A-EBC methods, according to:

$$\sigma_r = \frac{r_{95}}{t\sqrt{2}} \quad \text{(Eq. 3.29)}$$

Where t is the Student coefficient for ν degrees of freedom (ν = N-1, where N is the number of laboratories participating to the collaborative trial). The two repeatibilities are compared using a Chi square's test, in order to asses that the precision of the NIR multivariate method is comparable with the reference method's one.

The average values from each data set were compared with the true values determinate with the reference methods, in order to verify the predictability of the calibrations (PAPER I and II).

3.3 Software

All computation involving the calibration models (spectral data pretreatments, selection of the spectral ranges, construction of PLS regression models and validations) were carried out by OPUS software (version 5.5 or 6.5, Bruker Optics, Germany) or MATLAB software, Version 7.6.0 (The Mathworks Inc., Natick, MA, USA) with the PLS-Toolbox (Eigenvector Research, Inc., WA, USA) and in-house routines

3.4 Samples

In this thesis research project NIR spectroscopy has been applied to raw materials, intermediate and final products in the beer production.

Concerning raw materials, maize and barley samples have been analyzed, to assess their quality.

Up to 146 maize samples were supplied from industrial mills as grist. These samples can be considered representative of the ones available on the Italian market (PAPER I and II)

Concerning barley, 40 different barley samples were considered.

These two-row malting barley samples differ for variety, region and year of sowing. The spring varieties considered are Barke, Cheri, Henley, Scarlett, NFC Tipple, Publican, Prestige, Quench, Keops, Xanadu, Anaconda and Pewter, while the winter varieties considered are Puffin and Regina. The different samples were sowed during the four years of this thesis research (2008, 2009, 2010) in different regions of Europe: Italy, Finland, Lithuania, Sweden, Denmark, Austria, and Spain.

Then, these barley samples were malted in a micro-malting pilot-plant (Custom Laboratory Products, Keith, UK) in order to provide samples for a NIR monitoring of the malting process (PAPER IV).

This pilot-scale micromalting plant is provided with four independent steeping/germination tanks, each having the capacity to hold one drum filled with 3 kg of barley or four drums filled with 0.5 kg of barley each. In this work, for every malting run, 16 samples of 0.5 kg were processed. Each barley variety was split into ten samples of 0.5 kg and these were processed at the same time using all four tanks of the micromalting plant. After the malting process the single batches of 0.5 kg were collected to form a representative 5 kg sample for brewing. This experimental procedure avoids the variability due to the use of different steeping/germination tanks.

The steeping phase lasted 20 hours and it was divided into 3 steps:

Step 1: 5 h. of immersion in water at 18°C followed by 7 h. of air resting at 17°C

Step 2: 2 h. of immersion in water at 16°C followed by 5 h. of air resting at 16°C

Step 3: 1 h. of immersion in water at 16°C before starting of the germination.

The barley moisture during the germination was kept around 45% for all the varieties.

The germination phase lasted 4 days, during which the temperature was regularly decreasing: 16°C during the first day, 15°C during the second day and 14°C for the last two days. The green malts were dried using a kilning plant (Custom Product Laboratory, UK) consisting of 4 units and applying these drying temperatures:

55 °C for 15 h;

72 °C for 4 h and 30 min.;

82 °C for 3 h and 30 min.;

Cooling (to room temperature.)

The steeping, germination and kilning programs were suitable for the production of 'pilsner' malt type. The barley humidity during germination was maintained at $45 \pm 1\%$ for all varieties. These malt samples, together with other commercial samples have been analyzed to assess their quality, up to 316 samples. The commercial malt samples were supplied from industrial malthouses and mills and are representative of the ones available on the Italian market. Samples of malt

representative of the different types (i.e., pale, Munich, colored, and caramel) were considered (PAPER I and II).

Then, part of these malt samples were brewed in a micro-brewing pilot-plant (Braumaister, Feltre, I) in order to provide samples for a NIR monitoring of the brewing process (PAPER IV).

Each sample of barley malt was processed in a pilot scale plant programmed in order to produce 25 litres of wort suitable for "pilsner" beer type. Five kilograms of malt were milled in a two-roller mill (Engel, 120 Kg/h capacity) with a gap of 0.5 mm between the crushing rollers. The grist was then mixed with 23 litres of water. The properties of the brewing liquor were: 0.5 Mval, 15 mg/L of carbonate ($CO_3^=$), 30.5 mg/L of hydrogen-carbonate ($HCO_3^=$), 11 mg/L of dissolved CO_2 and pH 6.5. The milled malt was mashed in the brewing liquor at 52°C for 30 min. to allow the protein breakdown. The temperature was raised up to 65°C in 15 min. and maintained for 45 min. for the β-amylase activity. The temperature was increased to 72°C in 5 min. and maintained at this temperature for 20 min. to allow the α-amylase activity, testing the efficiency of saccharification by iodine solution test.

The last temperature step was a short rest at 76°C to inactivate the enzymes. Afterwards, the mash was transferred to a 30 L lauter-tun vessel. The first wort was collected from the lauter-tun, then the spent grains were sparged with 12 litres of water (78°C) for the washing out of the second wort. The total amount of wort was collected in a heated 30 L kettle for boiling: the temperature was raised to 100°C and manteined for 75 minutes. 100 grams of hops pellets (Saaz variety, 2.1% of α-acids) were added: 70% of hops was added at the beginning of boiling, and 30% was added 15 min. before the end of boiling.The wort was cooled to 18°C and transferred to the 30 L fermentation vessel (Spadoni, Orvieto, I). 11.5 grams of dry ale yeasts (Safale Fermentis, S-04) were put in 100 ml of sterile wort (12°P) at 18°C and gently stirred for 40 minutes in order to rehydrate the cells and to prepare the yeast to start the fermentation process without stress. After this treatment, the yeast suspension was gently transferred to the fermentation vessel. The fermentation temperature was maintained at 18°C for 5 days. When the gravity of the wort reached the 85% of the limit attenuation, the beer was bottled to continue the fermentation at 18°C. At the end of maturation, the bottles were kept at 4°C for two weeks and then analysed.

The obtained beer samples, together with other commercial samples have been analyzed to assess their quality, up to 50 samples.

The kind and number of samples used for each calibration will be better explained in the further samples.

3.5 Reference Methods

The reference methods used in this thesis comply with the standard procedures of Analytica EBC (European Brewery Convention) [82]. Each analysis is repeated twice, and the standard deviation between the two repetitions is lower than the SEL (Standard Error of Laboratory) calculated from the method repeatability. In addition, concerning malt, the reliability of analytical data is daily checked by analyzing a EBC Standard Malt simultaneously with the samples. The quality parameters of this standard malt result from a collaborative trials of 30 laboratories carried out by the ECB Analysis Committee. Moreover, every three months CERB laboratory participates on two collaborative trials, one for malt, called MAPS (Malt analytes Proficiency Testing Scheme) and one for beer, called BAPS (Malt analytes Proficiency Testing Scheme). Finally, most of the malt analyses are accredited following the international organizations of accrediation guidelines. In Italy, the Organization of Accreditation is ACCREDIA, which belongs to EA (European co-operation for Accreditation).

The quality parameters and the respective reference analyses considered for each kind of samples are:

a) Barley

a.1) Water Content (%)

The reference method is the "Analytica-EBC 3.2 Moisture Content of Barley", consisting of a standard drying procedure in which ground barley is dried at an exact defined temperature for a predetermined time. The moisture content of the barley is calculated in % from the loss in mass during drying.

a.2) The nitrogen content (% and %dm)

The reference method is the "Analytica-EBC 3.3.1 Total Nitrogen of Barley: Kjeldahl Method". Nitrogenous compounds in the barley are digested with hot sulphuric acid in the presence of a catalyst to give ammonium sulphate. This digest is made alkaline with a sodium hydroxide solution and released ammonia is distilled into an excess of boric acid solution. The ammonia is titrated with a standard acid solution.

b) Malt

b.1) Water content (%)

The reference method is the "Analytica-EBC 4.2 Moisture Content of Malt", which allows to the determination of the moisture content of all malts by loss in mass on drying under specified conditions

b.2) The nitrogen content (% and %dm)

The reference method is the "Analytica-EBC 4.3.1 Total Nitrogen of Malt: Kjeldahl Method" with the same principle used for the barley.

b.3) The Distatic Power (WK)

The reference method is the "Analytica EBC 4.12 Diastatic Power of Malt", and its aim is the determination of the combined activity of α- and β-amylase of malt under standardized reaction conditions. Malt enzymes are extracted with water at 40°C, then a standard starch solution is hydrolyzed by the malt enzyme extract, then the amount of reducing sugars formed by amylolytic action is estimated iodometrically. The result is calculated as grams of maltose which is produced under the specified conditions by 100 g of malt.

b.4) The Friability (%)

The reference method is the "Analytica EBC 4.15 Friability, Glassy Corns and Unmodified Grains of Malt by Friabilimeter". Whole malt grains are fragmented by the mechanical action of the friabilimeter's drum. Small fragments of physically modified material pass through the mesh of the drum whereas larger, unmodified, fragments are retained. The mass of unmodified fragments remaining after 8 minutes is determined and from this the friability is calculated.

b.5) Fine extract (% and %dm)

The most important analysis is the extract obtained from the Congress wort, which is measured by means of a pycnometer, refractometer, special hydrometer or precision density-measuring device. The reference method is the "Analytica-EBC 4.5.1 Extract of Malt: Congress Mash". The extract content of the wort is obtained from the specific gravity by means of the sugar table (Plato table) for 20°C. The Congress mash is performed on 50 g of malt very finely ground (0.2 mm). In accordance with EBC regulations, a DLFU disc mill is used which must be specially standardised for this purpose. Each 50 g of fine grind is mashed into 200 ml of distilled water at 45 to 46° C with constant stirring in a special mash beaker and mashed for 30 min with constant stirring at 45° C. The temperature in the mash vessel is then raised to 70° C in 25 min (1° C/min), then 100 ml of

water at 70° C added and the temperature maintained for an hour with stirring. During this time the saccharification is monitored. The mash is then cooled in 10 to 15 min to room temperature and the beaker contents made up to 450 g with distilled water. The entire contents are then filtered through a folder filter paper. The first 100 ml of the filtrate are returned to the filter and filtration is terminated when the filter cake appears dry. The wort obtained is called Congress wort and immediately analysed.

b.6) The wort viscosity (cP or mPa · s)

From the viscosity of the Congress wort conclusions can be drawn about the future behaviour of the wort during lautering and about the filtration of the beer. The reference method is the "Analytica-EBC 4.8 Viscosity of Laboratory Wort from Malt". The viscosity of the Congress wort at 20°C is determined using a calibrated viscometer of an appropriate type (glass capillary viscometer, rotary viscometer, falling ball viscometer).

b.7) The soluble nitrogen content (%dm)

The soluble nitrogen content refers to the nitrogen compounds which have dissolved in the Congress machine procedure. The reference method is the "Analytica-EBC 4.9.1 Soluble Nitrogen of Malt: Kjeldahl Method", which is the same procedure used for the determination of the Total Nitrogen content, but is applied to 20 ml of Congress wort instead of ground malt.

The Kolbach index (N_K) is the ratio between soluble (N_S) and total (N) nitrogen (both expressed as %dm) and it shows what percentage of the total nitrogen in the malt is dissolved in the Congress mashing procedure:

$$N_K = \frac{N_S \cdot 100}{N}$$ (Eq. 3.30)

b.8) The free amino nitrogen content (mg/L)

The amino nitrogen analysis measures low molecular weight nitrogen compounds like amino acids, ammonia and, in addition, the terminal α-amino nitrogen groups of peptides and proteins. The reference method is the "Analytica-EBC 4.10 Free Amino Nitrogen of Malt by Spectrophotometry". The scope is the determination of the free amino nitrogen content of malt using colorimetry with ninhydrin. A sample of malt is mashed following the Congress mash procedure. A sample of the Congress wort obtained is heated in the presence of ninhydrin at pH 6.7together with a standard

solution of glycine. The absorbance of both at 570 nm are measured against a reagent blank. Normal values are from 120 to 160 mg/L.

b.9) pH of the wort

The reference method is the "Analytica-EBC 8.17 pH of Wort", consisting in the determination of the pH of wort at 20°C using a pH meter and a suitable electrode system.

b.10) Fermentability (%)

The reference method is the "Analytitca EBC 4.11.1 Fermentability, Final Attenuation of Laboratory Wort from Malt: Reference Method", in which the fermentation of a boiled laboratory wort provides a measure of the attenuation for brewing. Filtered Congress wort is boiled in order to inactivate amylolytic enzymes. Then, the cooled wort is fermented for at least 24 hours by brewers yeasts, and the fermentability (apparent attenuation) is calculated from the difference between the specific gravity values of the wort before and after the fermentation.

d.11) Difference Fine/Coarse Extract (%)

It is the difference in yield between the extract obtained by applying the Congress mash to finely ground (F, 0.2 mm) and coarsely ground (C, 0.7 mm) malt. The reference method is the "Analytica EBC 4.5.2 Extract Difference of Malt: Congress Mash".

c) Beer

c.1) Alcohol content (% m/m and % V/V)

The reference method is the "Analytica-EBC 9.2.1 Alcohol in Beer by Distillation", in which the determination of the alcohol content of beer is determined by using a distillation procedure and by measuring the specific gravity at 20°C of the distillate. The beer is degassed and filtered avoiding loss of alcohol content from evaporation but ensuring that all carbon dioxide is removed so that it can not interfere in the analysis. The specific gravity at 20°C of the alcoholic distillate is determined after making it up to its original weight with water, and the alcohol content % m/m is calculated from this value. The specific gravity of the filtered beer is determined in order to convert alcohol % m/m to % V/V.

c.2) Original (% Plato), Real (% m/m) and Apparent (% m/m) Extract of Beer

The reference method is the "Analytica-EBC 9.4 Original, real and Apparent Extract and Original Gravity of Beer". These parameters are calculated from specific gravity determinations obtained on the beer and on the alcoholic distillate and beer residue after distillation. The real extract (% m/m) of the beer is calculated from the specific gravity of the distillation residue. The apparent extract (% m/m) of the beer is calculated from the specific gravity of the beer. The original extract (% Plato) and the original gravity (°Sacch) are calculated from the specific gravity of the distillation residue and the specific gravity of the beer distillate.

c.3) pH of Beer

The reference method is the "Analytica EBC 9.35 pH of Beer". The pH of decarbonated beer at 20°C is estimated using a pH meter and a suitable electrode system. Complete degassing of the beer sample must be achieved.

