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Pollen Analysis by FTIR Spectroscopy - A Feasibility Study for an Automated Method

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Preface

This thesis marks the end of a five-year master program in Industrial Economics at the Norwegian University of Life Sciences.

The thesis subject was suggested by Dr. Achim Kohler, and is part of the project of automated pollen monitoring at IMT (Department of Mathematical Sciences and Technology).

First of all, I want to thank my supervisors: Achim Kohler, for giving of his knowledge about spectroscopy, scattering and multivariate analysis, and generally being enthusiastic and including. Cecilia Marie Futsæther for eternal motivation and support, helping me find the literature I needed, and always being available. Tor Kristian Stevik for advice on the economic part.

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Murat Bagcioglu, thank you for being the best lab colleague, standing by my side despite your hand being mutated from pollen allergy. And thank you for your help with The Unscrambler X.

I also want to thank Kine for reading through parts of the thesis and correcting my somewhat broken English. And Anders, for not leaving me although I got married to my thesis for a while.

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Abstract

Pollen forecasting by optical microscopy remains is costly and time-consuming, and suffers from uncertainties connected to the visual inspection performed by the operator. An automated method for pollen analysis has been requested during the past few decades, but no attempt has proven to be sufficiently accurate and practical to implement. Simultaneously, prevalence of allergic rhinitis (hay fever) has increased noticeably the past century, reducing the quality of life for approximately 20 % of the global population. An improved method of pollen analysis therefore has the potential to better enhance the quality of life for pollen allergy sufferers.

This study examines the potential of FT-IR spectroscopy combined with multivariate analysis for a more accurate and detailed pollen count. Five different types of pollen was measured, and Principal Component Analysis (PCA) was applied on the infrared spectra, using the software The Unscrambler X, version 10.3. Instead of the commonly used KBr pellet, the pollen grains were embedded in paraffin between polyethylene foils, both for the practical implication as such foil easily can be used in pollen samplers, and to avoid scattering.

The study offers an analysis of socio-economic consequences of allergies, and identifies the market components, market interest, cost, and price for a pollen analysis service (PollenID) to be based in Ås, Norway.

Concerning the pollen analysis method, PCA plots show clear separation between pollen species, in addition to groupings among pollen of the same species. Due to the paraffin and foil embedding, scattering was almost completely avoided. The method needs further development to be fully automated, but demonstrates remarkable potential.

However, the market research indicates limited interest for pollen analysis, possibly because this service is not available today, and the target groups might not see the need for a customized pollen forecast. The estimated operation cost was estimated to 1.9 million NOK per year, and the price suggested was 200 NOK per sample, to make the analysis more available to the public.

Sammendrag

Pollenvarsling basert på optisk mikroskopi er en kostbar og tidkrevende metode, og medfører usikkerheter knyttet til menneskelige feil. En automatisert metode for pollenanalyse har vært etterspurt de siste tiår, men ingen forsøk har vist tilstrekkelig nøyaktighet og praktisk tilpasning. Samtidig har forekomsten av allergisk rhinit (høysnue) økt betraktelig det siste århundret, noe som medfører redusert livskvalitet for omtrent 20% av verdens befolkning. En bedre metode for pollenanalyse kan derfor bedre velferden for mange pollenallergikere.

I denne oppgaven utforskes mulighetene ved å bruke FTIR-spektroskopi kombinert med multivariabel analyse for å gi en mer nøyaktig og detaljert pollenanalyse. Fem ulike pollentyper ble målt, og Principal Component Analysis (PCA) ble benyttet på IR-spektraene, ved bruk av programvaren The Unscrambler X, versjon 10.3. I stedet for KBr-pellets, som vanligvis anvendes, ble pollenet lagt i parafin mellom plastfolie, dette øker den praktiske nytten, da denne type folie brukes i pollenfeller, og lysspredning kan også unngås på denne måten

I tillegg viser denne studien en samfunnsøkonomisk analyse av konsekvensene av allergi, og forsøker å kartlegge markedsaktører, og kostnad- og prisestimat for en pollenanalysetjeneste (PollenID), som er tenkt å være stasjonert i Ås, Norge.

Når det gjelder pollenanalysemetoden, viser PCA-plottene klare skiller mellom pollenarter, i tillegg til grupperinger av samme pollenart. Grunnet parafin- og folie innpakningen, ble lysspredning nesten fullstendig unngått. Metoden må videreutvikles for å være hel-automatisk, men viser et godt potensial.

Imidlertid indikerer markedsundersøkelsen at det er begrenset interesse for pollenanalyse, kanskje på grunn av at denne tjenesten ikke er tilgjengelig i dag, og målgruppene ikke ser behovet for et tilpasset pollenvarsel. Driftskostnadene ble anslått til 1,9 millioner kroner per år, og prisen foreslått var på 200 kroner per pollenprøve, for å gjøre mer tilgjengelig analysen for alle.

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Acronyms and abbreviations

NAAF – Norges Astma og Allergi Forbund (The Norwegian Asthma and Allergy Association)

Helserådet – the Norwegian Health Council

FTIR – Fourier Transform Infrared

PCA – Principal Cluster Analysis

IR – infrared

EMSC – Extended Multiplicative Signal Correlation

HCA – Hierarchical Cluster Analysis

SGA – Second Generation Antihistamine

FGA – First Generation Antihistamine

1. Introduction

The prevalence of allergy and asthma has increased considerably during the past century [1, 2]. Today, approximately 35 % of the global population suffers from allergic diseases [1]. The reason for the increase in allergy cases is not yet understood, and hypotheses vary greatly. Regardless of the cause, allergies are disruptive to the people who suffer from them, their families, and society as a whole. Seasonal allergic rhinitis, commonly known as hay fever, is one of the most common allergic diseases, affecting approximately 20 % of the population [3]. During the pollination season, those affected by allergies experience symptoms such as nasal congestion, ear inflammation (otitis media with effusion), sinus infection (sinusitis), coughing, sneezing, itchy, watery eyes and more. These symptoms can seriously impair labor and recreational activities, and result in sleeping disorders, causing fatigue, learning impairment and irritation. [4, 5] In addition to the patients' reduced quality of life, society's expenses related to pollen allergies are extensive. In Norway, the annually sum of direct and indirect costs amounts to 10 billion NOK [2], and nearly 9,5 billion USD (approximately 70 billion NOK[1]) in the United States [5].

The first advice for allergy patients is usually allergen avoidance [1, 2]. In the case of pollen, that is impossible most of the time. Medication is thus necessary. Most commonly used is antihistamine based medicine [1]. The medication should be started before the pollen season to give maximum effect during the season. Likewise, the effect of the strongest medication, corticosteroids injections, only last for 2-3 weeks, making it is necessary to receive the injection at the appropriate time. Pollen forecasting is essential for both medication and allergen avoidance. Some people might be able to plan their vacation and leave during for the pollen season, to a region with lower concentrations of the pollen to which they react.

Palynology, the study of pollen and spores, has been done the same way for almost a century providing data in medical, environmental and evolutionary studies [6, 7]. In aeroallergen studies, pollen is sampled at different sites in the country and sent for analysis by optical microscopy. This method requires qualified personnel with extensive knowledge about pollen morphology, it is time-consuming and potentially subjective. The needs for automation in the field of pollen monitoring was first addressed by Stilmann & Flenley in 1996 [8]. They requested a system that is able to cover more sites and larger counts, generate data quicker, and determine pollen types in more detail, preferable at species level, at a lower cost. Since then, some attempts of automated palynology were made, summarized by Holt & Bennett in 2014 [9]. Most of these attempts relied on image processing, that is, the same recognition principle as used by human palynologists. However, identification based on morphologic properties has its limitations: a pollen grain might be deformed, dried, or damaged,

[1] According to the currency calculation by google.com (14.12.14)

causing it to be rejected by the system as non-pollen. In addition, such a system would need a database for comparison, which would be based on human recognition. Therefore, the system might contain errors, as it can never be better than the human eye.

Another factor that can be used to distinguish between pollen types is their biochemical composition. It might be a more convenient method of identification, as the chemical composition does not change much between pollen grains of the same species. Fourier transform infrared (FTIR) spectroscopy is a scientific method used to gather information about the chemical composition of different substances [10, 11]. The method is based on a FTIR spectrometer which provides an infrared spectrum of a substance. A spectrum is a result of different vibrational modes within the molecule caused by infrared radiation. As every molecule vibrates slightly differently, the IR-spectrum of a given molecule can be used to identify that molecule. In an analogous way, the method has proven successful for identifying different pollen types in various studies [6, 12, 13]. However, only Dell'Anna et al. (2009) studied an automated system, using hierarchical cluster analysis (HCA) as classifier. The measurement accuracy was 84 % [13]. There is reason to believe that the accuracy could be improved by minimizing the problem of *scattering*, which is apparent in Dell'Annas spectra. Scattering of light causes false absorbance signals in the pollen spectrum, and thus aggravates its interpretation [14]. However, this problem might be eliminated by the right sample preparation.

This study examines the possibilities of an automated system for pollen analysis, based on FTIR spectroscopy. Both the technical feasibility and economic aspects of a pollen monitoring company in Ås, with the working title PollenID have been researched. The ability of separating pollen taxa using FTIR spectroscopy and principal component analysis (PCA) as classifier was investigated. For the proof of concept study of the alternative (FTIR-based) method, five different pollen types were measured. The measurements were performed on single grains, as this is closer to the real-life situation than measurement on multigrain. The conventional method of pollen analysis has been based on the contemporary pollen forecast in Norway, which is the standard method in the world [15-17]. The objective was to provide an overview of the conventional methodology and explore alternative methodology, while further development and the associated risks were not within the scope of the thesis.

In addition, economic research was conducted to explore both the socio-economic effects of allergies, and the pollen monitoring industry. In addition, annual operation costs for PollenID and price estimates were carried out. Market potential should be established, as even a perfect method is worthless if there is no interest. In the end, the vision is to reach more allergy sufferers with a more accurate and detailed pollen forecast. To achieve this, local health information distributors need to see the benefit of customized, local pollen forecasts. Municipalities, hospitals, and sports arenas are

therefore defined as the target group for this technology. Most of the market research in the paper at hand is based on Norway, and the immediate surroundings of Ås, where PollenID is thought to be established. The objective of the research was to provide an overview of the market, therefore each part is not explored in depth.

1.1 Outline of the thesis

The thesis is divided into four parts: 1. Background and theory, 2. Methodology, 3. Results, and 4. Discussion and conclusion.

Part one consists of three chapters. Chapter 2 provides information on pollen, allergy and pollen forecasting, chapter 3 explains the physics behind FTIR spectroscopy and scattering, and in chapter 4 the economic theory is described.

In part two, chapter 5, the methodology is considered.

Part three evaluates the results, which are divided into two parts; the technical aspects of the pollen analysis method, assessed in chapter 6, and the economic aspects, presented in chapter 7.

Part three entails the discussion of the findings, chapter 8, the conclusion in chapter 9, and some notes on further work, chapter 10.

Part I: Background and theory

2. Pollen and allergy

2.1 What is pollen?

The word "pollen" is Latin, and means "fine flour, dust". The size of the pollen grain varies from 5 to 250 µm in diameter, but usually is within 15-100 µm range [18]. Pollen grain is a male gamete of seed plants (spermatophytes). The main function of pollen is pollination: transport of a grain from male part of a plant to a female part, thereby enabling fertilization and reproduction. The transport is achieved either by the help of animals (usually insects), or by wind and water. To the naked eye, pollen grains appear as fine powder, but they can have different shapes, sizes, and wall patterns, which can be observed through a microscope [7].

Pollen grain consists of three parts; the grain wall, the vegetative cell and the generative cell (Figure 1). The two cells are located in the cytoplasmic core. During fertilization the generative cell becomes two sperm cells, while the vegetative cell develops pollen tube. Therefore, the vegetative cell is sometimes called the tube cell. During fertilization, the pollen tube emerges from one of the apertures in the grain wall, and provides transportation for the sperm cells [7].

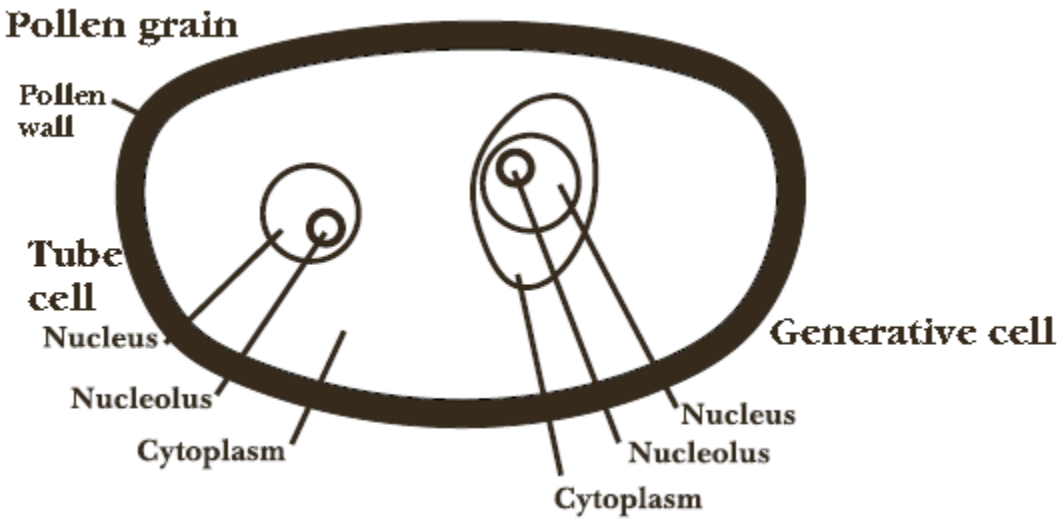


Figure 1: The structure of a pollen grain. Illustration from [7]

The pollen wall has two main layers: exine, the outer layer, and intine, the inner layer. The intine is mainly composed of cellulose and pectin. The exine consists mainly of *sporopollenins* [18]. Sporopollenin is a complex chemical substance that resembles polyethene, resistant to many physical

and chemical forces, and also to decay [7]. The durability of the pollen walls, and the preservation of the pollen grain itself, depends strongly on the presence of sporopollenin [7].
Figure 2 shows a flower's reproductive cycle and the origin of pollen. The pollen is produced in the *anthers* of the flower, by pollen mother cells. The anthers are located in the male part of the flower, in structures known as *stamen*, the male reproductive organ in plants [7]. At particular times, the pollen production is enormous: approximately 5.5 million pollen grains per catkin on birch, 4.5 million per catkin on alder, and 4 million per hazel catkin [2].

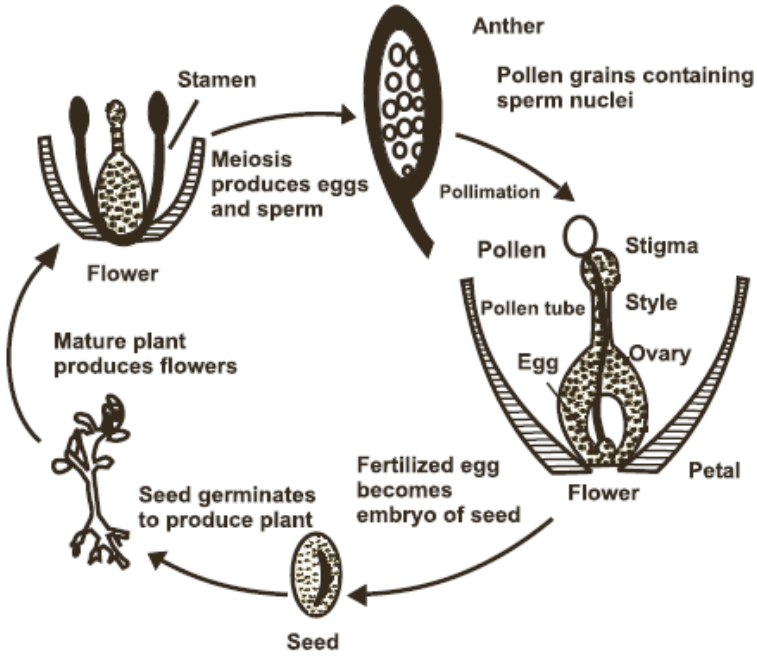


Figure 2: The life cycle of plants. Illustration from [7]

2.2 Why does pollen trigger allergic reactions?

It appears that proteins located on the exine are the allergenic triggers in pollen [2]. These proteins drop off the grain in case of contact with water, releasing a recognition mechanism to assure that the pollen has landed on a flower of the same species. What exactly in these proteins provokes the allergic reactions is not certain, but so far, evidence suggests that it could be the chemical bonds to carbohydrate compounds.

According to NAAF, there are three factors that determine whether a pollen type is of considerable allergenic importance [2]:

1. Pollen contains hay-fever allergens (allergy provoking proteins)
2. Pollen is adapted for wind pollination
3. The pollinating plant is widespread in the region

Among others, this excludes big and heavy pollen types, such as maize (*Zea mays*), even though it carries allergy triggering proteins. Although pine (*Pinus*) and spruce (*Picea*) pollen is spread by the wind in large amounts, they are also excluded, since the proteins on the pollen grains do not provoke allergic reactions. The reason some pollen proteins do trigger reactions and some do not, is unknown. In Norway, there are only six pollen types that meet all the conditions: alder (*Alnus*), hazel (*Corylus*), willow (*Salix*), birch (*Betula*), grass (*Poaceae*) and mugwort (*Artemisia*) [3].

Figure 3 shows the morphology of various common plants.

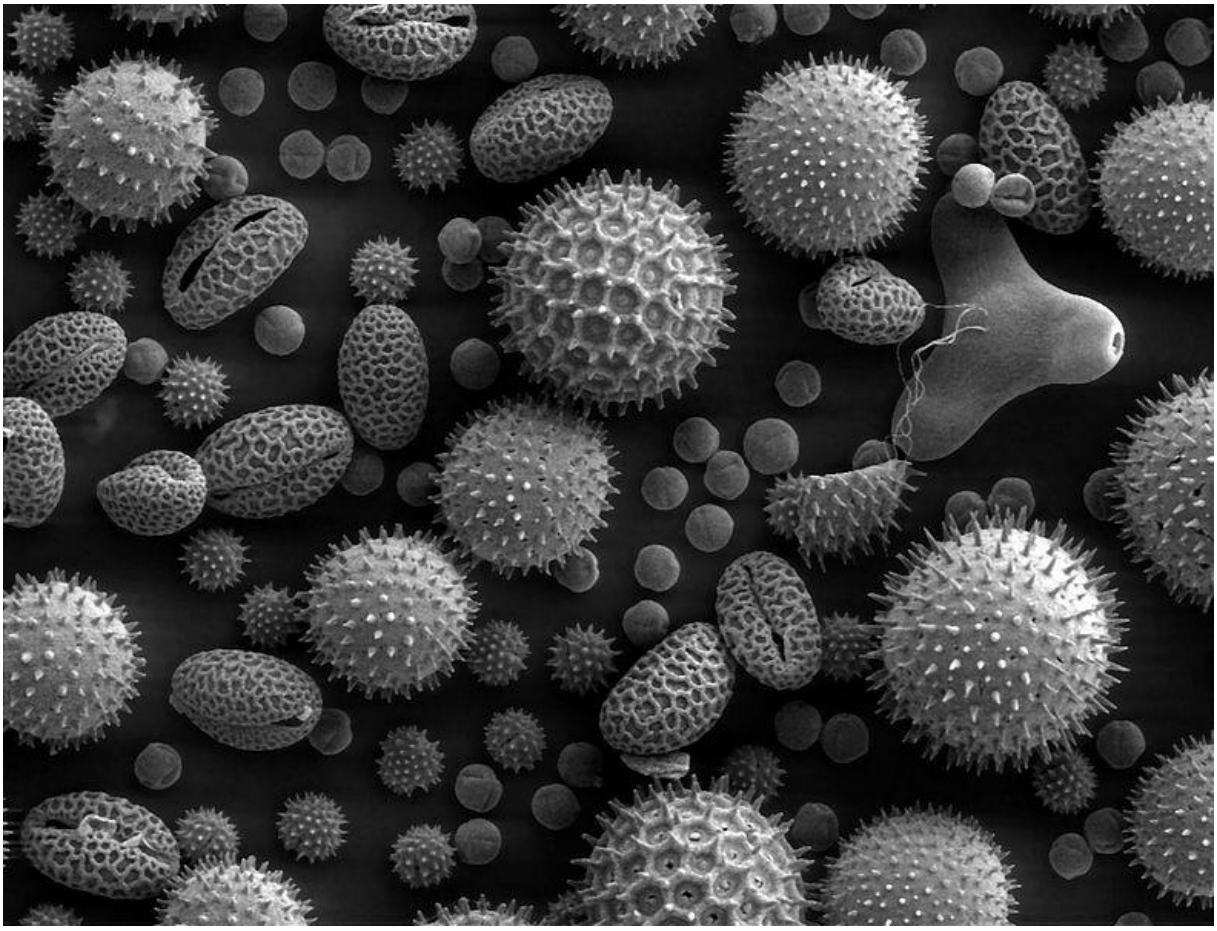


Figure 3: Picture of pollen grains from a variety of common plants, taken with a scanning electron microscope at Dartmouth College. Illustration from [19]

2.3 Definition of allergy and allergic rhinitis

The term allergy refers to the harmful effects of the immune system to otherwise harmless substances, called *allergens*. It is often used synonymously with hypersensitivity: an adverse response to an antigen or allergen [1]. In an allergic reaction, the body reacts to allergens with high production of

immunoglobulins (IgE), which will bind to the respiratory mucosa, the blood and the skin. This leads to an increased level of histamine, which gives the familiar symptoms; runny nose, itching, coughing, and difficulties with breathing. When the body has reacted to allergens once, it will remember and react equally next time the allergenic substance is encountered. There are numerous types of allergens, e.g. food, mites, animal fur and dandruff, and insect bites [1]. In this study, only allergy triggered by pollen – allergic rhinitis – has been considered.