The quality parameters and the respective reference analyses considered for each kind of samples will be better explained in the further chapters.

4. RESULTS AND DISCUSSION

4.1 Raw Materials – Barley and maize quality

Concerning the analysis of raw materials (Objectives A1 and A2) reasonable calibration models have been developed to determine the parameters of interest on malting barley and maize.

4.1.1 Maize

About the **maize**, NIR calibrations have been developed in order to estimate **the moisture and the lipid content** of grist. Concerning to maize grist, the request of water content is less than 13 %. However, at this level, which is the lower for the growth of micro-organism, enzymatic reaction can still take place and the fat composition due to lipase activity can continue. At 13% of water there can be danger since the flow properties already change in this range; this can lead to difficulties in air lifts and on emptying silos. The standard for fat content in maize grits is less than 1–1.3%. Most breweries want degreased maize products. The significance of the lipids in beer production has been extensively investigated [83]. Their influence on various properties of the beer, such as the taste and off-flavour, foam stability, gushing and the yeast viability, has been widely described. For these reasons, this thesis project focused mainly on these two parameters. In a first step, calibrations were developed by correlating the analytical data with spectra collected on milled samples (**PAPER I**). Maize grits (about 1 kg) were homogenized by means of a sample divider and finely ground by means of a DLFU type disk mill set at a distance between the disks of 0.2 mm. The flours were used to record the spectra and to carry out the reference analyses. For the determination of moisture content (%) 146 samples were used, while 95 maize samples were used for the determination of the total lipids content (both % as is and % dry matter). In the second step, the analytical data were correlated with the spectra collected directly on the maize grist (**PAPER II).** This step represents an improvement of the previous one, utilising the whole maize grits. The possibility of using unmilled samples can enable the users to mainly benefit from the several advantages of the NIR spectroscopy technique. These measurements are more rapid than the previous ones because no milling of the raw material is needed. NIR spectra of grist and flour samples were acquired and correlated with the relative humidity and fat content by PLS algorithm (Partial Least Squares). The calibration models were developed using the software OPUS 6.5, which through the OPTIMIZE tool allows the choice of the pretreatment and the spectral range most suitable for obtaining the best correlation with the

parameter of interest. Then, by choosing the proper number of principal components to describe the spectral matrix, and through the identification and the elimination of outliers, we obtained good calibrations, which have been validated by both cross-validation leave-one-out (CV loo) and test set validation by eliminating 33% of samples in calibration and using these samples to calculate the error of prediction (TS 33% out). Leave-one-out cross-validation could be indeed too optimistic to estimate the predictability, in terms of RMSECV, of the models. The calibrations were hence validated by means of an external validation, treating part of the complete sample set as a test set (33%). Such samples were chosen by operating a PCA on the complete sample set, equally dispersed on the score plot built by taking into account the first two components, to select a test set representative of all samples. Test set validation is hence carried out on a lower number of samples, but is supposed to be more realistic in evaluating the predictability of the models. The values of errors of prediction obtained for all the calibrations with the two kinds of validation are similar, and this indicates that the model is stable and enough reliable in its good predictability.

The calibration models relative to maize grist are characterized by lower values of R and higher values of the RMSEP (both CV and TS) parameters than the ones obtained from the finely ground cereals. This behaviour, which is observed for all the analytes, can be understood by considering that the light beam can have a better interaction with all the parts of the seeds when they are milled and of course the samples in form of flour are more homogeneous than the grist.

In addition, these calibration models were validated following the guidelines of the international organizations of accreditation. In Italy, the organization is ACCREDIA, which belongs to EA (European co-operation for Accreditation). For this reason, 10 spectra of the same sample were acquired, and the calibration models have been applied on these spectra to determine the parameters of interest. First, it was verified that the average predicted values fell within the range defined by the value chemically determined and the expanded uncertainty of the method. Subsequently, the standard deviations calculated on the 10 predicted values were compared using Chi square's test with the reference methods' one, with good results (**PAPER I and II**).

All the results are shown in **Table 4.1.1**.

Parameter	Range	Maize Flour		Maize Grist	
		RMSEP CV loo (TS 33% out)	R CV loo (TS 33% out)	RMSEP CV loo (TS 33% out)	R CV loo (TS 33% out)
Moisture %	12.424–15.213	0.074 (0.080)	0.99 (0.98)	0.127 (0.152)	0.97 (0.96)
Total Lipids % as is	0.472–1.306	0.042 (0.044)	0.96 (0.95)	0.065 (0.066)	0.92 (0.90)
Total Lipids % dry matter	0.551–1.525	0.054 (0.055)	0.95 (0.94)	0.070 (0.079)	0.93 (0.90)

Table 4.1.1 - *Results of NIR calibrations for the determination of the quality parameter of maize (RMSEP: Root Mean Square Error of Prediction, R: correlation coefficient, CV loo: Cross-Validation leave-one-out, TS: Test Set Validation).*

The repeatabilities of NIR methods match those of the standard A-EBC methods, for both moisture content and total lipids, as is (total lipids, dry matter, was not validated by the A-EBC collaborative trials), and extended uncertainties were calculated from the reproducibility values of the standard A-EBC methods. All differences between NIR-determined and true values were smaller than the uncertainties of NIR methods.

This is the most important part of these results. In fact, NIR calibrations for the determination of moisture and fat content in maize grist is not something new itself, but this comparison with the repeatibilities of the A-EBC reference methods make these calibration models specific analytical method for breweries and cereal industries, suitable for the evaluation of the quality of brewing raw materials.

4.1.2 Barley

Concerning **malting barley**, NIR spectra were acquired on 40 samples of whole grains and used to develop calibrations able to correlate these spectra with the parameters of **moisture (%) and total nitrogen content (% dm)**.

The water content is the most important parameter in barley evaluation. In fact, barley is a "live product" and it is necessary to control its moisture content in order to ensure its preservation. The absorption of water beyond determined limits must be avoided, because the presence of water can cause the activation of hydrolytic enzymes, leading to unwanted transformations. For this reason, the moisture content of barley has to be between 12 and 15%. Barley must have moisture content below 15% for long term storage. Very moist barley must be dried because it cannot be stored for long and it would quickly lose its ability to germinate properly. Moreover, high moisture content has a great influence on the mould and fungi attack. On the other hand, the drying process for the long-term storage should be not very intense in order to avoid the damaging of the enzymes.

Moreover, the determination of the moisture content has a commercial value, because the amounts of the other components are related to the dry weight.

The protein content of barley has an important role in malt and beer production. For this reason, it is important to control the protein content in barley. The protein content of barley can vary from 8 to 16%, but the normal commercial requirement for malting barley is a maximum of 11.5% protein in the dry matter. In fact, protein rich barleys are more difficult to process and produce a higher malting loss. Moreover, the potential extract obtainable from malt decreases in almost the same

extent (0.7 to 1%) as the protein content of the barley increases. About 30% of the proteins are stored as transport proteins in the cell walls of the endosperm and regulate the mass transfer. Only about a third of this protein passes into the finished beer. Although the amount of protein in beer is relatively small, it can have an important effect on its quality. Thus protein make a considerable contribution to the head retention but can on the other hand have an important influence on the occurrence of haze in beer. The protein content (% dm) can be calculated from the nitrogen (% dm) by multiplying this value for 6.25. The normal commercial requirement for the nitrogen content is a maximum of 1.84 % protein in the dry matter.

The process followed to develop the calibrations was the same adopted for the calibrations on maize. The calibration models were developed using the software OPUS 6.5, which through the OPTIMIZE tool allows the choice of the pretreatment and the spectral range most suitable for obtaining the best correlation with the parameter of interest (**Table 4.1.2**). Then, the best number of principal components to describe the spectral matrix has been chosen by plotting the RMSECV value versus the number of principal components, as shown in **Fig. 4.1.2 (moisture) and 4.1.4 (total nitrogen)**. After this step, 3 outliers in the calibration model for the determination of total nitrogen and 2 in the calibration for the determination of moisture, have been identified and the eliminated. Finally, good calibration models have been obtained, which have been validated by cross-validation leave-one-out (CV loo) In this case, it was not possible to apply the test set validation, because the number of the calibration samples was not enough high. The plots of predicted versus true values are shown in **Fig. 4.1.1 (moisture) and 4.1.3 (total nitrogen)**. All the results of the validation are shown in **Table 4.1.2**.

The agreement between the model and the reference method was evaluated by verifying that at least 95 % of the true values fall into range defined by the predicted values +/- the R_{95} of the reference method. All the results are shown in **Table 4.1.2**. Also in this case, this comparison with the A-EBC reference methods is the most interesting part of the results, because it makes these calibration models specific analytical method for breweries and cereal industries, suitable for the evaluation of the quality of brewing raw materials. In the Analytica EBC, NIR standard methods for the determination of moisture and total nitrogen content on barley are available (Method 3.13). The errors of prediction of the two calibration models developed in this project are much lower than the reproducibility values of these two standard methods.

PARAMETER	Range of true values (range of predicted values)	N°SAMPLES (N° of samples after outliers remotion)	PRETREATMENT	SPECTRAL RANGE	PCs	RMSECV	R^2 (R)	r_{95} (sd)	R_{95} (Ue)	% of samples outside the range
Moisture %	10.1-13.4 (10.3-13.6)	40 (38)	Multiplicative Scattering Correction	11501.7 - 5450	7	0.2	94.66 (0.98)	0.14 (0.05)	0.75 (0.53)	0
Total nitrogen %dm	1.38-2.05 (1.46-2.00)	40 (37)	Constant Offset Elimination	6101.8-5450 4601.4-4246.6	6	0.05	92.26 (0.96)	0.04 (0.015)	0.10 (0.07)	0