The term allergic rhinitis literally means "inflammation of the nose", and it is recognized by rhinorrhea, sneezing, and nose congestion [1]. Seasonal allergic rhinitis (hay fever) is caused by the seasonal appearance of allergenic particles in the air, such as pollen. If left untreated, hay fever may lead to asthma.

2.4 Prevalence of allergy in Europe today

Approximately 35 % of the population suffer from allergic diseases [1]. During the last century, cases of seasonal allergic rhinitis (hay fever) in Europe have increased by 10-20 %. According to the European White Paper (1997), today's prevalence is about 10 %, with slight variations between countries. For children it is somewhat higher, about 15 %. This seem to have changed since 1997, and NAAF (the Norwegian Asthma and Allergy Association) operates with 20 % prevalence [3]. In addition, it seems that many individuals are undiagnosed, or do not seek medical help for the symptoms [20]. The cause of the inflation in allergic patients is widely discussed, and a number of hypotheses are considered. The European White Paper suggests that there are three main factors:

- **Outdoor pollution.** The prevalence of air pollutants, such as sulphur oxides (SO_2), particulate matter, NO_3 and O_3 has increased the past century, in consistency with the increase in industry and traffic. In addition to allergy to these substances themselves, exposure to pollutants seem to favor IgE sensitization and development of allergic rhinitis [1]. A study from Japan shows considerably higher prevalence of hay fever in residents alongside intercity main roads than in those living further away, despite equal levels of cedar pollen [1].
- **Indoor pollution.** The level of some allergens encountered indoors has increased the past 60 years: house-dust mites, pets, tobacco smoke, and humidity and mold. House-dust mites are the most common allergen, and the level has increased with changes in housing habits, such as wall-to-wall carpeting, heating and ventilation.
- **Changes in life-style and hygienic conditions.** The change in nutritional intake, especially considering processed food, might have some influence on the increased level of allergic cases. Another hypothesis suggest that a more hygienic environment may actually increase the

chances of developing allergy. Children are not as exposed to bacteria as before and therefore they might not develop the natural responses to allergens.

In the years after European White Paper was written, a new hypothesis has emerged:

- **Climate changes.** Climate change is likely to result in substantial weather variations, such as temperature, humidity and precipitation, thus shifting the production and distribution of allergenic pollen. It is expected that shifts in aeroallergen human exposures due to global climate change might also increase the severity of allergies. According to Shea et al. (2008), the concentration of hazel, birch and grass has increased the past 30 years [21].

2.5 Allergy treatment

According to the European White Paper, there are three main principles for allergy treatment [1]:

- Allergen avoidance
- Pharmacological treatment
- Immunotherapy

This corresponds to what NAAF recommends [3]. However, pollen is difficult to avoid, and since having annual vacations corresponding to the pollen season seems impossible, medication is usually necessary. According to Helserådet (the Norwegian Health Council) there are three steps of pharmacological treatment of allergic rhinitis [2]:

- Prevent the symptoms from occurring: preventive treatment
- Relieve the symptoms when they occur: symptomatic treatment
- Cure the allergy by "vaccination": immunotherapy

For preventive treatment, it is sufficient to start the treatment a few days before the expected pollination season [2, 3]. Patients with allergy to grass pollen can start the medication later than patients allergic to other types of pollen. Most people with allergic rhinitis benefit from taking *antihistamines*. There are several medicines on the market, available with and without prescription. If the antihistamine treatment is insufficient, it can be combined with other medicines, to relieve the allergy symptoms. Examples of such medicines are nasal cortisol spray and eye drops. If the combination of medication is not enough, treatment by corticosteroids is advisable, either ingested or as depot preparation intramuscularly (injections). The corticosteroids injections cannot be given too early in the season, as the effect only last for two to three weeks. It is only used if the effects of other

medications are absent, to limit the use of cortisone. If the patient is heavily troubled, treatment by antihistamines and symptom relieving medications is continued after the corticosteroid injections.

If the allergy symptoms are lighter, daily treatment is unnecessary. Symptomatic treatment is given as required, when the symptoms are bothersome, or larger amounts of pollen are expected to be encountered (in accordance to the pollen forecast, or before a countryside trip) [2]. The medicines are the same as for preventive treatment, primarily antihistamines.

Immunotherapy is a treatment method where the patient is given a small dose of the allergen(s) responsible for the allergic reaction in the form of tablets (only for grass pollen) or injections. The treatment period is three to five years [1]. The tablets should be taken every day, and the injections are given once a week the first eight weeks, and then every second month for three to five years. The aim is to change the body's reaction to allergens [22]. According to Helserådet, many patients are cured by immunotherapy, and for many others the symptoms are reduced, and symptomatic treatment is enough after immunotherapy. The report does not state what is meant by "many". In addition, "a few" patients do not experience any effects from the treatment. The European White Paper cites that "immunotherapy is effective in some patients with allergic rhinitis". Furthermore, there is a risk associated with the treatment, if not treated with the right allergens and in appropriate doses and durations. Severe adverse effects have been reported, including a few deaths [1]. However, the treatment is under development, and improved versions are expected within few years [2, 3].

In Norway, it is also common to consult alternative medicine, such as acupuncture or homeopaths, for advice and treatment of allergic rhinitis. As these methods have no documented scientific effects, they will not be discussed further in this thesis.

Still, the most critical factor of allergy treatment is diagnosis. A British study states that only 62 % of adults with nasal symptoms had ever consulted a doctor [1]. It is also common among patients with allergic rhinitis to self medicate without prescription [5].

2.6 Pollen forecasting

The pollen forecast is made by counting pollen grains under an optical microscope [7, 17, 18, 23, 24]. A Burkard or whirling arms trap is commonly used for pollen sampling outside [17]. The pollen sample is then brought to a scientific institution, where the pollen identification and count is performed by scientific researchers [3, 15, 23, 24]. The researchers usually hold a higher degree in biology (or something of the sort), and will herby be referred to as *palynologists.* Not every pollen

grain is counted, only a known proportion of the sampling slide area, then the count is multiplied by a correction factor [17]. The pollen concentration is usually reported as number of pollen grains per cubic meter of air [17]. Figure 4 shows the pollen calendar for Norway, which is a result of years of pollen counting.

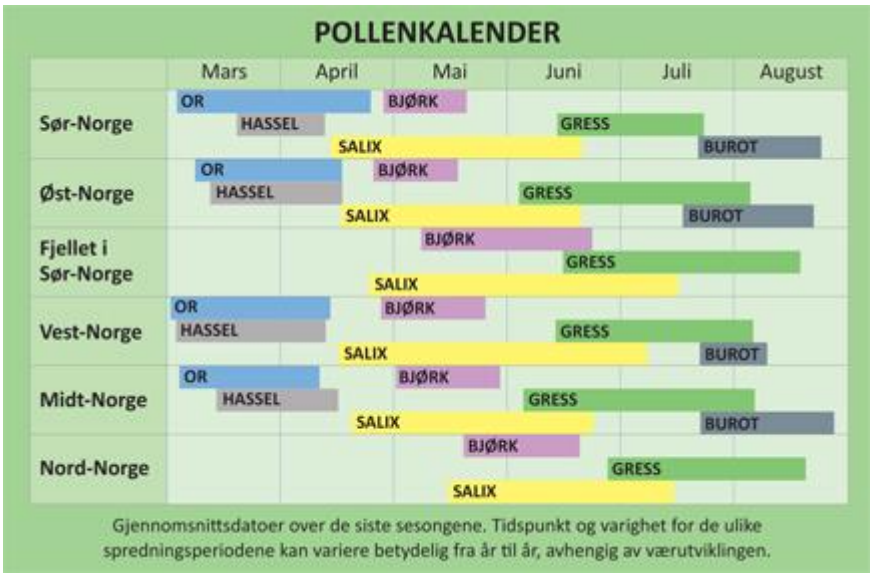


Figure 4: Pollen calendar for Norway. It describes when and where the pollen concentration of different species is expected to be high throughout the year. The six most common pollen types are displayed: alder (or), hazel (hassel), willow (salix), birch (bjørk), grasses (gress) and mugwort (burot). Illustration from [16].

2.7 Criteria for automated pollen analysis

In 1996, Stillman & Flenley addressed the need for automation in palynology [8]. The areas requested in the paper are as follows: more sites, fine resolution, larger counts, objectivity, speed, and finer determination [8]. In other words, Stillman & Fenley requested a larger volume of pollen counting, that is, more sites sampled and more pollen grains counted per sample [9]. They requested less time spent on each sample, reducing labor costs and generate data sooner and more efficiently. They addressed the needs of reducing analyst biasing, and improving consistency across all levels of pollen analysis [9]. Finally, they requested finer determination levels of pollen, preferably at species level, a job that is too laborious by manual palynology, but would give far more valuable information. They also stated one quantitative criteria, the automated system should be able to identify at least 40 different taxa to "be useful at all" [8]. In 1996, the only attempt of an automated palynology system managed to classify six taxa at 90 % accuracy [8].

Almost two decades later (2014), Holt & Bennett summarized the development of automated palynology so far, based on Stillman & Fernleys requests [9]. Holt & Bennett also extend the criteria and concretized them. According to Holt & Bennett, the automated system should be able to operate

24/7. It should, optimally, provide faster pollen counts than human palynologists, but even at the same speed it would provide benefits. The system should require little modification of existing sample collection and preparation techniques, and impart no new limitations. For instance, if the method required that no nonpollen particles were present in the sample for the count to be accurate, the time saved in counting would be absorbed in cleaning the samples. Holt & Bennett also establish an accuracy aim of 95 %.

Of all the post-1996 attempts published on an automated palynology system, more than 50 % include more than six taxa [9]. However, only seven attempts include classification of 10 taxa or more. 25 attempts are mentioned by Holt & Bennett, and it is therefore assumed that these are the most successful ones. The classification accuracy ranges from 60 – 100 %. The highest success rates are achieved by a combination of image processing and a learning-based classifier. Ronnenberger et al. (2002) obtain the most impressive results: 26 taxa identified at 92 % accuracy, by image processing (FL) (based on morphologic features). Ticay-Rivas et al. (2011) identified 17 pollen types, at 94 %, also by image processing. Only one attempt with FTIR was carried out: Dell'Anna et al (2009) classified 11 pollen types, at 84 % accuracy. Hierarchal cluster analysis (HCA) was used as classifier for Dell'Annas measurements.

3. FTIR spectroscopy

3.1 Infrared radiation (IR)

Infrared radiation has wavelengths between 780 nm and 100 μm, and lies between visible light and microwaves in the electromagnetic spectrum [25]. Heat radiation is in the infrared region. The electromagnetic waves are defined by wavelength, λ, and frequency, v. The wavelength is inversely proportional to the frequency:

$$v = c/\lambda \qquad (1)$$

Where c is the speed of light and λ is the wavelenght

The energy, however, is directly related to the frequency:

$$E = hv \qquad (2)$$

Where h is Planck's constant and v is the frequency

In the infrared region, a unit called wavenumber, denoted $\bar{v}$, is generally used instead of wavelength [11]. Wavenumber is the number of waves per unit length, usually given in reciprocal centimeters. An increase in wavenumber corresponds to an increase in energy. The unit is calculated from the inverse of the wavelength, and is expressed in cm^{-1}: $\bar{v} = \frac{1}{\lambda}$

In wavenumbers, the infrared region ranges from 12 820 cm^{-1} to 33 cm^{-1}. The region is divided into three subregions: near, mid and far infrared. The mid infrared region is presented in this study ranges from 4000 cm^{-1} to 400 cm^{-1}.

3.2 Absorption

Absorption occurs when a photon collides with an atom, and its energy is equal to the energy gap between two energy levels. The atom then absorbs the photon, and we say that the atom is excited [25]. The different radiation types in the electromagnetic spectrum give different types of energy transitions in the atom [11]. Absorption of x-rays can be bond breaking, while absorption of ultraviolet and visible light excites an electron to the next energy state. Infrared photons cause vibrations in the atom. Only selected frequencies of infrared radiation are absorbed [11], the ones that exactly matches that of the bond motion. However, not all bonds are capable of absorbing infrared radiation, only those with a dipole moment that changes as a function of time [11]. Symmetric bonds are inactive in IR, as

the vibration caused produces no change in the dipole moment. Not every molecule is IR-active, as a vibration must cause a change in dipole moment for the molecule to be active in IR.

3.3 Vibrational modes

The absorption of infrared radiation gives energy changes of 8 to 40 kJ/mol, which corresponds to the stretching and bending frequencies in covalent molecules [11]. The frequencies of the absorbed radiation match the natural vibrational frequencies of the molecule, and increase the amplitude of the vibrational motions [11]. The modes of stretching and bending are shown in Figure 5.

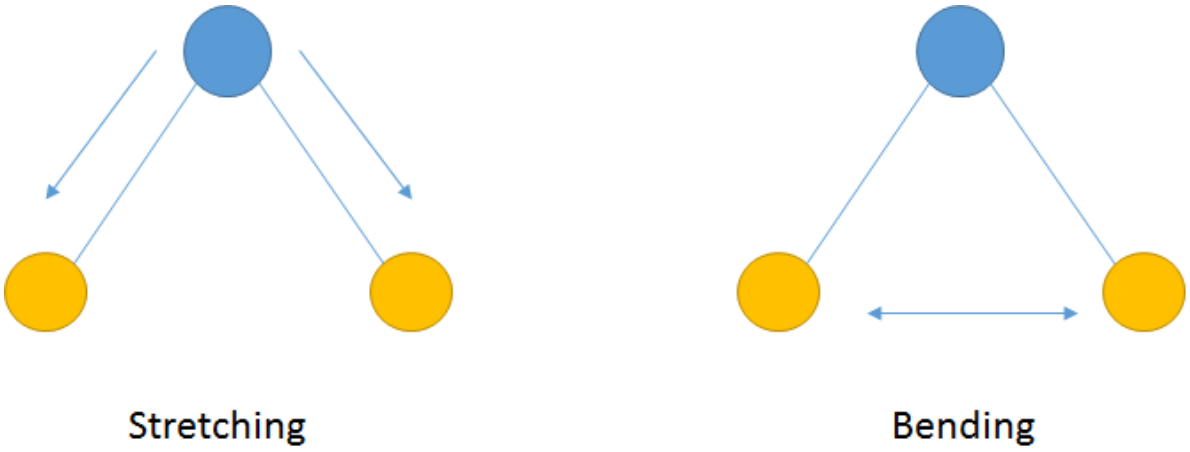


Figure 5: The modes of stretching and bending. Own illustration, after [11]

The mode of stretching comes in two variations: symmetric and antisymmetric. The symmetric stretch is shown in Figure 5, in the antisymmetric stretch the arrows would point in opposite directions. The symmetric and asymmetric stretch corresponds to vibrations at different wavenumbers. For example, CH2 gives rise to a symmetric stretching vibration at 2853 cm^{-1}, and an antisymmetric stretch at 2926 cm^{-1} [11].

Stretching and bending are the simplest modes, but there are also more complex modes. Scissoring, rocking, wagging and twisting are variations of the bending mode [11]. All the modes mentioned are *fundamental absorptions*, i.e. caused by excitations from the ground state to the lowest energy state. The spectra are often complicated by the presence of overtones, combination bands and difference bands [11].

The vibrational modes can be calculated considering the atoms' degrees of freedom. A molecule with *n* atoms has *3n* degrees of freedom, three of which correspond to the rotational motion of the atom,

and three translational. Therefore, the number of ways a nonlinear molecule can vibrate is *3n – 6* [10]. A linear molecule, on the other hand, has *3n – 5* degrees of freedom.

3.4 Implications of the molecular vibration modes

As every molecule has vibration modes that are slightly different, the IR-spectrum of a given molecule can be used to identify that molecule [10]. As a general approximation, only a few atoms in the molecule vibrate for a given frequency, and the frequency is characteristic of the specific functional group (in which the motion is centered), which appear along the spectrum as peaks [10]. Thus, it is possible to identify different chemical functional groups in the molecule. The wavenumbers of the functional group-region range from 4000 to 1500 cm^{-1}, and the bands within this region are called *characteristic group frequencies* [10]. Another part of the spectrum, consisting of vibrations below 1500 cm^{-1}, is called the fingerprint region, as these vibrations are used to distinguish one molecule from another. For bands in this region, we find significant motion in a few atoms and their frequencies varies from one molecule to another. Below 1000 cm^{-1} we find the skeletal modes, which involve significant displacement of many of the atoms in the molecule [10]. This region is not commonly used to identify the chemical composition of the molecule, but might be useful for differentiating between similar molecules.

Table 1 illustrates how the vibrational bonds are spread out over the infrared region. Only the stretching vibrations are included for clarity.

Table 1: Common absorption bands in approximate regions. From [11].

Frequency (cm^{-1})

4000	2500	2000	1800	1650	1550 650
O – H C – H	C≡C	Very few	C=O	C=N	C – C1
	C≡N	bonds			C – O
				C=C	C – N
N – H	X=C=Y				C – C
				N=O	N=O

3.5 Absorbance and transmittance

Absorbance refers to a substance's capacity of absorbing radiation, while the *transmittance* is the fraction of incident radiation that passes through the sample. The concepts are related by the equation

$$A = log_{10}\left(\frac{1}{T}\right) \qquad (3)$$

Where A is the absorbance, T is transmittance and log_{10} is the base 10 logarithm.

Moreover, the transmittance of any sample equals the radiation from the sample, I, over the radiation at the front of the sample, I_0 [10]:

$$T = \frac{I}{I_0} \qquad (4)$$

Beer-Lambert Law, the fundamental law of quantitative absorption spectroscopy [10], states that the absorbance of a sample of thickness l (cm) is given as follows:

$$A = log_{10}\left(\frac{I_0}{I}\right) = \varepsilon l c \qquad (5)$$

Where I_0 is the incident radiation, I is the radiation leaving the sample, ε is the molar absorptivity, l is the sample thickness and c is molar concentration.

The absorbance of any component is proportional to its concentration in the sample [10]. However, it is usually the transmittance that is measured, as there is no technology for measuring the absorbance directly.

3.6 The FTIR spectrometer

In Fourier Transform Infrared (FTIR) spectrometers, the transmittance spectra of different substances are measured and converted into absorbance spectra by Fourier transformation [11]. Transmission in the spectrometer is illustrated in Figure 6. The infrared beam passes through the sample and is detected by a detector which is located behind the sample [26].

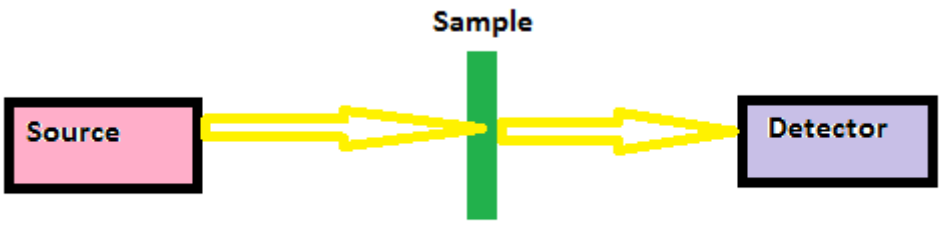


Figure 6: Transmission in the spectrometer. Own illustration, after [26]

The key component of the FTIR spectrometer is the two-beamed *interferometer* [10], which is shown in Figure 7. It modulates the pattern called the *interferogram*, which is the radiation of each wavelength in the infrared spectrum at a different frequency [10]. It is essentially an intensity versus time-plot [11]. The spectrometer acquires the interferogram in less than a second, and is therefore

capable of collecting several interferograms and accumulate them. This will result in spectra with better signal-to-noise ratio after the Fourier transform is performed.