Table 4.1.2 - Results of NIR calibrations for the determination of the quality parameter of barley (PCs: Principal Components, RMSECV: Root Mean Square Error of Cross Validation, R: correlation coefficient, r_{95}: repeatability, sd: standard deviation, R_{95}: reproducibility, Ue: extended uncertainty)
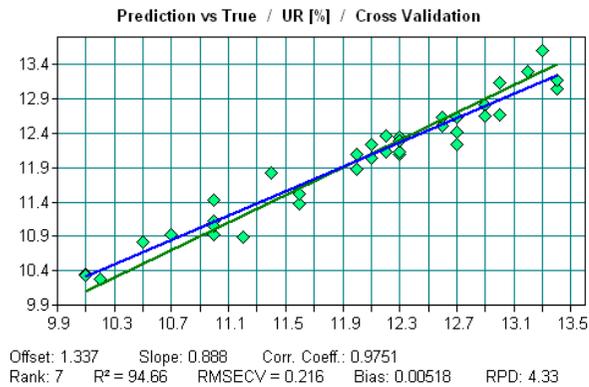
Figure 4.1.1 – Predicted vs true Barley Moisture %
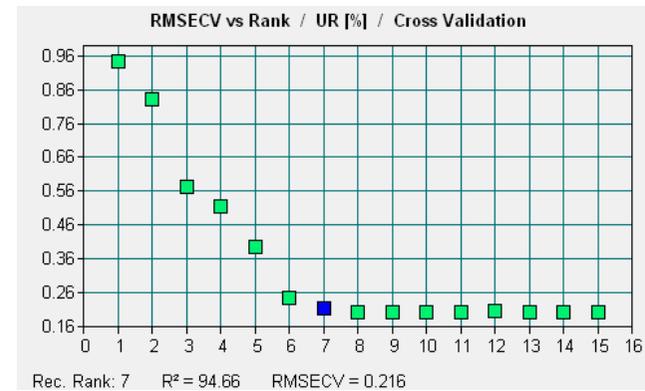
Figure 4.1.2 – RMSECV vs rank Barley Moisture %
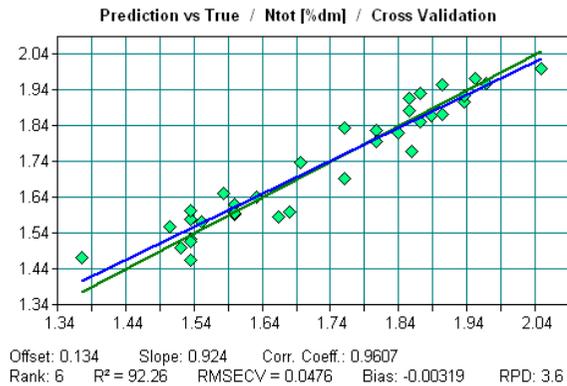
Figure 4.1.3 – Predicted vs true Barley Total Nitrogen %dm
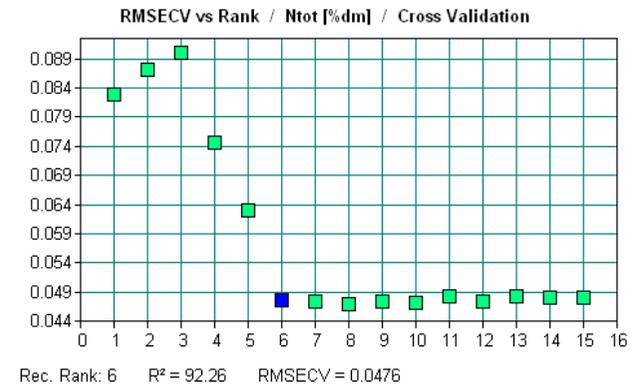
Figure 4.1.4 – RMSECV vs rank Barley Total Nitrogen %dm

113

4.2 Monitoring the malting process

Concerning the objective A3, a process control using NIR spectroscopy applied to malting process has been implemented.

4.2.1 Moisture

During **malting process**, the spectra were collected daily on samples of germinating barley at-line, i. e. outside the production line but during the process, in real time. At the same time of the spectra acquisition, the germinating barley **water content** was determined.

This parameter is crucial in the malting process. In fact, during the steeping, the seed absorbs water and its initial moisture of 14-15% increases. Once the humidity is around 30%, the embryo of the kernel starts the process of germination, synthesizing growth hormones (gibberellins), which stimulate the formation of hydrolytic enzymes. These enzymes begin to degrade various reserve substances such as starch, proteins and β-glucans. Water passes first into the embryo region but later also through the sides of the husks into the grain. Water uptake depends on the steeping time, steeping temperature, kernel size, barley variety and the barley harvest year. Following the guidelines for micromalting of the EBC barley and malt committee [84], the water content should be between 44 and 46 % at the end of steeping and from 42 to 44 % at the end of germination. It is really important to monitor this parameter during germination, in order to ensure a good malt modification. As specified in the "MATERIALS AND METHODS" section, the micro-malting pilot-plant (Custom Laboratory Products, Keith, UK) used for this project is provided with four independent steeping/germination tanks, each having the capacity to hold four drums filled with 0.5 kg of barley each (Fig 4.2.1). It was possible to measure the water content by weighting the drums containing the malting barley every day from the end of the steeping to the end of germination. At the same time of this determination, NIR spectra were collected on the germinating barley.

Fig. 4.2.1 - Independent steeping/germination tanks with the four drums

Subsequently, a calibration model was developed to relate the spectra acquired on germinating barley with their moisture. The calibration model, obtained using 228 spectra and as many corresponding relative humidity values, was generated by PLS algorithm using the software OPUS 6.5. Through the OPTIMIZE tool the best spectral pretreatment and range and the number of principal components most suitable to describe the spectral matrix were chosen (Table 4.2.1). After the identification and the elimination of outliers the calibration model was validated by cross validation, showing a good degree of predictability. Indeed, the value of R was 0.92 and the prediction error was lower than 2% (**POSTER I, Table 4.2.1 and Fig. 4.2.2**). There is not a reference method to compare the predictive performance of the calibration model. For this reason, an external validation, treating part of the complete sample set as a test set (33%) was performed, in order to verify if the cross-validation was too optimistic. The values of errors of prediction and coefficient of correlation obtained by test-set validation are similar to the cross-validation's ones (**Table 4.2.1 and Fig. 4.2.3**), and this indicates that the model is stable and enough reliable in its good predictability.

Validation	N°SAMPLES in calibration (outliers)	Range True (Predicted)	PRETREATMENT	SPECTRAL RANGE (cm^{-1})	PCs	RMSECV/ RMSEP	R^2 (R)
CV-loo	217 (11)	38.13-52.76 (37.74-51.74)	FD + SLS	115.01.7 – 4246.6	8	0.75	92.4 (0.96)
TS 33% out (71 samples)	144 (13)	38.44-52.76 (38.3-51.88)	FD + SLS	115.01.7 – 4246.6	8	0.79	91.9 (0.96)

Table 4.2.1 - Results of NIR calibrations for the determination of the moisture % of green malt (PCs: Principal Components, RMSECV/RMSEP: Root Mean Square Error of Cross-Validation/Prediction, R: correlation coefficient).
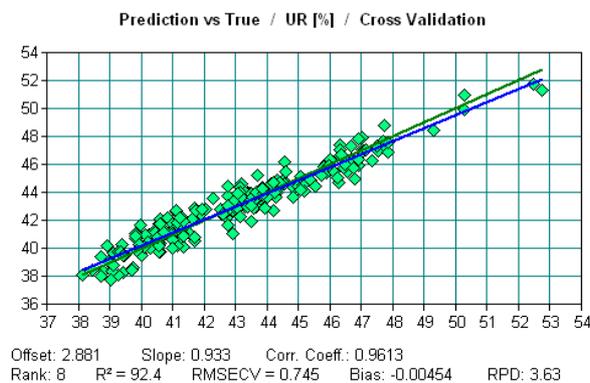
Figure 4.2.2 – Predicted vs true Moisture % of the green malt Cross-Validation
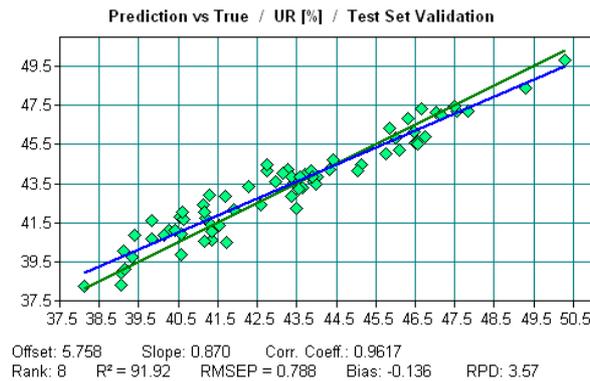
Figure 4.2.3 – Predicted vs true Moisture % of the green malt Test-Set Validation

This result suggests that it is possible, during the malting process, measure the moisture content on germinating barley using an at-line, accurate and not destructive NIR method. This method can be extremely useful in malting plants which have not the possibility of weighting the drums containing the green malt.

4.2.2 Malt Quality Parameters

Furthermore, calibration models were developed to monitor other important parameters to evaluate the performance of malting barley by NIR spectra. These models were developed on the assumption that some important parameters for the assessment of malt quality, which analytically are determined on the dried malt, are "visible" to the NIR already on the green malt. Then, 67 spectra of **green malt** collected during the **last day of germination** were correlated with some analytical parameters determined on the corresponding malts after drying by PLS algorithm. The selected parameters are the most important in the evaluation of malt quality and are:

- **Extract (% and % dm)**
- **Diastatic Power (WK)**
- **Fermentability (%)**
- **Viscosity (cP)**
- **pH**
- **Friability (%)**
- **Total Nitrogen (% and % dm)**
- **Soluble Nitrogen (% dm)**
- **Kolbach Index**
- **FAN (mg/L)**

116

These parameters can provide an indication of the carbohydrates and protein breakdown during malting. The extract is most important parameter in malt quality evaluation. It is reported as a percentage both on an "as is" basis and also related to dry weight. The extract yield on a dry weight basis is of much greater importance than the "as is" value, since the latter depends on the water content of the malt. The extract collected through a Congress mash is very important because it is strictly correlated with the malt yield during mashing, so the brewers use this value to calculate the amount of malt that they need to obtain the desired Plato. The extract measures the quantity of soluble matter in a Congress wort. The parameters mainly correlated with the carbohydrates metabolism are the Diastatic Power, which measures the activity of the α- and β-amylase, the fermentability, which depends on the quantity of fermentable sugars in the Congress wort and the viscosity, which is strictly correlated with the polysaccharides such as starch, dextrin, β-glucan, and pentosan content. High values of extract, Diastatic Power and fermentability indicate a well modified malt, while a under modified malt has a high value of viscosity.

The nitrogen content, expressed as a percentage both on an "as is" basis and also related to dry weight, can provide some information about the enzymatic activity and it is strictly correlated with the extract yield. In fact, every percent of additional protein results in approximately one percent less extract. Therefore, malts with high protein content have low values of extract and friability, and high amount of FAN, soluble nitrogen and β-glucan, and high values of diastatic power and viscosity. This value depends on barley variety, harvest and growing area but also on the agronomic conditions like nitrogen fertilization. The parameters more correlated with the protein breakdown during malting are the Kolbach Index and the soluble nitrogen and FAN content. High values of these parameters indicate an intense activity if the proteases during malting. The friability is a physical method to evaluate the modification of the malt, which provides information about both the carbohydrates and protein breakdown. In fact, undermodified malts have low values of friability and a high content of β-glucans and protein complexes. The pH of the Congress wort is an useful parameter to consider in the further phases of brewing.

Also in this case, the OPUS software was used for the set up of the calibration models and the OPTIMIZE tool allows the choice of the best spectral pretreatment, range and number of principal components (**Table 4.2.2**). The calibration models obtained were validated through cross-validation (CV), because the number of samples in calibration was not enough high to perform the external validation. Instead, the number of samples in the calibration set was enough high to ensure reliable calibrations, following the indication of "The American Society for Testing and Materials" [72], which requires a minimum of 6k (k = rank, or number of principal components) spectra in calibration after elimination of outliers.

The results of the cross-validations are shown in **Table 4.2.2**. The best calibrations were obtained for Fine Extract (% dm), Kolbach Index, Viscosity (cP) and Friability (%) with the highest R values.

Parameter	N°SAMPLES in calibration (outliers)	Range True (Predicted)	PRETREATMENT	SPECTRAL RANGE (cm^{-1})	PCs	RMSECV	R^2 (R)
Fine Extract (% dm)	60 (7)	74.4-85.3 (75.6-84.1)	First Derivative + Straight Line Subtraction	11501.7-7498.1 5453.8-4246.6	7	0.76	87.58 (0.94)
Fine Extract (%)	63 (4)	72.9-81.3 (71.8-80.7)	Constant Offset Elimination	7501.9-6098 5453.8-4246.6	9	0.84	83.46 (0.92)
Fermentability (%)	63 (4)	76.4-85.9 (75.4-85.3)	First Derivative + Straight Line Subtraction	7501.9-5450 4601.4-4246.6	4	1.0	82.50 (0.91)
Diastatic Power (WK)	63 (4)	234-488 (253-517)	Vector Standard Normalisation	7501.9-6098 5453.8-4246.6	5	42	68.82 (0.83)
Viscosity (cP)	63 (4)	1.47-1.70 (1.45-1.67)	Constant Offset Elimination	11501.7-4246.6	8	0.06	91.34 (0.96)
Total Nitrogen (% dm)	64 (3)	1.25-1.97 (1.35-1.97)	Constant Offset Elimination	11501.9-6098 5453.8-4246.6	8	0.07	82.11 (0.91)
Total Nitrogen (%)	64 (3)	1.30-1.85 (1.35-1.84)	Constant Offset Elimination	11501.9-6098 5453.8-4246.6	7	0.06	80.42 (0.90)
Soluble Nitrogen (% dm)	63 (4)	0.53-0.82 (0.52-0.79)	First Derivative + Multiplicative Scattering Correction	11501.9-7498.1 6101.8-5450 4601.4-4246.6	4	0.04	72.43 (0.85)
Kolbach Index	60 (7)	29.8-52.9 (29.7-50.8)	Second Derivative	6101.8-5450 4601.4-4246.6	6	1.9	86.84 (0.93)
FAN (mg/L)	62 (5)	101-193 (100-181)	First Derivative	6101.8-5450 4601.4-4246.6	4	10	78.32 (0.89)
pH	63 (4)	5.84-6.15 (5.89-6.14)	Straight Line Subtraction	11501.7-6098	7	0.05	50.53 (0.72)
Friability (%)	62 (5)	39-98 (41-96)	First Derivative	7501.9-4246.6	8	5	90.98 (0.95)

Table 4.2.2 *- Results of NIR calibrations for the determination of quality parameters of green malt (PCs: Principal Components, RMSECV: Root Mean Square Error of Cross-Validation, R: correlation coefficient).*

The plot of predicted versus true values obtained for the friability is shown in Fig. 4.2.4 as example.
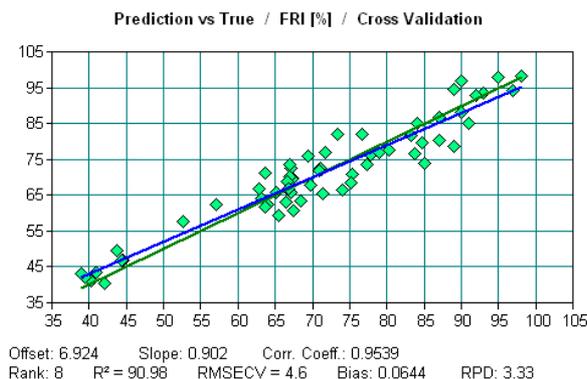
Figure 4.2.4 – Predicted vs true Friability % of the green malt

For the parameters considered the agreement between the calibration models and the reference methods was evaluated, and the results are shown in **Table 4.2.3**. This was not possible for the extract and the total nitrogen % "as is", because the reproducibility values for these two parameters were not available the Analytica EBC. As can be seen in Table 4.2.3, for the main part of the parameters considered more than the 95% of the true values fall into range defined by the predicted values +/- the R_{95} of the reference method. This is not true just for the Fine Extract %dm and for the Kolbach Index. In fact, in these two cases, just 56 true values on 60 fall into the range, namely the 93%. Anyway, the agreement with the reference method can be considered good even in these two cases.

The results shown in Table 2 are very encouraging and show the possibility of using NIR monitoring of the germination process, in order to assess how they are evolving the most important quality parameters of malt. This possibility would be extremely important for maltsters, which could change the process causing acceleration or deceleration of germination varying the parameters of humidity and temperature.

Also in this case, this comparison with the A-EBC reference methods is very important, because it gives an indication of the good predictability of the calibration models, which have an error comparable with the official analytical method used by breweries and cereal industries for the evaluation of the quality of brewing raw materials.

Parameter	RMSECV	r_{95}	sd	R_{95}	Ue	% of samples outside the range
Fine Extract % dm)	0.76	0.58	0.20	1.2	0.81	**7**
Fermentability (%)	1.0	0.7	0.22	2.8	2	0
Diastatic Power (WK)	42	21	7	78	55	2
Viscosity (cP)	0.06	0.06	0.02	0.19	0.14	0
Total Nitrogen (% dm)	0.07	0.05	0.02	0.13	0.09	3
Soluble Nitrogen (% dm)	0.04	0.04	0.01	0.09	0.06	0
Kolbach Index	1.9	1.2	0.4	3.4	2.4	**7**
FAN (mg/L)	10	13	4	22	16	3
pH	0.05	0.02	0.01	0.12	0.09	0
Friability (%)	5	4	1	8	6	5

Table 4.2.3 - *Comparison between the reference methods and the NIR calibrations for the determination of quality parameters of green malt (, RMSECV: Root Mean Square Error of Cross Validation, r_{95}: repeatability, sd: standard deviation, R_{95}: reproducibility, Ue: extended uncertainty).*

4.3 Malt quality

Still inside the objective A3, several calibration models were developed to allow a full assessment of the **malt quality** by NIR spectroscopy.

First of all, we focused on the determination of the most important parameters for the evaluation of malt quality, the **moisture (%) and total nitrogen (% and %dm) content**.

The water content depends on the type of malt. Normal values are around 3.0 – 3.5% for pale malt and 1.0 – 4.5% for dark malt. In any case, the commercially acceptable limit is usually 5%. This determination is very important for commercial reasons, because the brewers would like to buy malt with the lowest water content as possible. Moreover, this parameter allows obtaining the amount of dry matter, which is relevant for brewers. On the other hand, the moisture content is very important for malt quality. In fact, if this value is too high, that is the malt is slack. Slack malt can lose its aroma on storage and will not break up normally when milled. Furthermore the presence of water causes the reactivation of hydrolytic enzymes, resulting in undesired transformation. For this reason, the malt has to be carefully stored. In fact, dry malt is a hygroscopic product and it is necessary to avoid moisture absorption. A high value of moisture can be due to an irregular kilning or to bad storage conditions, for example to an accidental access of water in the silo, and it can lead to low yield.

The nitrogen content also depends on barley variety, harvest and growing area but also on the agronomic conditions like nitrogen fertilization. Like as for barley, the protein content has an important role in beer production, and for this reason its determination is very important in the assessment of malt quality. Usually, it is up to 0.5% lower in malt than in barley, and it can provide some information about the enzymatic activity during malting. It is usually expressed as protein content (calculated as N x 6.25). The protein content in malt should be between 10 and 11%. It must not exceed the 11% because of its strictly correlation with the extract yield. In fact, every percent of additional protein results in approximately one percent less extract. Therefore, malts with high protein content have low values of extract and friability, indicating a low modification during malting. Moreover, a high content of protein is correlated with high amount of di-methyl-sulphide (DMS), free-amino-nitrogen (FAN), soluble nitrogen and β-glucan, and high values of diastatic power, colour and viscosity. Concerning beer, if the nitrogen content is too high, the brewers will have problems of low yield and filterability of the wort and beer turbidity. On the other hand, protein content lower than 10% can lead problems of yeast nutrition, insufficient beer foam stability and low enzymatic activity.

Malt samples were supplied from industrial malthouses and are representative of the ones available on the Italian market. Samples of malt representative of the different types (i.e., pale, Munich, coloured, and caramel) were considered.

In a first step, calibrations were developed by correlating the analytical data with spectra collected on milled samples (**PAPER I**). Malt grains (about 1 kg) were homogenized by means of a sample divider and finely ground by means of a DLFU type disk mill set at a distance between the disks of 0.2 mm. The flours were used to record the spectra and to carry out the reference analyses. In particular, the following data sets were used to set the calibrations: 284 malt samples (among which were 13 Munich, 8 coloured, 2 caramel, and 1 wheat) for malt moisture content; 275 malt samples (among which were 13 Munich, 7 coloured, 2 caramel, and 1 wheat) for malt total nitrogen content, both as is and dry matter.

In the second step, the analytical data were correlated with the spectra collected directly on the maize grist (**PAPER II**). This step represents an improvement of the previous one, utilising the whole malt grains in order to provide more rapid measurements because no milling of the raw material is needed. In this case the cereals were not milled to record the NIR spectra, but two aliquots (about 500 g each one) of malt grains samples were separated after homogenisation by means of a sample divider: one was used to record the spectra, while the other one was finely ground to measure the moisture and total nitrogen content in the malt.

NIR spectra of grains and flour samples were acquired and correlated with the relative humidity and nitrogen content by PLS algorithm (Partial Least Squares). The calibration models were developed using the software OPUS 6.5, which through the OPTIMIZE tool allows the choice of the pretreatment and the spectral range most suitable for obtaining the best correlation with the parameter of interest. Then, the proper number of principal components was chosen and the outliers were identified and the eliminated. The calibration models obtained were validated by both cross-validation leave-one-out (CV loo) and test set validation by eliminating 33% of samples in calibration and using these samples to calculate the error of prediction (TS 33% out). Leave-one-out cross-validation could be indeed too optimistic to estimate the predictability, in terms of RMSECV, of the models. The calibrations were hence validated by means of an external validation, treating part of the complete sample set as a test set (33%). Such samples were chosen by operating a PCA on the complete sample set, equally dispersed on the score plot built by taking into account the first two components, to select a test set representative of all samples. Test set validation is hence carried out on a lower number of samples, but is supposed to be more realistic in evaluating the predictability of the models, because of the high number of samples in calibration.

The values of errors of prediction obtained for all the calibrations with the two kinds of validation are similar, and this indicates that the model is stable and enough reliable in its good predictability.

As for the calibration models developed on maize flour and maize grist, the light beam had a better interaction with all the parts of the seeds when they are milled and of course the samples in form of flour are more homogeneous than the whole grains, so the calibration models relative to malt grains are characterized by lower values of R and higher values of the RMSEP (both CV and TS) parameters than the ones obtained from the flours.

In addition, these calibration models were validated following the guidelines of the international organizations of accreditation. In Italy, the organization is ACCREDIA, which belongs to EA (European co-operation for Accreditation). For this reason, 10 spectra of the same sample were acquired, and the calibration models have been applied on these spectra to determine the parameters of interest. First, it was verified that the average predicted values fell within the range defined by the value chemically determined and the expanded uncertainty of the method. Subsequently, the standard deviations calculated on the 10 predicted values were compared using Chi square's test with the reference methods' one, with good results (**PAPER I and II**).

All the results are shown in **Table 4.3.1**.

Parameter	Range	Malt Flour		Malt Grain	
		RMSEP CV loo (TS 33% out)	R CV loo (TS 33% out)	RMSEP CV loo (TS 33% out)	R CV loo (TS 33% out)
Moisture %	0.521 – 7.155	0.097 (0.100)	0.989 (0.991)	0.132 (0.165)	0.979 (0.976)
Total Nitrogen % as is	1.234 – 1.930	0.026 (0.042)	0.964 (0.924)	0.043 (0.048)	0.883 (0.848)
Total Nitrogen % dry matter	1.296 – 2.034	0.024 (0.029)	0.970 (0.950)	0.046 (0.053)	0.854 (0.843)

Table 4.3.1 - Results of NIR calibrations for the determination of the moisture and total nitrogen content of barley malt RMSEP: Root Mean Square Error of Prediction, R: correlation coefficient, CV loo: Cross-Validation leave-one-out, TS: Test Set Validation).

In addition, various calibration models have been developed for the determination of other malt quality parameters from NIR spectra of malt flour. Compared to the models of the first year of PhD **(POSTER II)**, some calibrations have been improved implementing new spectra acquired during 2008, 2009 and 2010 and especially using advanced chemometric methods that have allowed refinement of the models, mainly concerning the selection of the spectral bands (interval-PLS algorithm) and new spectral pretreatments (Extended Multivariate Scattering Correction). The calibration models were developed through PLS algorithm, using the software MATLAB 7.6 R2008a. The different pretreatments or combinations of them were compared manually. The choice of spectral range has been performed applying the interval-PLS algorithm (PLS-toolbox). The

identification of outliers was conducted using the same three different parameters used by OPUS and described in the MATERIALS AND METHODS section. The best number of principal components was chosen by plotting the error of prediction versus the number of principal components and selecting the one leading to the lowest error.

The parameters considered are the following:

- **Extract (% and % dm)**
- **Fermentability (%)**
- **Viscosity (cP)**
- **pH**
- **Friability (%)**
- **Soluble Nitrogen (% dm)**
- **Kolbach Index**
- **FAN (mg/L)**

The more interesting aspect resulting from the use of the software MATLAB 7.6 R2008a is the possibility of perform different kinds of validation. In fact, as explained in **PAPER III**, one single performance criterion of validation often is not sufficient to judge a Near Infrared calibration model in the proper way which can lead to over- or under-estimation of the model quality. In fact, the error of prediction is a statistic, which can change according to the type or number of samples considered in the validation. For this reason, we decided to compare the simple leave-one-sample-out cross-validation (CV) with a more challenging CV with leave-33%-samples-out, where the re-samplings were repeated 200 times. The selection of the samples excluded was random. This kind of validation was performed instead of the classic test-set validation considered in the previous results in order to verify how the RMSECV-values change by the kind of samples used to calculate it. Because the error will change according to chance the two re-samplings were repeated 200 times.

For each i-set of 200 containing the 33% of samples, the error of prediction was calculated. Finally, for each principal component 200 different values of error of prediction have been calculated, according to the type of validation samples. It was therefore possible to calculate a mean (blue) and an uncertainty (red) of the error of prediction for each principal component (**Fig. 4.3.1**).
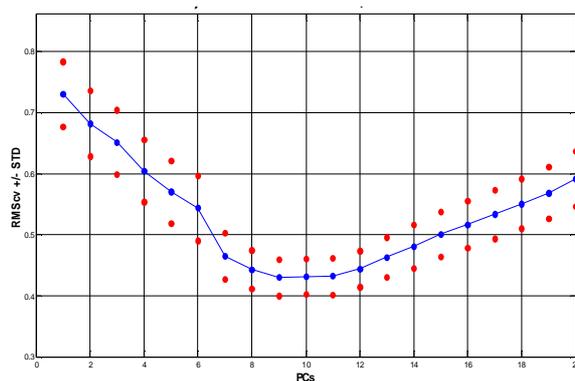
Fig. 4.3.1 - Mean (blue) and an uncertainty (red) of the error of prediction for each principal component.