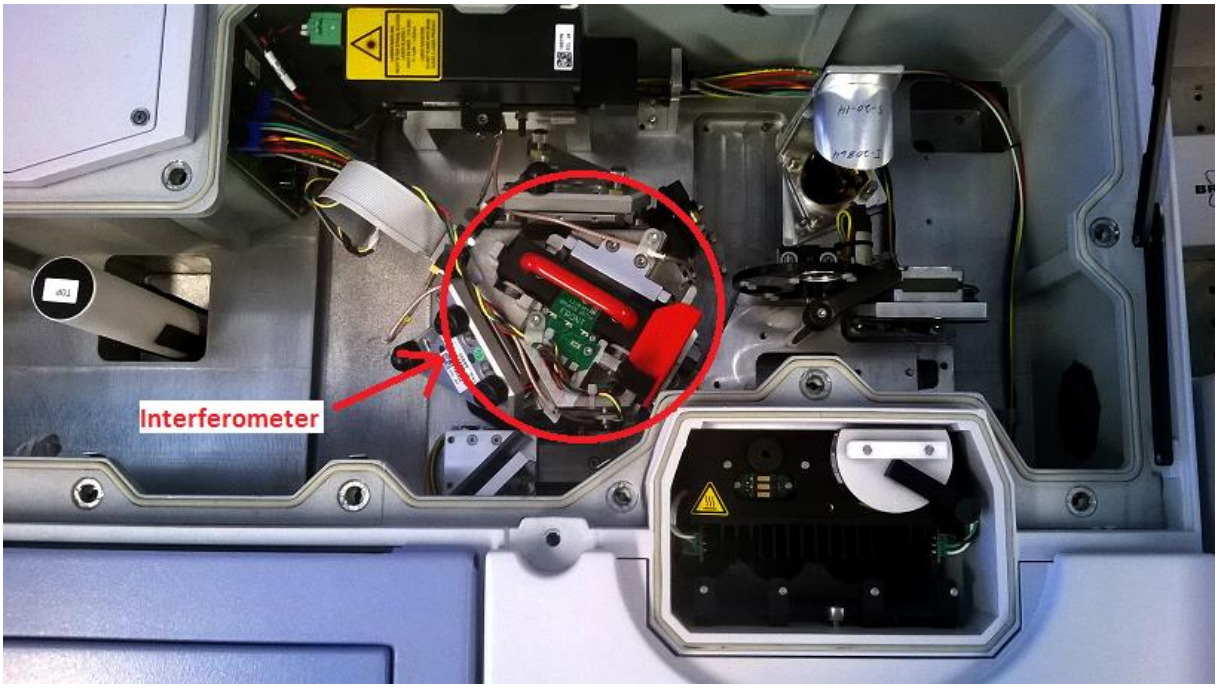


Figure 7: The interferometer inside the FTIR spectrometer.

3.7 Scattering

When light interacts with biological material, it can be absorbed or scattered. Since the wavelength of the infrared light is in the same order of magnitude as the size of pollen, scattering phenomena are strong in the infrared spectroscopy of pollen [14, 27]. Since scattering signatures in the infrared spectra of pollen overlap strongly with absorption bands, the interpretation and analysis of spectral data becomes more complicated.

Scattering of light happens all around us. It makes the sky blue and the clouds white. Scattering is often accompanied by absorption [28]. The leaves on the tree appear green because the red light is absorbed by the atoms in the leaf, and the green light is scattered. Both scattering and absorption remove energy from the light beam: we say that the beam is attenuated [28]. The amount and distribution of the light scattered by a particle strongly depends on the characteristics of the particle, such as its shape, size and chemical composition [29].

The scattering and absorption of infrared light at a pollen sample measured in a FTIR spectrometer is illustrated in Figure 8. The incident infrared light, I_0, impinges on a pollen particle. The infrared light

is then either scattered, shown by the blue arrows, absorbed, as illustrated by the red area, or transmitted, as indicated by the green arrows. The transmitted light, *I*, is measured by a detector.

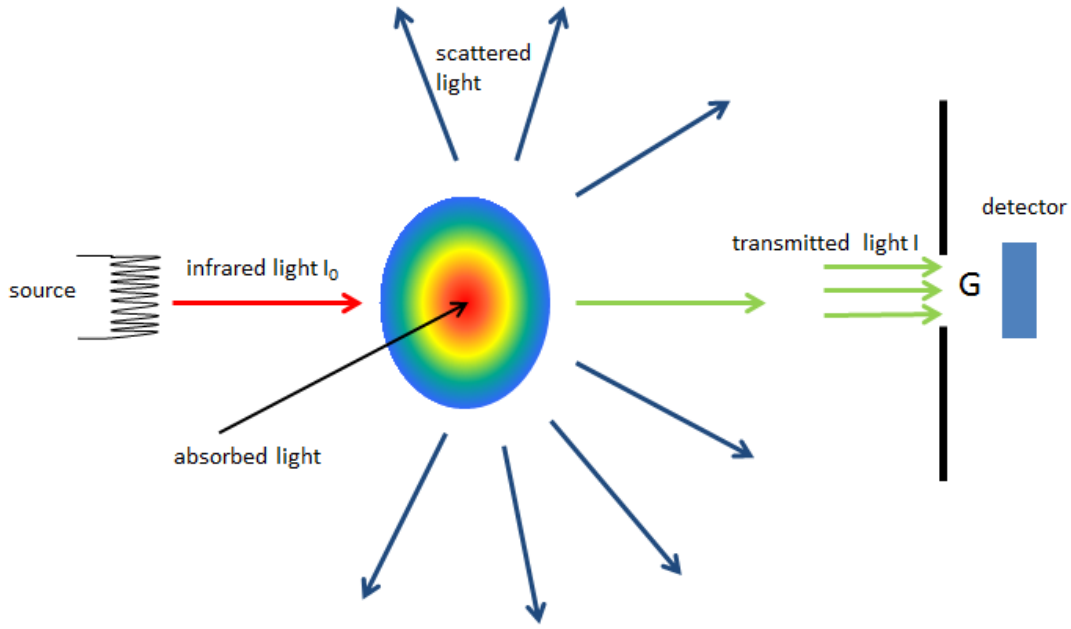


Figure 8: Scattering in a spectrometer. Illustration from [14]

As described in section 3.5 "Absorbance and transmittance", the transmittance is the fraction between *I* and I_0, and the absorbance is calculated by the 10 logarithm of the transmittance (eq. 3 and 4). Thus, when recording the absorbance spectrum *A* by a spectrometer, loss of light expressed as high values in the absorbance may be due to scattering and absorption. Therefore, the researcher interpreting the spectra is at a loss to decide whether a high absorbance value relates to scattering or absorbance. Several scattering phenomena such as the scattering of light at thin films and the scattering of light at spherical particles (Mie scattering) are described in the literature [14, 28-30]. Both thin film scattering and Mie scattering are very relevant for the experimental setup used in this thesis.

Thin film scattering is illustrated in Figure 9. The incident light, I_0, impinges on the front of the film, where it is absorbed (red area) transmitted (*I*), and scattered, (I_{sca}). The scattered light from thin films can be described exactly, and can be removed from the results. Internal reflections and interference between reflected and transmitted beams lead to sinusoidal oscillations which appear as irregular wavy structures along the spectra: *fringes*. Dispersive artefacts as they appear in Mie scattering have not been observed in the scattering of infrared light at thin films [31]. Therefore, the correction or interpretation of absorbance spectra containing fringes is less problematic than the correction and interpretation of absorbance spectra of single cells showing Mie scattering.

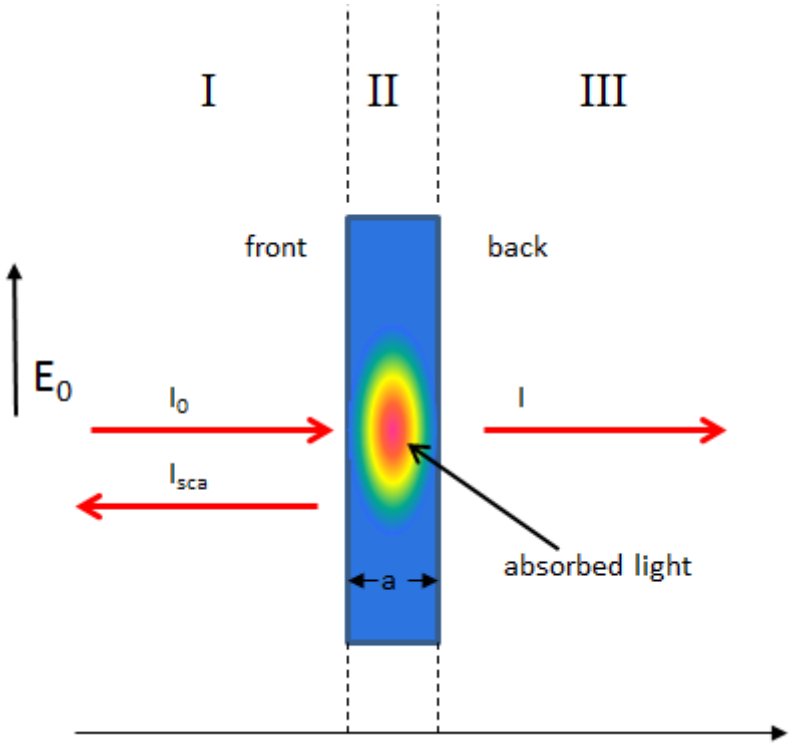


Figure 9: Scattering on a thin film. Illustration from [14]

Mie scattering represents a major problem in infrared microspectroscopy of single cells. Gustav Mie described for the first time the scattering of light at spherical particles analytically [28]. He gave an analytical expression for the extinction efficiency $Q_{ext}(\tilde{\nu})$ which is a dimensionless quantity describing the ratio of the extinction cross section $\sigma_{ext}(\tilde{\nu})$ and the geometrical cross section $g = \pi r^2$ according to

$$\sigma_{ext}(\tilde{\nu}) = \pi a^2 Q_{ext}(\tilde{\nu}) \qquad (6)$$

Where $Q_{ext}(\tilde{\nu})$ is the extinction efficiency and a is the radius.

The absorbance spectrum is obtained from the extinction efficiency according to

$$T = 1 - \frac{\sigma_{ext}(\tilde{\nu})}{G} \qquad (7)$$

Where $\sigma_{ext}(\tilde{\nu})$ is the extinction cross section and G is the area of the aperture in front of the detector (see Figure 8).

The absorbance spectrum, A, is related to the transmittance, T, as showed in equation 3.
In the first approximation, A is related to $Q_{ext}(\tilde{\nu})$ as following:

$$A \approx \frac{\pi a^2}{G \ln(10)} Q_{ext}(\tilde{\nu}) \qquad (8)$$

Where $Q_{ext}(\tilde{\nu})$ is the extinction efficiency, *a* is the radius and *G* is the area of the aperture in front of the detector.

The exact Mie formalism is complicated, but handy approximation formula for the extinction efficiency $Q_{ext}(\tilde{\nu})$ have been provided in the literature.
In the case of |m – 1| << 1, $Q_{ext}(\tilde{\nu})$ can be approximated as shown by Van De Hulst [28]:

$$Q_{ext}(\tilde{\nu}) \approx 2 - 4e^{-\varrho \tan\beta}\frac{\cos\beta}{\rho}sin(\rho-\beta) - 4e^{-\rho\tan\beta}\left(\frac{\cos\beta}{\rho}\right)^2 cos(\rho-2\beta) + 4\left(\frac{\cos\beta}{\rho}\right)^2 cos(2\beta) \quad (9)$$

Where

$\rho = 4\pi a\tilde{\nu}(n-1)$ and $tan\,\beta = n'/(1-n)$

To be able to evaluate equation 9, it is necessary to know the real part of the refractive index, *n*, and the imaginary part of the refraction index, *n'*.

The imaginary part of the refractive index *n'* can be calculated from the pure absorbance spectrum according to

$$A = -\log_{10}(T) = 4\pi n' a\tilde{\nu}/\ln(10) \quad (9)$$

Where T is the transmittance, n' is the imaginary part of the refractive index, a is the area of the cross section and $\tilde{\nu}$ is the wavenumber of the incident light.

Thus, in order to predict the scatter extinction and the measured absorbance spectrum *A*, we need as input the pure absorbance spectrum. The problem is an inverse problem, which is difficult to solve. Various approaches based on EMSC or iterative algorithms have been suggested, but with limited success [27, 30, 32].

In an approach presented by Kohler *et al*. 2008, the refractive index, *n*, is considered constant (when using EMSC) [30]. Therefore, the dispersive artefact is not corrected, only the strong Mie oscillations. In addition, several approximations have to be done for the formulas to be applicable. Thus, it can be argued that the better option would be to avoid Mie scattering experimentally.

4. Economic calculations and definitions

4.1 Pricing

Setting the price for the product is not an easy task. There are three main strategies to consider when setting a price: penetration, parity and skimming pricing strategies [33]. Following a *penetration pricing strategy*, the price of the product is set below its value, often lower than competitor's products, to increase initial sales. A *parity pricing strategy* indicates that the price is set at or near competitor levels, to avoid a pricing response. No competitive advantage will be created either. *Skimming pricing strategy* refers to a strategy where the price is set above the products value. This might jeopardize the sales, if costumers cannot afford the product, or competitors are offering the same service at a lower price. On the other hand, fewer units must be sold to cover the costs, and a higher price might help create a prestige image.

4.2 Break-even point

The break-even point describes how many units must be sold to cover the production costs [34]. The BEP-formula is shown below:

$$x = \frac{FC}{p-v} \qquad (10)$$

Where *FC* is fixed costs, *p* is the price, *v* is the variable cost per unit, and *x* is the quantity sold.

Part II: Methodology

5. Materials and methods

5.1 Sample preparation

The pollen types used were collected from *Fagus sylvatica* (European beech), *Betula pendula* (Silver birch), and *Quercus robur* (Penduculate oak) of the Fagales (beech) order, and *Juniperus chinesis* (Chinese juniper) and *Cupressus sempervirens* (Mediterranean cypress) of the Pinales (Pine) order. Pollen samples were obtained through fieldwork at the Botanical Garden of the Faculty of Science, University of Zagreb, during 2012 pollination seasons. The pollen samples were collected directly from plants at flowering time. The details are provided in Zimmermann & Kohler (2014) [35]. The fresh pollen was stored at -15˚C, and were measured without any chemical treatment.

The pollen samples were embedded in a Vaseline-type paraffin between two 40 μm polyethylene foils, which are standard embedding materials for use in IR spectrometry. Polyethylene foil is comparable to the polyester foil, while paraffin is a commonly used adhesive substance in the Burkard trap [36]. It is the only adhesive substance that is mostly transparent in the infrared region. However, both paraffin and polyethylene have some absorption bands in the infrared. These absorption bands overlap with spectral bands of the pollen and could complicate the spectral analysis. First, the foil was cut into two 5 x 5 cm pieces, and then the paraffin was applied with a spatula to both pieces. Approximately 1 mg of paraffin was used per piece of foil. The pieces were put together and compressed, to even out the paraffin layer. Then the pieces of foil were separated again, and the pollen were transferred to the foil with a spatula. The foils were brought back together, ready for infrared measurement. A special plastic sample holder for the foils was manufactured at IMT. The holder was made to stretch the foil, to minimize the appearance of air bubbles in the sample. The equipment used to prepare the samples is shown in Figure 10, and the prepared sample in Figure 11.

In one case, a 3 mm ZnSe slide was used for measurement of pollen, in order to demonstrate the scattering phenomena on pollen grains.

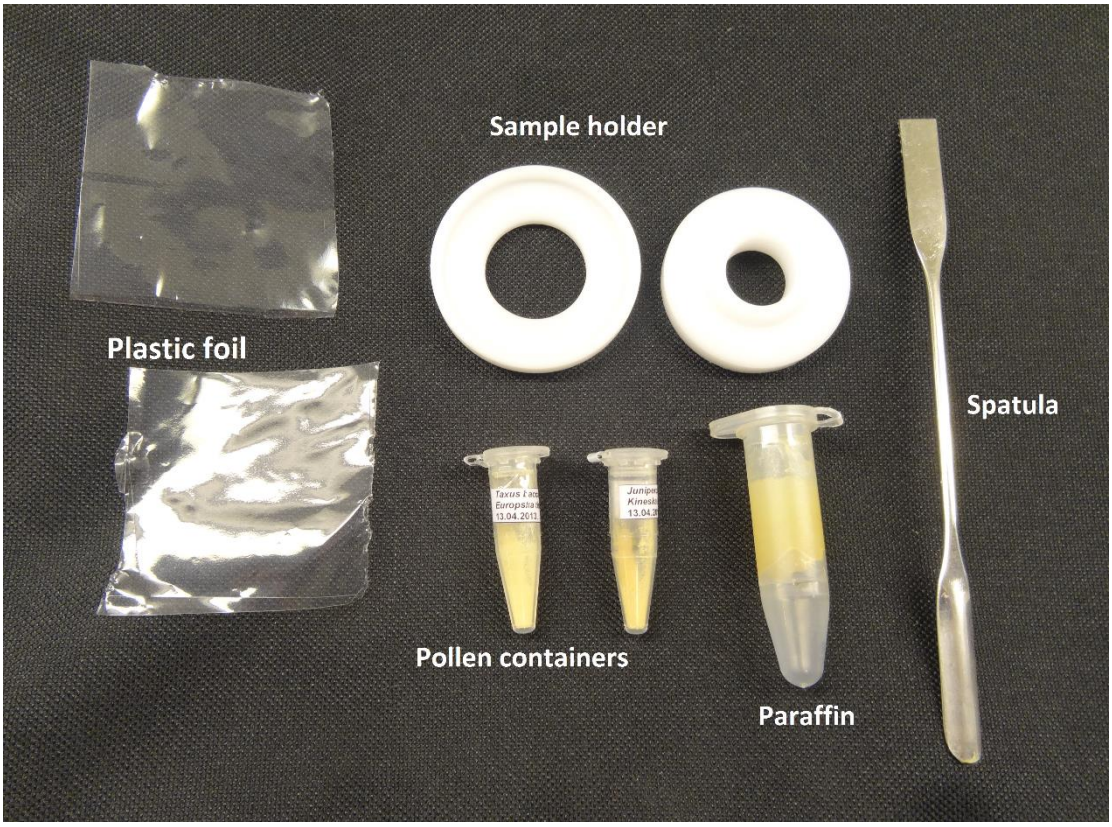


Figure 10: The equipment used to prepare the samples

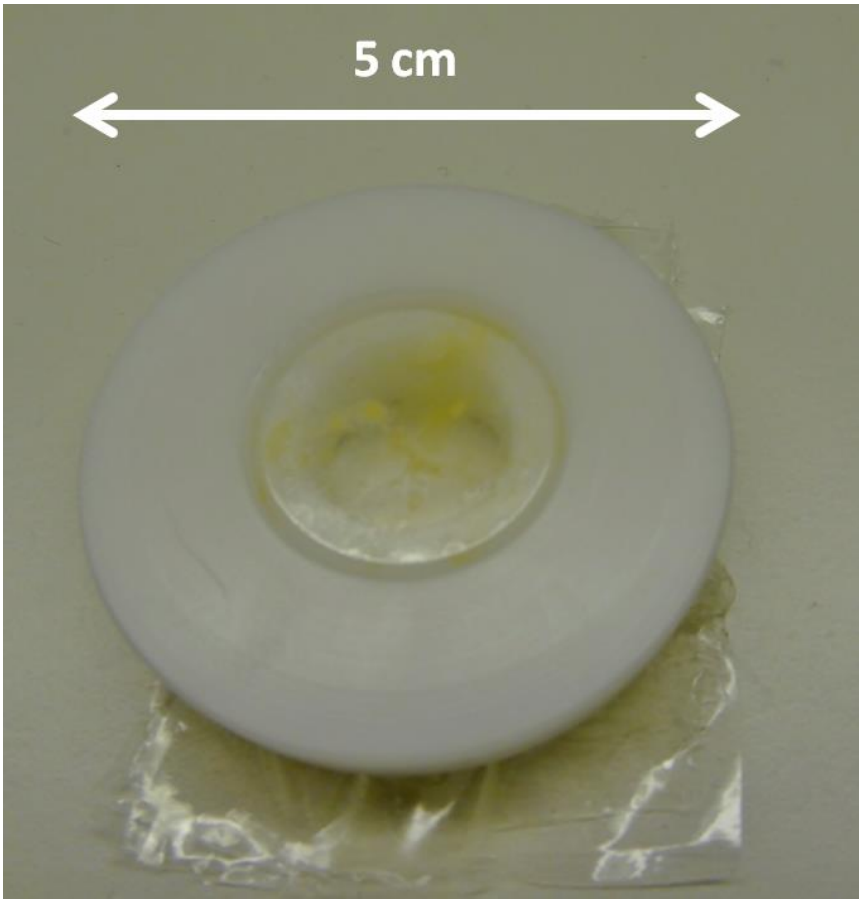


Figure 11: Prepared pollen sample

5.2 FTIR spectroscopy

A VERTEX 70 Fourier transform infrared (FTIR) spectrometer with a HYPERION 3000 IR microscope (Bruker Optik GmbH, Germany) was used for the infrared spectroscopy. The spectra were recorded in transmission mode with a spectral resolution of 4 cm^{-1}. 50 – 120 pollen samples per sample were recorded. The system is equipped with a globar mid-IR source and a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector. The spectra were measured in the 4000-600 cm^{-1} spectral range, with 64 scans for both background and sample spectra, and using 15× objective, with 25×25 µm aperture sizes. Background (reference) spectra of air were recorded once, before starting each measurement using the sample-free setup. The Bruker system was controlled with OPUS 7.5 software (Bruker Optik GmbH, Germany). Figure 12 shows the sample ready for measurement.