The results for the Viscosity (cP), FAN (mg/L) and Friability (%) parameters, for which from 166 to 239 samples, are shown in Table 4.3.2. In the cross-validation leave 33% out, the i-set which is predicted the worse leads to the higher error of prediction, which can be calculated adding the Mean RMSECV to its Standard Deviation (sd). This worse possibility is also the one considered in the comparison between the range of the true and the predicted values.

It can be easily seen that the errors of prediction obtained using the two types of validation are the same. This means that the cross-validation in these models does not overestimate their predictability, then the models can be considered stable and reliable.

PARAMETER		Viscosity (cP)	FAN (mg/L)	Friability (%)
PRETREATMENT		First Derivative + Multiplicative Scattering Correction	Extended Multiplicative Scattering Correction	Extended Multiplicative Scattering Correction
SPECTRAL RANGE (cm^{-1})		9000-4000	9000-4000	9000-4000
PCs		11	13	10
N°SAMPLES in calibration (outliers)		222 (17)	101 (15)	176 (20)
Cross Validation leave-one-out	N°SAMPLES in validation	1	1	1
	Range True (Predicted)	1.45-1.58 (1.45-1.56)	102-168 (109-168)	78.8-99.4 (80.5-100.4)
	RMSECV	0.0193	5.1	2.1
	R	0.73	0.91	0.85
Cross Validation leave-33%-out (x 200)	N°SAMPLES in validation	73	33	58
	Range True (Predicted)	1.45-1.58 (1.47-1.58)	102-167 (109-166)	78.8-97.4 (81.1-98.1)
	Mean RMSECV ± sd	0.0173 ± 0.0013	5.9 ± 0.8	2.17 ± 0.16
	Mean R ± sd	0.683 ± 0.049	0.887 ± 0.037	0.842 ± 0.033

Table 4.3.2 - Results of NIR calibrations for the determination of malt Viscosity, FAN and Friability, validated by Cross-Validation (leave-one-out and leave-33%.out) (PCs: Principal Components, RMSECV: Root Mean Square Error of Cross-Validation, R: correlation coefficient).

This statement is confirmed by checking the agreement between the calibration models and the reference methods. From the results shown in **Table 4.3.3**, it can be seen that for all the parameters considered more than the 95% of the true values fall into range defined by the predicted values +/- the R_{95} of the reference method.

Parameter	r_{95} (sd)	R_{95} (Ue)	% of samples outside the range CV loo	% of samples outside the range CV 33%out
Viscosity (cP)	0.04 (0.02)	0.14 (0.09)	0	0
FAN mg/L	13 (4)	22 (16)	0	0
Friability (%)	2.4 (0.8)	4.9 (3.5)	1	2

Table 4.3.3 - Comparison between the reference methods and the NIR calibrations (Viscosity, FAN and Friability) (r_{95}: repeatability, sd: standard deviation, R_{95}: reproducibility, Ue: extended uncertainty, CV: Cross-Validation)

The results for the Fine Extract (%dm), Fermentability (%), pH and Soluble Nitrogen (%dm) parameters, for which from 318 to 334 samples, are shown in Table 4.3.4.

PARAMETER		Fine Extract (%dm)	Fermentability (%)	pH	Soluble Nitrogen (%dm)
PRETREATMENT		Extended Multiplicative Scattering Correction	Extended Multiplicative Scattering Correction	Extended Mult. Scattering Correction	Extended Mult. Scattering Correction
SPECTRAL RANGE (cm^{-1})		7163-6781 6006-5624 4463-4981	9000-4000	8320-7940 6000-5600 5320-4850 4460-4000	9091-9709 7934-6009 5620-4081
PCs		11	13	14	12
N°SAMPLES in calibration (outliers)		295 (30)	288 (30)	304 (30)	310 (17)
Cross Validation leave-1-out	N°SAMPLES in validation	1	1	1	1
	Range True (Predicted)	79.14-84.10 79.51-83.50	77.78-83.40 78.74-82.95	5.76-6.18 5.81-6.15	0.53-0.83 0.56-0.80
	RMSECV	0.60	0.67	0.04	0.02
	R	0.73	0.72	0.75	0.82
Cross Validation leave-33%-out (x 200)	N°SAMPLES in validation	98	95	101	103
	Range True (Predicted)	79.25-83.57 79.19-83.66	77.78-83.35 78.39-82.73	5.73-6.18 5.84-6.13	0.57-0.75 0.60-0.76
	Mean RMSECV ± sd	0.6091 ± 0.0349	0.6993 ± 0.0426	0.0461 ± 0.0070	0.0237 ± 0.0014
	Mean R ± sd	0.7233 ± 0.0374	0.7008 ± 0.0444	0.7192 ± 0.0456	0.8058 ± 0.0329

Table 4.3.4 *- Results of NIR calibrations for the determination of malt Fine Extract, Fermentability, pH and Soluble Nitrogen validated by Cross-Validation (leave-one-out and leave-33%out) (PCs: Principal Components, RMSECV: Root Mean Square Error of Cross-Validation, R: correlation coefficient).*

The results for the Fine Extract % and Kolbach Index can be seen in **POSTER III** and **PAPER III**, respectively. Also in this case, the considerations considered for the previous parameters can be made.

Then, a "year-according" test-set validation was applied on these four calibration models. In fact, it this case it was possible to check if the calibration models have stable predictive performances during the time because the samples used to set up these calibration models have been analyzed form 2006 to 2009. Three test set validations, where the validation samples were chosen according to the year of collection and analysis, were applied, in the order of check if the calibration model

has a stable predictive performance on samples collected in different years. Three different data blocks were defined and used for the validation: 2006, 2007 and 2008+2009. Two of these three data blocks were used to develop the calibration and the third one was used as validation-set. This "extrapolation" test should clarify the long-term effects on NIR calibrations and give a good indication of the model performance for coming years. The results are shown in **Table 4.3.5**.

		Fine Extract (%dm)		Fermentability (%)		pH	Soluble Nitrogen (%dm)	
PRETREATMENT		Extended Multiplicative Scattering Correction		Extended Multiplicative Scattering Correction		Extended Mult. Scattering Correction	Extended Multiplicative Scattering Correction	
SPECTRAL RANGE (cm^{-1})		7163-6781 6006-5624 4463-4981		9000-4000		8320-7940 6000-5600 5320-4850 4460-4000	9091-9709 7934-6009 5620-4081	
Test Set Validation 2006 out	**N°SAMPLES in calibration**	184		187		194	194	
	N° SAMPLES in validation	111		101		110	116	
	Range True	79.14-82.86		78.01-83.40		5.86-6.18	0.53-0.76	
	Best PCs/CV PCs	10	11	14	13	14	11	12
	Range Predicted	80.0-83.18	80.12-83.37	79.43-82.91	79.50-82.75	5.87-6.14	0.56-0.75	0.56-0.76
	RMSEP	0.69	0.76	0.73	0.76	0.05	0.02	0.03
	R	0.62	0.59	0.67	0.65	0.72	0.79	0.74
Test Set Validation 2007 out	**N°SAMPLES in calibration**	171		166		176	185	
	N° SAMPLES in validation	124		122		128	125	
	Range True	79.14-84.10		77.78-83.40		5.73-6.12	0.59-0.76	
	Best PCs/CV PCs	12	11	11	13	14	11	12
	Range Predicted	79.28-83.15	79.28-82.90	78.82-83.40	78.68-83.70	5.71-6.15	0.60-0.74	0.60-0.78
	RMSEP	0.70	0.77	0.80	0.86	0.05	0.02	0.03
	R	0.74	0.72	0.68	0.65	0.78	0.73	0.70
Test Set Validation 2008+2009 out	**N°SAMPLES in calibration**	235		223		238	244	
	N° SAMPLES in validation	60		65		66	666	
	Range True	79.41-83.28		79.00-83.10		5.80-6.11	0.61-0.83	
	Best PCs/ CV PCs	10	11	9	13	14	12	
	Range Predicted	79.67-82.24	79.43-82.11	79.12-82.36	79.14-82.57	5.82-6.07	0.60-0.68	
	RMSEP	0.76	0.80	0.79	0.86	0.06	0.03	
	R	0.59	0.60	0.68	0.63	0.56	0.72	

The best number of principal components was chosen by plotting the error of prediction versus the number of principal components and selecting the one leading to the lowest error. If this number is different from the one found by the cross-validations, both the results are shown in order to compare them with the cross-validations results.

A better discussion about these data can be done by looking at the agreement between the calibration models and the reference methods, shown in **Table 4.3.6**.

Parameter	Fine Extract (% dm)	Fermentability (%)	pH	Soluble Nitrogen (% dm)
r_{95} (sd)	0.58 (0.20)	0.7 (0.2)	0.02 (0.01)	0.04 (0.01)
R_{95} (Ue)	1.2 (0.8)	2.8 (2.0)	0.12 (0.09)	0.09 (0.06)
% of samples outside the range CV leave-one-out	3	0	1	0
% of samples outside the range CV 33%out	5	0	1	0
% of samples outside the range TS 2006 out	11PC =6 / 10PC=12	0	1	0
% of samples outside the range TS 2007 out	11PC=10 / 12PC=6	0	3	0
% of samples outside the range TS 2008/2009 out	11PC=15 / 10PC=12	0	1	0

Table 4.3.6 - *Comparison between the reference methods and the NIR calibrations (Fine Extract, Fermentability, pH and Soluble Nitrogen) (r_{95}: repeatability, sd: standard deviation, R_{95}: reproducibility, Ue: extended uncertainty, CV = Cross-Validation, TS: Test Set Validation)*

The results demonstrate that one single performance criterion often is not sufficient to judge a Near Infrared calibration model in the proper way which can lead to over- or under-estimation of the model quality. Leave-one-sample-out cross validation is too optimistic, because excluding one sample has a low perturbing effect on the model. The second type of cross-validation examined, the leave-33%-out, gives a more realistic idea of the predictive power of the model, and it is necessary

to be conservative and consider at least the higher errors of prediction (mean RMSECV + sd) in the description of the predictability of the models.

Then, using three year-blocks as test-sets in order to obtain an indication of the model performance for coming years, it was evident that the calibration models have lower predictive powers than in the cross-validations and do not give a equally good prediction on the three data sets.

About Fine Extract, the errors of prediction obtained by test-set validation are appreciably higher than the ones calculated by cross-validations, and this statement is confirmed in table 4.3.6, looking at the % of samples outside the range defined by the predicted values +/- the R_{95} of the reference method. It is difficult to state how the model will predict the fine extract values of unknown samples analyzed, for example, in 2010, but it is necessary to be conservative and consider an error of prediction between 0.7 and 0.8 and an agreement with the reference method lower than the 95%.

Also about Fermentability, the errors of prediction obtained by test-set validation are appreciably higher than the ones calculated by cross-validations, and it can be realistic to consider this predictive power on future unknown samples. However, as shown in table 4.3.6, the agreement with the reference method is good for all the kinds of validation considered. About the pH and the Soluble Nitrogen, the differences between the errors of prediction obtained by internal or external validation are not significant. This indicates a very good stability and reliability of the models.

There results are confirmed by the data shown in **table 4.3.6**, then the estimates produced with these calibration models agree with those produced by the reference methods as well as a second laboratory repeating the reference measurements would agree.

The overall conclusion is that we likely need to be modest in reporting the expected prediction error.

In addition, these calibration models were validated following the guidelines of the international organizations of accreditation. In Italy, the organization is ACCREDIA, which belongs to EA (European co-operation for Accreditation). For this reason, 10 spectra of the same sample were acquired, and the calibration models have been applied on these spectra to determine the parameters of interest. This procedure is repeated for 3 samples. For each distribution of 10 predicted values, the normality test was performed by means of the Shapiro-Wilk test with a probability level of p = 95% (α = 5%). Moreover, anomalous data were identified by means of the Huber test, which is based on the evaluation of the median and is one of the most robust methods.

First, it was verified that the average predicted values fell within the range defined by the values chemically determined and the standard deviation of the method.

Subsequently, the variances (sd^2) calculated on the 10 predicted values were compared using Chi square's test with the reference methods' one. All the results are shown in **Table 4.3.7 and 4.3.8**,

where for each sample it has been reported the variance of the reference method, the variance calculated from the 10 repeated values, and the probability (P) that the $H\chi^2$ hypothesis is true.

$H\chi^2$ is the hypothesis that the considered data come from a normal distribution with variance V (reference method's variance), against the alternative that the data come from a normal distribution with a different variance. The result is $H\chi^2 = 0$ (with a probability = P) if the null hypothesis (variance is V) cannot be rejected at the 5% significance level, or H = 1 if the null hypothesis can be rejected at the 5% level.

Moreover, for each sample, the "true" value chemically determined is reported ± the repeatability of the method, and it is compared with the mean of the 10 predicted values ± the calculated repeatability.