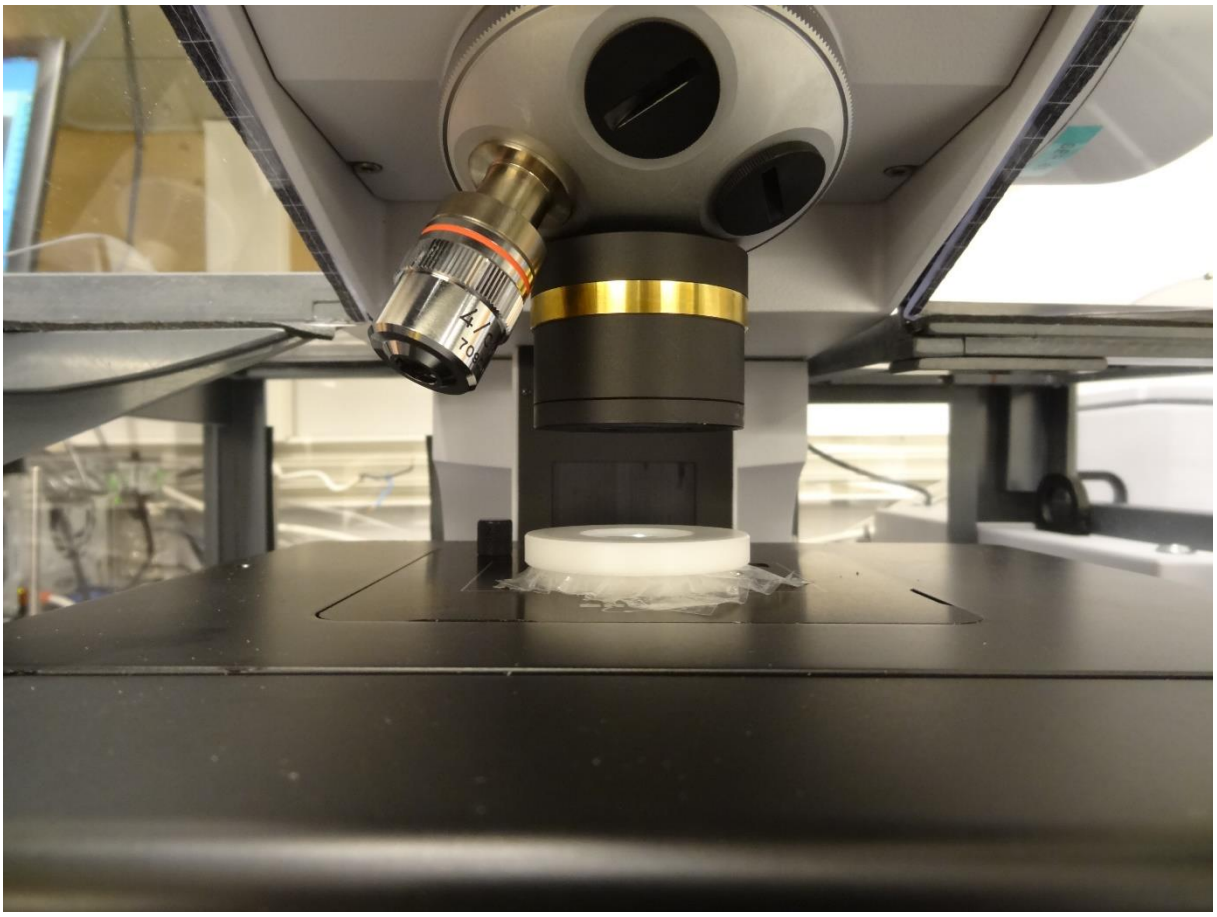


Figure 12: Pollen sample in the spectrometer

5.3 Data analysis

Unwanted variations may complicate the data analysis of the raw spectra of pollen. Various transformation techniques or model based pre-processing can be applied to reduce the effects of these variations. Calculating the second derivative spectra of the spectra removes baseline effects due to scattering and variations in the light source [37]. In addition, it resolves overlying bands. For second derivate calculations, the Savitzky-Golay algorithm was used. The Savitzky-Golay algorithm fits a polynomial of a predefined order to each absorbance value, *channel*, in the spectrum taking into account a predefined number of neighboring points, *window size*. In addition, it employs weighting of all neighboring points in the fitting process. The derivative in each channel is obtained by derivation of the polynomial fit to each channel in the spectrum. The bigger the window size use for the fitting of the polynomial, the higher is the smoothing effect. A small window size is more prone to noise, but it retains peaks and fine structure in the spectra, while a bigger window size is appropriate when the larger structures are more essential in the spectra. In our analysis, the second derivative was used with a window size of 31 points and a polynomial of order 2. In addition, smoothing by Savitzky-Golay algorithm was applied to two sets (without calculating the second derivative), with a window size of 11 or 13.

Differences in optical path length and sample thickness and different physical variations in the spectra can be separated and quantified by the use of model-based pre-processing techniques, such as extended multiplicative signal correlation (EMSC). EMSC normalizes spectra with respect to a reference spectrum and removes physical effects [38]. For the purpose of data analysis, the software "The Unscrambler X" version 10.3 from CAMO, Oslo, Norway, was used. The raw spectra were transformed into the second derivative by Savitzky-Golay algorithm. Then EMSC was applied. The spectral region of 1900 cm^{-1} to 900 cm^{-1} was selected for data analysis. The saturation region of the polyethylene foils and paraffin, 1500-1300 cm^{-1}, was excluded from the spectral region. For the set called "all spectra, original data", the saturation region 1500-1300 cm^{-1} was included, and the spectra was only smoothed by Savitzky-Golay algorithm with a window size of 11 points. For the set "chosen spectra, original data" the second derivative was not calculated, but the spectra were smoothed by Savitzky-Golay algorithm with a window size of 13 points.

Multivariate analysis of the spectral data was done by principal component analysis (PCA). By PCA the main variation patterns in the sample and variable space are revealed [39]. There is a high co-linearity in FTIR data of biological materials, and PCA is excellent for handling this problem. The high co-linearity is related to the fact that several bands are connected with the same biomolecules, as the spectral bands in infrared cover many variables. PCA reduces the number of dimensions in the data, without much loss of information [39]. The new variables are called principal components (PCs),

and explain most of the variability in the data. The first PC, PC1, explains most of the variability in the data, PC2 the second most, etc. Just as each sample had a value for each of the wavelength variables in the original data, each of the PCs will have a value referred to as *score* [39]. Similarities and differences between variable groups will appear in the score plot. Another interesting plot is the *loading*-plot that follows each PC. The loading explains how the PC relates to the original spectral variables (i.e. wavenumbers) [39]. Plotting loadings as line plots reveals variables that contribute to a given PC. When a spectral band has a strong contribution to a given PC, this band typically shows a negative or positive peak in the loading line plot, depending on the orientation of the PC. Thus, spectral bands and chemical differences causing differences in sample patterns observed in the score plots, can be interpreting with the help of the loading plot.

5.4 Market research and economic estimates

The market research was primarily conducted as a secondary research, where the objective was to map the pollen monitoring industry. Information was gathered from companies' webpages, data sheets, articles, and by personal correspondence. Due to time limitations, providing a wide range of information about the market was preferred to exploring a few factors in depth, e.g. by conducting a survey about the demand for a pollen analysis service.

The operational costs were determined in accordance with information provided from senior engineer at IMT, Andreas S. Flø and financial manager at IMT, Anita Haugen Habbestad. The price sensitivity was attempted established by interviewing representatives from the defined target groups. Remaining economic estimates was conducted accordingly to information provided in this thesis.

Part III: Results

6. Results: technical aspects

6.1 Scattering

In order to demonstrate the effects of scattering, spectral measurements of single pollen grains were performed without embedding. To this purpose, the pollen samples were placed on a 3 mm ZnSe slide. Three different spectra are shown in Figure 13. All the spectra are from cypress pollen, but as can be seen, there are big differences between the spectra caused by scatter effects. Broad and almost periodic oscillation structures due to Mie scattering are visible. Since the Mie scattering depends on the pollen morphology and it is known that the pollen morphology can vary within the same type of pollen, it is obvious that the identification of pollen samples is impaired by strong scattering effects in the spectroscopy of single pollen.

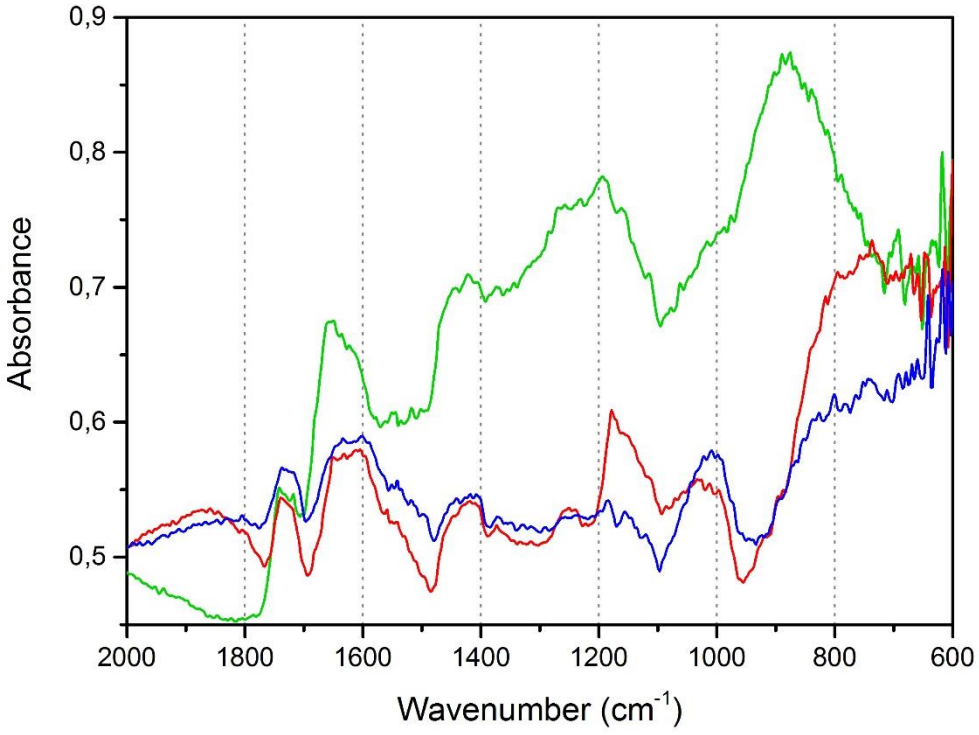


Figure 13: Scattered spectra of Cypressus sempervirens

Figure 14 shows the true absorbance spectrum of *Cypressus sempervirens*. The red graph is the spectrum measured in a KBr pellet, and the blue graph is the spectrum recorded in this study, with the paraffin and polyethylene foil embedding. The spectrum shows no signs of scatter contributions. Three peaks are especially apparent in the spectrum: the peak at 1750 cm^{-1} associated with lipids, the signal at 1650 cm^{-1} related to proteins and a peak around 1035 cm^{-1}, corresponding to carbohydrates. In

addition, Figure 14 shows the infrared spectrum of the embedding materials: paraffin and polyethylene. This will be further discussed in the next section, section 6.2 “Cut-out region”.