Looking at the results about the Viscosity (cP), FAN (mg/L) and Friability (%), (Table 4.3.7) it can be seen that the NIR method for FAN determination has a variance statistically comparable with the reference method's one, with probability values close to 1. This is true also for the NIR method for Viscosity determination, for 2 of the 3 samples analyzed. In the case of Friability, the probability that the variance calculated is statistically similar to the reference method's one is not close to 1 in the three samples considered. In fact, the obtained variance values are higher or lower than the reference method's one, but the null hypothesis is confirmed for the 3 samples, so the two variance values are statistically similar at the 5% significance level.

Sample		Viscosity (cP)	FAN (mg/L)	Friability (%)
	Reference Variance (sd^2)	**0.00014**	**19.44**	**0.64**
Sample 1	Variance Predicted	0.00004	18.37	0.33
	P for Hχ^2=0 (α=0.95)	0.2943	0.9915	0.2188
	Predicted value $\pm$ r$_{95}$	1.54 $\pm$ 0.02	147 $\pm$ 13	96.0 $\pm$ 1.7
	True Value $\pm$ r$_{95meth}$	1.55 $\pm$ 0.04	149 $\pm$ 13	97.5 $\pm$ 2.4
Sample 2	Variance Predicted	0.00010	18.37	0.82
	P for Hχ^2=0 (α=0.95)	0.9505	0.9915	0.4621
	Predicted value $\pm$ r$_{95}$	1.51 $\pm$ 0.03	141 $\pm$ 13	85.1 $\pm$ 2.7
	True Value	1.50 $\pm$ 0.04	141 $\pm$ 13	87.5 $\pm$ 2.4
Sample 3	Variance Predicted	0.00010	15.60	0.43
	P for Hχ^2=0 (α=0.95)	0.9505	0.7221	0.4548
	Predicted value $\pm$ r$_{95}$	1.52 $\pm$ 0.03	119 $\pm$ 12	88.5 $\pm$ 1.9
	True Value	1.51 $\pm$ 0.04	115 $\pm$ 13	87.6 $\pm$ 2.4

Table 4.3.7 – Comparison with the reference methods' variance and the NIR methods' ones for the determination of malt Viscosity, FAN and Friability, by Chi square test (sd: Standard Deviation, P: probability, r$_{95}$: repeatability).

Concerning the results about the Fine Extract (%dm) (Table 4.3.8), the NIR method has a variance statistically comparable with the reference method's one, with probability values close to 1, only for one of the samples examined. For the other 2 samples the variance is higher than the reference method's one, but the null hypothesis is still confirmed.

Also the NIR methods for the determination of pH and Soluble Nitrogen (%dm), show a variance statistically comparable with the reference method's one, with probability values close to 1, only for one of the samples examined. Anyway, both these situations are different from the previous one because for the other 2 samples the variance is lower than the reference method's one, but the null hypothesis is still confirmed. In the case of Fermentability, the probability that the variance calculated is statistically similar to the reference method's one is not close to 1 in the three samples considered. In fact, the obtained variance values are higher or lower than the reference method's one, but the null hypothesis is confirmed for the 3 samples, so the two variance values are statistically similar at the 5% significance level.

Sample		Fine Extract (% dm)	Ferm.(%)	pH	Soluble Nitrogen (% dm)
	Reference Variance	**0.0385**	**0.04**	**1.4161e-004**	**1.9321e-004**
Sample 1	Variance Predicted	0.0595	0.0457	9.6040e-005	1.8769e-004
	P for Hχ^2=0 (α=0.95)	0.2151	0.6785	0.3884	0.9947
	Predicted value	82.1 ± 0.7	81.8 ± 0.6	5.95 ± 0.03	0.66 ± 0.04
	True Value	81.5 ± 0.6	82.3 ± 0.7	5.94 ± 0.02	0.65 ± 0.04
Sample 2	Variance Predicted	0.0366	0.0281	1.0404e-004	1.0201e-004
	P for Hχ2=0 (α=0.95)	0.9775	0.5526	0.9735	0.3736
	Predicted value	81.7 ± 0.6	79.7 ± 0.5	5.80 ± 0.03	0.60 ± 0.03
	True Value	82.0 ± 0.6	80.0 ± 0.7	5.79 ± 0.02	0.62 ± 0.04
Sample 3	Variance Predicted	0.0520	0.0582	7.9210e-005	1.3689e-004
	P for Hχ2=0 (α=0.95)	0.3785	0.2982	0.1987	0.5229
	Predicted value	82.8 ± 0.7	80.3 0.7	5.83 ± 0.03	0.65 ± 0.03
	True Value	83.0 ± 0.6	81.0 ± 0.7	5.84 ± 0.02	0.66 ± 0.04

Table 4.3.8 *– Comparison with the reference methods' variance and the NIR methods' ones for the determination of malt Fine Extract, Fermentability, pH and Soluble Nitrogen, by Chi square test : (sd: Standard Deviation, P: probability, r$_{95}$: repeatability).*

This is the most important part of these results.

In fact, NIR calibrations for the determination of quality parameters on malt are already available, but this comparison with the repeatability values of the A-EBC reference methods confirm the reliability of the predictive power of the models.

4.4 Malting Quality of barley

Regarding the objective A4, correlation models between the NIR spectra acquired on the samples of barley and the quality parameters of the corresponding malt was searched. The aims of this research was to defined the **barley attitude to malting** using NIR spectroscopy. 40 barley samples were malted in micromalting pilot plant and then analyzed for the same quality parameters considered in the chapter 4.2:

- **Extract (% and % dm)**
- **Diastatic Power (WK)**
- **Fermentability (%)**
- **Viscosity (cP)**
- **pH**
- **Friability (%)**
- **Total Nitrogen (% and % dm)**
- **Soluble Nitrogen (% dm)**
- **Kolbach Index**
- **FAN (mg/l)**

The importance of these parameters has been already discussed, and it is clear that they can be considered key parameters in evolution of malt quality. Consequently, it is extremely useful the possibility of knowing if a barley will provide a malt with a good extract and well modified (friability) thanks to the action of the enzymes breaking the polysaccharides (Diastatic Power) and protein (Kolbach Index). The FT-NIR spectra collected on barley whole grains were correlated to the quality parameters determined on the respective malt samples by PLS regression. The idea was to predict from barley spectra the quality of the malt which is possible to obtain from the same barley samples. The calibration models are supposed to permit a preliminary screening of barleys in order to individuate which are suitable for malting.

Also in this case, the OPUS software was used for the set up of the calibration models and the OPTIMIZE tool allows the choice of the best spectral pretreatment, range and number of principal components (Table 4.4.1). After outliers elimination, the calibration models obtained were validated through cross-validation (CV), because 40 samples in calibration were not enough to perform the external validation.

The calibration models obtained using OPUS showed a good degree of predictability, even if these models have been developed using just 40 samples. The results of the cross-validations are shown

in **Table 4.4.1** and are very encouraging. The best calibrations were obtained for Fine Extract (% dm), Total Nitrogen (% dm) and Friability (%) with the highest R values.

Parameter	N°SAMPLES in calibration (outliers)	Range True (Predicted)	PRETREATMENT	SPECTRAL RANGE (cm^{-1})	PCs	RMSECV	R^2 (R)
Fine Extract (% dm)	35 (5)	77.9-84.7 (77.9-83.7)	Vector Standard Normalisation	6101.8-5450 4601.8-4246.6	7	0.61	90.71 (0.95)
Fine Extract (%)	39 (1)	74.1-82.8 (79.5-83.4)	First Derivative + Vector Standard Normalisation	6101.8-4246.6	7	1.01	82.86 (0.91)
Fermentability (%)	37 (3)	78.1-84.8 (78.9-85.3)	Min.-Max. Normalisation	6101.8-5450 4601.8-4246.6	4	1.0	76.42 (0.87)
Diastatic Power (WK)	36 (4)	211-488 (216-488)	Multiplicative Scattering Correction	5453.8-4246.6	6	37	84.02 (0.92)
Viscosity (cP)	38 (2)	1.47-1.70 (1.45-1.67)	Vector Standard Normalisation	6101.8-5450 4601.8-4246.6	7	0.04	70.58 (0.84)
Total Nitrogen (% dm)	37 (3)	1.39-1.92 (1.40-1.90)	Vector Standard Normalisation	6101.8-5450 4601.8-4246.6	7	0.06	86.60 (0.93)
Total Nitrogen (%)	36 (4)	1.30-1.84 (1.31-1.90)	Min.-Max. Normalisation	6101.8-5450 4601.8-4246.6	9	0.06	87.98 (0.94)
Soluble Nitrogen (% dm)	36 (4)	0.59-0.77 (0.58-0.76)	Constant Offset Elimination	7501.9-4246.6	9	0.02	74.87 (0.87)
Kolbach Index	34 (6)	35.1-52.9 (35.2-51.5)	Constant Offset Elimination	7501.9-6098 4601.4-4246.6	6	2.0	83.07 (0.91)
FAN (mg/l)	35 (5)	122-172 (131-171)	Straight Line Subtraction	5453.8-4246.6	6	8	60.55 (0.78)
pH	37 (3)	5.87-6.15 (5.85-6.12)	Constant Offset Elimination	7501.9-6098 5453.8-4246.6	8	0.03	75.24 (0.87)
Friability (%)	35 (5)	63-98 (64-99)	Min.-Max. Normalisation	5453.8-4246.6	7	4	89.63 (0.95)

Table 4.4.1 - *Results of NIR calibrations for the determination of malting quality parameters of barley (PCs: Principal Components, RMSECV: Root Mean Square Error of Cross-Validation, R: correlation coefficient).*

Also in this case, the agreement between the calibration models and the reference methods was evaluated for all the parameters considered apart from extract and the total nitrogen % "as is", and the results are shown in Table 4.4.2.

It can be seen that for all the parameters considered, apart from the Kolbach Index, more than the 95% of the true values fall into range defined by the predicted values +/- the R$_{95}$ of the reference method. In the case of the Kolbach Index determination, just the 88% of samples fall into the range of agreement with the reference method. But this can be easily explained considering that the

Kolbach Index is a ratio between the soluble and the total nitrogen, so it includes the errors of the calibration models for the determination of these two parameters.

Anyway, the agreement with the reference method can be considered good, because for all the quality parameters apart from the Kolbach index, the estimates produced with the multivariate NIR models agree with those produced by the reference methods as well as a second laboratory repeating the reference measurement would agree. For this reason, the results shown in Table 4.4.2 can be considered very encouraging, even if the number of samples in calibration is not enough high to ensure the reliability of the results.

Parameter	RMSECV	r_{95}	sd	R_{95}	Ue	% of samples outside the range
Fine Extract (% dm)	0.61	0.58	0.20	1.2	0.81	3
Fermentability (%)	1.0	0.7	0.22	2.8	2	0
Diastatic Power (WK)	37	19	7	72	51	3
Viscosity (cP)	0.04	0.05	0.02	0.16	0.12	0
Total Nitrogen (% dm)	0.06	0.05	0.02	0.13	0.09	3
Soluble Nitrogen (% dm)	0.02	0.04	0.01	0.09	0.06	0
Kolbach Index	2.0	1.2	0.4	3.4	2.4	**12**
FAN (mg/l)	8	13	4	22	16	0
pH	0.03	0.02	0.01	0.12	0.09	0
Friability (%)	4	3	1	6	4	5

Table 4.4.2 - Comparison between the reference methods and the NIR calibrations for the determination of malting quality parameters of barley (RMSECV: Root Mean Error of Cross-Validation, r_{95}: repeatability, sd: standard deviation, R_{95}: reproducibility, Ue: extended uncertainty).

However, determining these parameters directly on barley as "potential" of malt features can be difficult, perhaps because they are strictly related to the process. The risk is to develop calibration models suitable for a standardized process, but may not for a different processes. This aspect should be deepened in the future, including in the calibration malt samples obtained with a different program.

However, beyond the determination of the individual parameters, what can be interesting is a combination of them, expressing the **"Malting Quality" (MQ) of a barley**, namely its suitableness to be malted. This parameter should be able to discriminate a malting barley, namely a barley suitable for use in the malting industry, from a feed barley. In fact a malting barley, as opposed to feed barley, demands a premium at the farm gate. However, in return, it must be of high quality and be able to germinate evenly and rapidly. It must exceed a range of malting quality (MQ) parameters laid down by the processing (malting, brewing and distilling) industries. Meeting the exacting specifications laid down by brewer and distiller customers is critical to the business of any maltster and is dependent on the barley used. The determination of the MQ of barley is thus a critical issue, as the accuracy of its assessment determines if the barley is suitable to be taken into the plant, influencing its price and subsequent quality of the end product. MQ is a complex character. In this project, the MQ of a barley has been expressed as the overall quality of the respective malt, calculated following the indication of the "Two years variety summary 2007/2008 - Spring & Winter Barley" of the EBC Barley and Malt Committee [85] for the Quality Index (QI) of malt.

The Quality Index is an estimation of the overall malt quality. It takes into account five parameters: fine extract (ext), Kolbach Index (KI), Diastatic Power (DP), Fermentability (Ferm) and Viscosity (Visco). Quality index is the sum of the differences between the sample examined and the reference sample; each parameter being balanced by its specific weight (40% for the extract, 15% for each other) and by the extended uncertainty of this given analysis, defined once on a large sample number. The viscosity term is subtracted, and the other parameters are summed:

$$MQ = QI = \left(\frac{(ext-ext_{std}) \cdot 0.40}{1.24}\right) + \left(\frac{(KI-KI_{std}) \cdot 0.15}{3.4}\right) + \left(\frac{(DP-DP_{std}) \cdot 0.15}{3.4}\right) - \left(\frac{(Visco-Visco_{std}) \cdot 0.15}{0.16}\right) + \left(\frac{(Ferm-Ferm_{std}) \cdot 0.15}{2.8}\right)$$
(Eq. 4.4.1)

The standard sample considered was the 17th EBC Standard Malt, which was daily analyzed simultaneously with the samples in order to ensure the reliability of analytical data. In fact, the quality parameters of this standard malt result from a collaborative trials of 30 laboratories carried out by the ECB Analysis Committee, and the analytical data daily obtained by the laboratory of CERB on this malt must match the given values +/- the extended uncertainty. The values of the individual quality parameters considered for the 17th EBC Standard Malt are:

-   Fine Extract = 82.3 %dm.
-   Diastatic Power = 339 WK.
-   Fermentability = 82.3%.

- Viscosity = 1.53cP.
- Kolbach Index = 38.

If the quality index of the malt analysed is as good as the 17th EBC Standard Malt's one, the contribute will be 0 for each parameter. Then the individual samples are shown as a +/- percentage of that standard value. This percentage will be greater than 0 for malts with a quality index better than the standard and lower than 0 for malts with a quality index worse than the standard.

Also in this case, the quality index of the malts was correlated by PLS algorithm with the NIR spectra of the respective barley samples, by using OPUS program, in order to express the MQ of the barley samples. The results of the cross-validation are shown in **Table 4.4.3** and the plot of predicted versus true values is shown in Fig. 4.4.1.

Parameter	N°SAMPLES in calibration (outliers)	Range True (Predicted)	PRETREATMENT	SPECTRAL RANGE (cm^{-1})	PCs	RMSECV	R^2 (R)
MQ	37 (3)	-3.04-0.28 (-3.04-0.12)	Multiplicative Scattering Correction	6101.8-4246.6	7	0.42	85.50 (0.92)

Table 4.4.3 - Results of NIR calibrations for the determination of Malting Quality of barley (PCs: Principal Components, RMSECV: Root Mean Square Error of Cross-Validation, R: correlation coefficient).
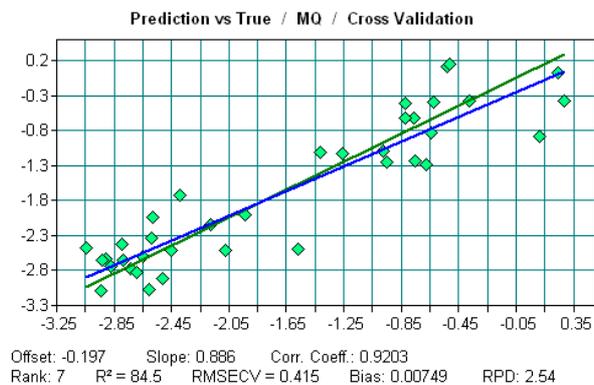
Figure 4.4.1 – Predicted vs true Malting Quality of barley.

The first conclusion is that the barleys considered in this project after malting give malts with an overall quality lower than the 17th EBC Standard Malt, and this is one of the reasons for which this model needs the implementation of more samples. In fact, in this case the range of true (and predicted) values is from -3.04 to 0.28 %, so it is unbalanced toward the "bad malting quality", instead of being centered on the 0 as it is supposed to be. The second reason is that the number of samples in the calibration set was enough high to ensure the reliability of the model, following the indication of "The American Society for Testing and Materials" [72], which requires a minimum of

6k (k = rank, or number of principal components) spectra in calibration after elimination of outliers. Moreover, there are not reference methods to compare the error of prediction of the model in order to evaluate the predictability of the model. On the other hand, the correlation coefficient is higher than 90, and for this reason it can been said that this model is enough good to discriminate a barley suitable for malting from a barley which is not. Moreover, the number of samples in calibration is enough high to give a realistic idea of the real predictive power of this kind of application.

4.5 Beer quality and monitoring the beer process

Regarding the objective A5 or control by NIR of beer fermentation, it was decided to assess the quality of fermenting wort.

Five samples of beer was produced as explained in the MATERIALS AND METHODS section, and two times for each day of fermentation a sample of fermenting wort was collected in order to perform the reference analysis and to acquire the NIR spectra.

The quality parameters considered are the **alcohol (% v/v) content, the pH, the real (% m/m), the apparent (% m/m) and the original extract (%P).**

The apparent extract (% m/m) of the fermenting wort is calculated from its specific gravity, and it is supposed to decrease during fermentation.

The fermenting wort samples are then distillated in order to monitor the production of alcohol (V/V) during fermentation. The real extract (% m/m) of the fermenting wort is calculated from the specific gravity of the distillation residue. The original extract (% Plato) is calculated from the specific alcohol content and from the real extract.

The pH of fermenting wort at 20°C is estimated using a pH meter and a suitable electrode system this value is supposed to decrease during fermentation.

These parameters were then correlated with the spectra of samples of the fermenting wort acquired at-line in parallel with analytical determinations in diffuse reflectance through quartz cuvette and reflective gold.

Again, these correlations between the analytical and spectroscopic data were generated by PLS algorithm using the software OPUS 6.5. Through the OPTIMIZE tool the best spectral pretreatment and range and the number of principal components most suitable to describe the spectral matrix were chosen. After the identification and the elimination of outliers the calibration models were validated by cross validation, showing a good predictability, with values of R was greater than 0.98, apart from pH which shows a R value of 0.91. the results are shown in **Table 4.5.1**.

For the parameters considered the agreement between the calibration models and the reference methods was not evaluated. In fact, there are not reference methods for the determination of the alcohol content and the real and original extract on wort.

Parameter	N°SAMPLES in calibration (outliers)	Range True (Predicted)	PRETREATMENT	SPECTRAL RANGE (cm^{-1})	PCs	RMSECV	R^2 (R)
Apparent Extract (%)	53 (47)	2.11-13.57 (2.38-13.40)	Straight Line Subtraction	11501.7-7498.1 6101.8-5453.8	5	0.52	97.69 (0.99)
Real Extract (%)	53 (49)	3.91-13.57 (3.94-13.33)	Multiplicative Scattering Correction	11501.7-7498.1 6101.8-5450 4601.4-4246.6	6	0.52	96.61 (0.98)
Original Extract (%P)	53 (48)	6.7-20.2 (6.4-20.8)	First Derivative + Multiplicative Scattering Correction	11501.7-7498.1 6101.8-5450	5	0.6	95.04 (0.97)
Alcohol Content (%V/V)	53 (47)	0-5.05 (0-5.28)	Constant Offset Elimination	6101.8-5450	5	0.18	99.02 (1.0)
pH	53 (48)	4.17-5.36 (4.20-5.20)	Multiplicative Scattering Correction	11501.7-7498.1 5453.8-4246.6	6	0.14	83.41 (0.91)

Table 4.5.1 - *Results of NIR calibrations for the determination of quality parameters of fermenting wort (PCs: Principal Components, RMSECV: Root Mean Square Error of Cross-Validation, R: correlation coefficient).*

Moreover, there are not methods for the determination of apparent extract and pH on fermenting wort. Considering the A-EBC method for the determination of pH in the wort, the reproducibility is 0.12, and so is lower than the RMSECV if the NIR method. This lead to the 35 % of samples falling outside the range defined by the predicted values +/- the R$_{95}$ of the reference method. The plot of predicted versus true values obtained for the pH is shown in Fig. 4.5.1 as example.
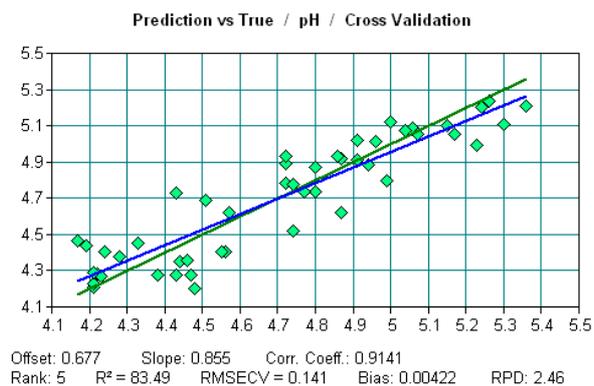
Figure 4.5.1 – Predicted vs true pH of fermenting wort

Concerning the Apparent Extract, the reproducibility of the reference method for wort is 0.08, so is much lower than the one determined by NIR. Anyway, the normal values for apparent extract of the

wort go from 8 to 14 %, and probably the different range of application, including fermenting wort, makes this comparison impossible. The plot of predicted versus true values obtained for the apparent extract % is shown in Fig. 4.5.2 as example.
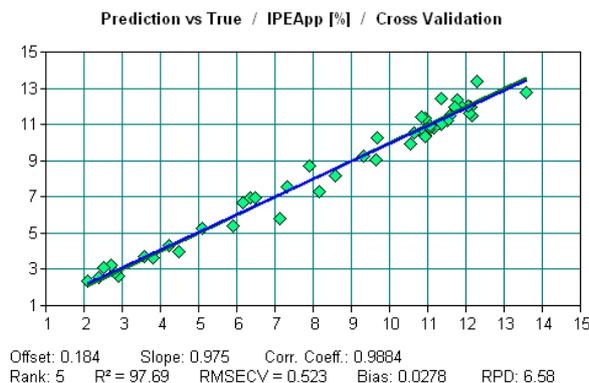
Figure 4.5.2 – Predicted vs true Apparent Extract (%) of fermenting wort

The obtained **beer** samples, together with other commercial samples have been analyzed to assess their quality. Finally, 50 beer samples have been analyzed for the following quality parameters: **alcohol (% v/v) content, real (% m/m), apparent (% m/m) and original extract (%P)**. Also in this case, these parameters were then correlated with the spectra of samples of the beer acquired in diffuse reflectance through quartz cuvette and reflective gold.

Again, these correlations between the analytical and spectroscopic data were generated by PLS algorithm using the software OPUS 6.5 and the OPTIMIZE tool to select the best spectral pretreatment and range. After the selection of the number of principal components most suitable to describe the spectral matrix and the identification and the elimination of outliers, the calibration models were validated by cross validation, showing a good predictability, with values of R was greater than 0.97, as shown in **Table 4.5.2**.

The results are similar to the ones obtained on fermenting wort.

For the parameters considered the agreement between the calibration models and the reference methods was evaluated.

Surprising, looking at the good values of the correlation coefficients, the reproducibility values of the standard methods are significantly lower than the RMSECV values of the NIR methods. This leads to a % of samples between 12 and 35 falling outside the range defined by the predicted values +/- the R_{95} of the reference method, as can be seen at table 4.5.3. The RMSECV value is even much higher than the reproducibility of the NIR method proposed by the Analytica EBC for the determination of the alcohol content in beer, which is 0.09.

Parameter	N°SAMPLES in calibration (outliers)	Range True (Predicted)	PRETREATMENT	SPECTRAL RANGE (cm^{-1})	PCs	RMSECV	R^2 (R)
Apparent Extract (%)	50 (46)	0-6.1 (0-6.3)	Standard Vector Normalization	6101.8-5450	5	0.4	93.76 (0.97)
Real Extract (%)	50 (46)	0.9-9.6 (1.0-9.9)	Multiplicative Scattering Correction	6101.8-5450	5	0.3	96.35 (0.98)
Original Extract (%P)	50 (45)	6.7-20.2 (6.5-20.7)	First Derivative + Multiplicative Scattering Correction	6101.8-5450	3	0.5	97.62 (0.99)
Alcohol Content (%V/V)	50 (46)	0.3-9.4 (0.1-9.6)	First Derivative	6101.8-5450	3	0.4	94.07 (0.97)

Table 4.5.2 - Results of NIR calibrations for the determination of quality parameters of beer ((PCs: Principal Components, RMSECV: Root Mean Square Error of Cross-Validation, R: correlation coefficient).

Parameter	RMSECV	r$_{95}$	sd	R$_{95}$	Ue	% of samples outside the range
Apparent Extract (%)	0.35	0.018	0.006	0.08	0.06	**35**
Real Extract (%)	0.32	0.020	0.007	0.09	0.06	38
Original Extract (%P)	0.48	0.15	0.05	0.38	0.27	21
Alcohol Content (%V/V)	0.40	0.06	0.02	0.18	0.12	32

Table 4.5.3 - Comparison between the reference methods and the NIR calibrations for the determination of quality parameters of beer (RMSECV: Root Mean Error of Cross-Validation, r$_{95}$: repeatability, sd: standard deviation, R$_{95}$: reproducibility, Ue: extended uncertainty).

Anyway, the good values of the correlation coefficients lead to the conclusion that probably the predictive power of the calibration models can not be improved, for example by the introduction of new samples. This means that with these spectra, probably the best correlation with the reference data was found. Probably the instrument's mode of acquisition used in this project (diffuse reflectance) was not the best one for liquid samples.

5. CONCLUSIONS

This PhD thesis research project developed innovative applications of near-infrared spectroscopy in reflectance (NIR) in the production chain of beer.

Concerning **raw materials**, and more precisely **maize**, three calibration models were developed for the determination of **moisture (%) and fat content (both % and %dm)** from both grist and flour spectra, with good predictive performances. The reliability and stability of these models was confirmed by applying both internal and external validations and obtaining comparable results. Moreover, the precision of the calibration models was checked by a statistical comparison with the reference methods. This is the most important part of these results. In fact, NIR calibrations for the determination of moisture and fat content in maize grist is not something new itself, but this comparison with the repeatability values of the A-EBC reference methods make these calibration models specific as analytical method for breweries, suitable for the evaluation of the quality of brewing raw materials (**PAPERS I and II**).

Concerning **malting barley**, two NIR calibration models were developed in order to correlate NIR spectra of whole grains with the parameters of **moisture and total nitrogen content**. The agreement between the model and the reference method was evaluated by verifying that at least 95 % of the true values fall into range defined by the predicted values +/- the R_{95} of the reference method. This demostrated that the estimates produced with the multivariate NIR models agree with those produced by the reference method as well as a second laboratory repeating the reference measurement would agree. This is confirmed also for the NIR methods for the determination of moisture and total nitrogen content proposed by the Analytica EBC. Also in this case, this comparison with the A-EBC reference methods is the most interesting part of the results, because it makes these calibration models specific analytical method for breweries, suitable for the evaluation of the quality of brewing raw materials. In fact, these NIR methods for the determination of moisture and total nitrogen were already available in the Analytica EBC. Even if the calibration models developed in this project show a better predictive power than the A-EBC NIR methods, this application can not be considered something new. It was developed in this project for the sake of completeness.

Concerning the **monitoring of the malting process**, a calibration model was developed to correlate the spectra acquired on germinating barley with their **moisture**. The calibration model, obtained using 228 spectra and as many corresponding relative humidity values, was generated by PLS algorithm and validated by cross validation, showing a good degree of predictability. There is not a

reference method to compare the predictive performance of the calibration model. For this reason, an external validation, treating part of the complete sample set as a test set (33%) was performed, in order to verify if the cross-validation was too optimistic. The values of errors of prediction and coefficient of correlation obtained by test-set validation are similar to the cross-validation's ones (**Table 4.2.1 and Fig. 4.2.3**), and this indicates that the model is stable and enough reliable in its good predictability (**POSTER II**).

This result suggests that it is possible, during the malting process, measure the moisture content on germinating barley using an at-line, accurate and not destructive NIR method.

Furthermore, calibration models were developed to monitor other important parameters to evaluate the performance of malting barley by NIR spectra. These models were developed on the assumption that some important parameters for the assessment of malt quality, which analytically are determined on the dried malt, are "visible" to the NIR already on the **green malt**.

For this reason, NIR spectra of green malt collected during the last day of germination were correlated with some analytical parameters determined on the corresponding malts after drying by PLS algorithm. The calibration models developed showed a good degree of predictability.

For the parameters considered the agreement between the calibration models and the reference methods was evaluated, and the results demonstrated that at least 93% of the true values fall into range defined by the predicted values +/- the R_{95} of the reference methods. So, the agreement with the reference method can be considered good, especially considering that the reference analysis were performed on finished malt, but the NIR spectra were collected on the green-malt before kilning. These results are very encouraging and show **the possibility of using NIR monitoring for the germination process, in order to assess how they are evolving the most important quality parameters of malt.** This possibility would be extremely important for maltsters, which could change the process causing acceleration or deceleration of germination varying the parameters of humidity and temperature.

Concerning **malt**, several calibration models were developed to allow a full assessment of the malt quality by NIR spectroscopy.

First of all, we focused on the determination of the most important parameters for the evaluation of malt quality, the **moisture (%) and total nitrogen (% and %dm) content**. The NIR calibration models developed for the determination of these two parameters show a good predictive power, reliability and stability confirmed by both internal and external validation (**PAPERS I and II**).

Anyway, as explained in **PAPER III**, one single performance criterion of validation often is not sufficient to judge a Near Infrared calibration model in the proper way which can lead to over- or under-estimation of the model quality. In fact, the error of prediction is statistic, which can change

according to the type or number of samples considered in the validation. For this reason, we decided to compare the simple leave-one-sample-out cross-validation (CV) with a more challenging CV with leave-33%-samples-out, where the re-samplings were repeated 200 times. The selection of the samples excluded was random. This kind of validation was performed instead of the classic test-set validation considered in the previous results in order to verify how the RMSECV-values change by the kind of samples used to calculate it. Because the error will change according to chance the two re-samplings were repeated 200 times. This kind of validation is something new and was applied on the parameters Viscosity (cP), FAN (mg/l) and Friability (%). The results show that the errors of prediction obtained using the two types of validation are the same. This means that the cross-validation in these models does not overestimate their predictability, then the models can be considered stable and reliable. Then, a more extreme kind of validation was applied for the parameters Fine Extract (%dm), Fermentability (%), pH and Soluble Nitrogen (%dm), which can be called a "year-according" test-set validation. In fact, for these four calibration models it was possible to check if the predictive performance is stable during the time because the samples used to set up these calibration models have been analyzed form 2006 to 2009. Three test set validations, where the validation samples were chosen according to the year of collection and analysis, were applied, in the order of check if the calibration model has a stable predictive performance on samples collected in different years. Three different data blocks were defined and used for the validation: 2006, 2007 and 2008+2009. Two of these three data blocks were used to develop the calibration and the third one was used as validation-set. This "extrapolation" test should clarify the long-term effects on NIR calibrations and give a good indication of the model performance for coming years. The results demonstrate that one single performance criterion often is not sufficient to judge a Near Infrared calibration model in the proper way which can lead to over- or under-estimation of the model quality. Leave-one-sample-out cross validation is too optimistic, because excluding one sample has a low perturbing effect on the model. The second type of cross-validation examined, the leave-33%-out, gives a more realistic idea of the predictive power of the model, and it is necessary to be conservative and consider at least the higher errors of prediction (mean RMSECV + sd) in the description of the predictability of the models.

Then, using three year-blocks as test-sets in order to obtain an indication of the model performance for coming years, it was evident that the calibration models have lower predictive power than in the cross-validations and do not give a equally good prediction on the three data sets.

About **Fine Extract**, the errors of prediction obtained by test-set validation are appreciably higher than the ones calculated by cross-validations. It is difficult to state how the model will predict the fine extract values of unknown samples analyzed, for example, in 2010, but it is necessary to be

conservative and consider an error of prediction higher than the one calculated by cross-validation and an agreement with the reference method lower than the 95%.

Also about **Fermentability**, the errors of prediction obtained by test-set validation are appreciably higher than the ones calculated by cross-validations, and it can be realistic to consider this predictive power on future unknown samples. However, the agreement with the reference method is good for all the kinds of validation considered. About the **pH** and the **Soluble Nitrogen**, the differences between the errors of prediction obtained by internal or external validation are not significant. This indicates a very good stability and reliability of the models.

Moreover, the estimates produced with these calibration models agree with those produced by the reference methods as well as a second laboratory repeating the reference measurements would agree. The overall conclusion is that we likely need to be modest in reporting the expected prediction error. The kind of validation used is something new and it is extremely useful in order to estimate the real predictive power of the proposed calibration models and to understand the long-term effects. Moreover, the precision of all the calibration models considered was estimated and statistically compared with the reference methods, with good results.

This comparison with the A-EBC reference methods makes these calibration models specific analytical methods for breweries, suitable for the evaluation of the quality of brewing raw materials with the same precision of the reference method.

Concerning the **"malting quality" of barley**, the purpose of this project was to measure, through this rapid, non-destructive and reliable method, a parameter able to indicate whether if barley germination will be rapid and uniform and to know if a certain type of barley is suitable for the production of beer and spirits. For this reason, barley samples were malted in micromalting pilot plant and then analyzed for the same quality parameters. The FT-NIR spectra collected on barley whole grains were correlated to the quality parameters determined on the respective malt samples by PLS regression. The idea was to predict from barley spectra the quality of the malt which is possible to obtain from the same barley samples. The calibration models were supposed to permit a preliminary screening of barleys in order to individuate which are suitable for malting. The results obtained were very encouraging, with correlation coefficient always higher than 0.78. Also in this case, the agreement with the reference methods was assessed. The results can be considered good, because for all the quality parameters apart from the Kolbach index, the estimates produced with the multivariate NIR models agree with those produced by the reference methods as well as a second laboratory repeating the reference measurement would agree. For this reason, the results can be considered very encouraging, even if the number of samples in calibration is not enough to ensure the reliability of the results. However, determining these parameters directly on barley as

147

"potential" of malt features can be difficult, perhaps because they are strictly related to the process. The risk is to develop calibration models suitable for a standardized process, but may not for a different processes. This aspect should be deepened in the future, including in the calibration malt samples obtained with a different program.

However, beyond the determination of the individual parameters, it was defined a combination of them, expressing the "Malting Quality" (MQ) of a barley, namely its suitableness to be malted. This parameter should be able to discriminate a malting barley, namely a barley suitable for use in the malting industry, from a feed barley. MQ is a complex character. In this project, the MQ of a barley has been expressed as the overall quality of the respective malt, calculated following the indication of the "Two years variety summary 2007/2008 - Spring & Winter Barley" of the EBC Barley and Malt Committee [85] for the Quality Index (QI) of malt.

The MQ values of the individual samples are shown as a +/- percentage of that standard value. This percentage will be greater than 0 for malts with a quality index better than the standard and lower than 0 for malts with a quality index worse than the standard.

Also in this case, the quality index of the malts was correlated by PLS algorithm with the NIR spectra of the respective barley samples, by using OPUS program, in order to express the MQ of the barley samples. The results show that this model needs the implementation of more samples. In fact, the barleys considered in this project after malting give malts with an overall quality lower than the 17th EBC Standard Malt, and the range of true (and predicted) values is from -3.04 to 0.28 %, so it is unbalanced toward the "bad malting quality", instead of being centered on the 0 as it is supposed to be. Furthermore, the number of samples in the calibration set was enough high to ensure the reliability of the model, following the indication of "The American Society for Testing and Materials" [72]. Moreover, there are not reference methods to compare the error of prediction of the model in order to evaluate the predictability of the model.

On the other hand, the correlation coefficient is higher than 90, and for this reason it can been assessed that this model is enough good to discriminate a barley suitable for malting from a barley which is not. **This application is new and can be extremely useful for breweries and cereal industries, for the evaluation of the quality of brewing raw materials.**

Concerning **the monitoring of fermentation**, NIR methods were developed for the evaluation of quality parameters in the fermenting wort. The predictive power is good, with correlation coefficients always higher than 0.91, but it is not possible a comparison with the reference methods because they are not available. Concerning the assessment of beer quality by NIR, calibration models have been developed for the evaluation of the main quality parameters. Surprising, even if

good values of the correlation coefficients were obtained, the reproducibility values of the standard methods are significantly lower than the RMSECV values of the NIR methods.

This is true also for the NIR method proposed by the Analytica EBC for the determination of the alcohol content in beer. This probably means that the instrument's mode of acquisition used in this project (diffuse reflectance) was not the best one for liquid samples.

In conclusion, the calibration models developed during this PhD thesis research project allows innovative applications of near-infrared spectroscopy in reflectance (NIR) in beer production chain.

It is possible to check the quality of the raw materials like barley, maize and malt using a rapid, non-destructive and reliable method, with a low error of prediction and with a repeatability comparable with the one of the reference method. Then, these new calibration models allow to monitor the malting process, measuring important quality parameters during germination. Moreover, it is possible to obtain an estimation of the "malting quality" (MQ) of barley, to predict whether if its germination will be rapid and uniform and if a barley is suitable for the production of beer. Finally, the NIR technique can be applied to monitor the brewing process, using correlations between NIR spectra of beer and some important analytical parameters.

These innovative results are now available for the operators involved in the beer production chain.

The suitability of NIR for innovative quality control in specific parameters for the brewing industries confirms what is already studied for the cereal food chain, and open new interesting perspectives for other ones outside of the brewing industries.

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7. LIST OF PUBLICATIONS

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