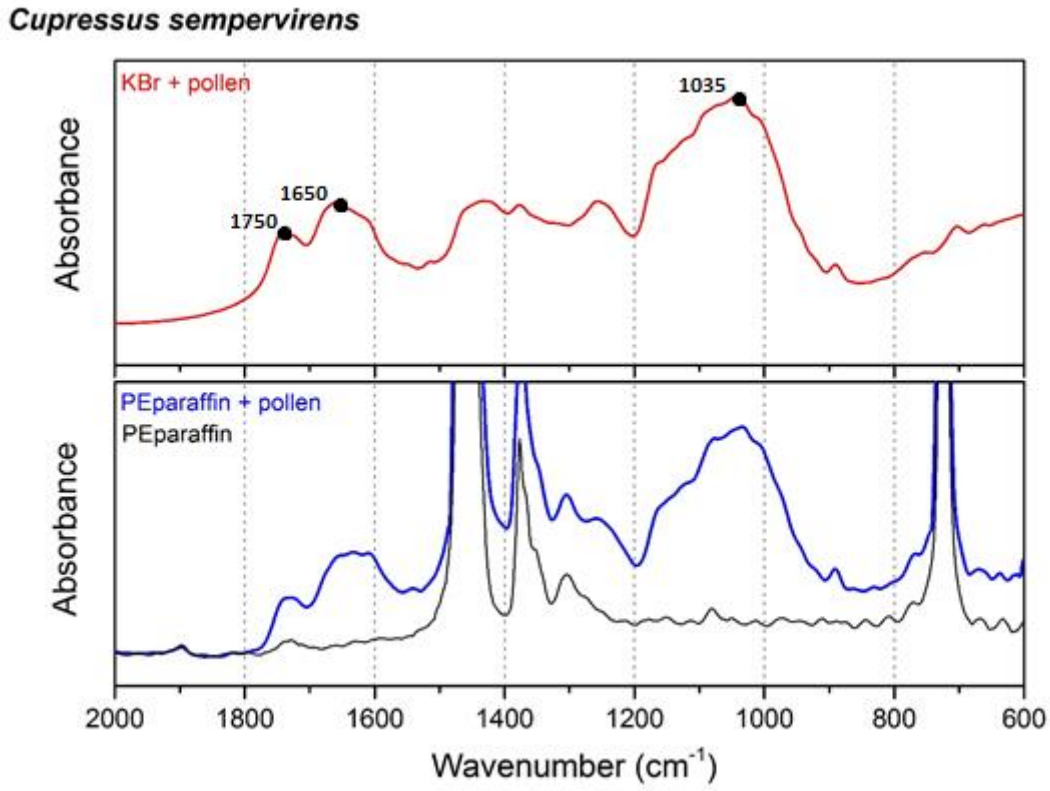


*Figure 14: Absorbance spectrum of Cypressus semperviren and strong vibrational bands.. **Above:** Measured in a KBr pellet. **Below:** Measured in foil and paraffin embedding. The spectrum of only paraffin is also displyaed.*

6.2 Cut-out region

Figure 15 shows the infrared spectrum of *Betula pendula* (silver birch). The red graph is the absorbance spectrum of birch in a KBr pellet, the blue graph below shows the spectrum of birch measured in paraffin and foil embedding. The gray graph is the absorbance spectrum of paraffin and polyethylene. The two *betula*-spectra are approximately identical with the exception of the saturation regions due to paraffin and polyethylene foil (approx. 1500-1300 cm^{-1} and 800-600 cm^{-1}). The peak at 1750 cm^{-1} corresponding to the C=O stretch associated with lipids is apparent in both spectra, so is the peak at 1650 cm^{-1} associated with proteins (amide I). The vibrations in the carbohydrate region, 1200-900 cm^{-1} is also similar in both spectra. In conclusion, Figure 15 shows that only a small amount of information is lost due to the embedding.

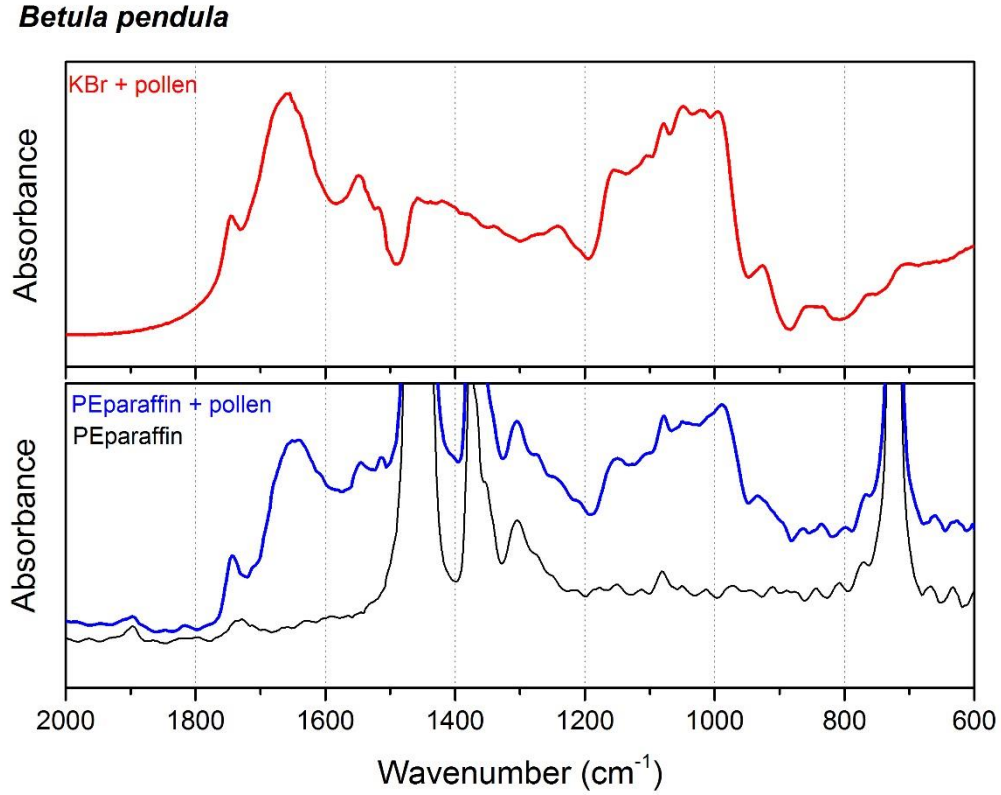


Figure 15: IR-spectrum of Betula pendula. ***Above:*** *Measured in a KBr pellet.* ***Below:*** *Measured in paraffin and foil (blue). The absorbance spectrum of paraffin is illustrated by the grey graph.*

In accordance with the information given above, only the spectral regions between 1900-1500 and 1300-900 cm^{-1} were analyzed. The region 4000-1900 cm^{-1} was left out as it contained a saturation region from the polyethylene foil and paraffin and since it is known that the region below 1800 cm^{-1} is sufficient for pollen identification [35, 40]. The region from 1500 cm^{-1} to 1300 cm^{-1} was cut out due to polyethylene/paraffin saturation, as illustrated in Figure 16, Furthermore, the 900-600 cm^{-1} region was cut due to the same saturation situation.

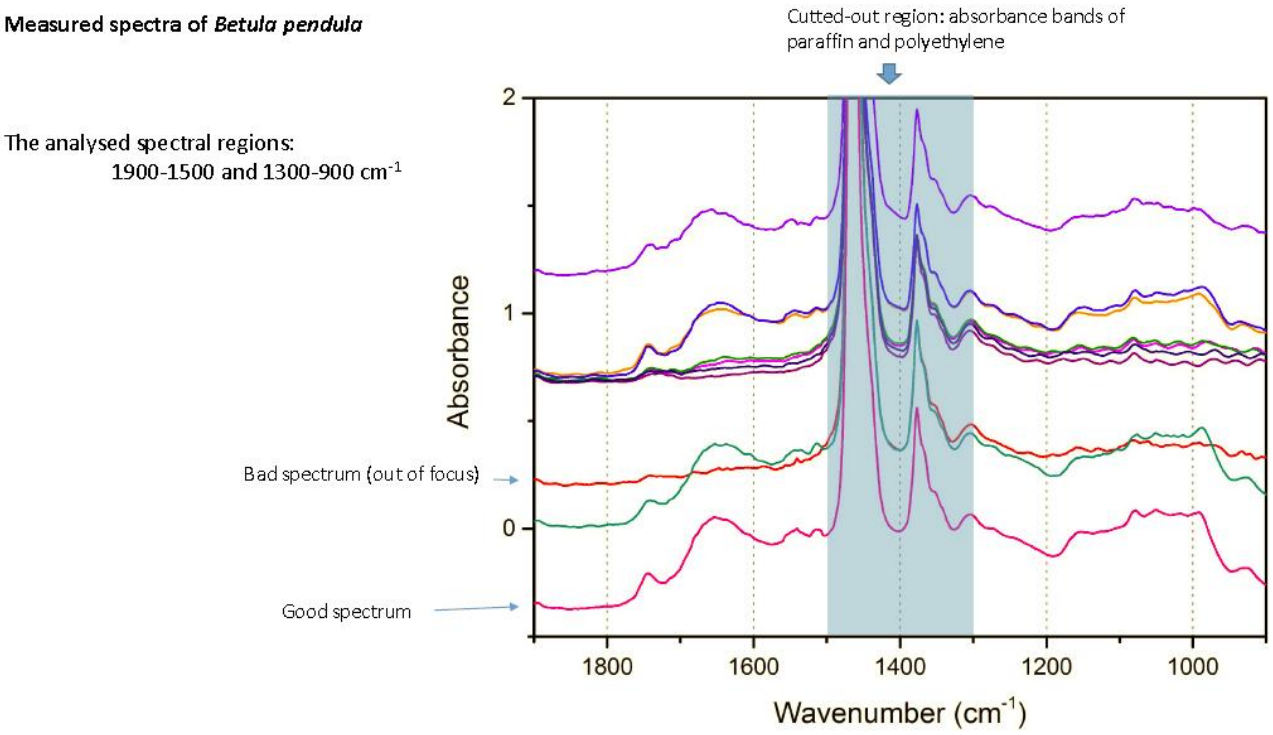


Figure 16: Cut-out region 1500-1300 cm^{-1}, and illustration of "good" and "bad" spectra.

6.3 Pre-processing

In Figure 17 the raw spectra of each pollen species are shown. As mentioned above, the saturation regions of the polyethylene foils and paraffin (4000-1900 cm^{-1}, 1500-1300 cm^{-1}, and 900-600 cm^{-1}) was removed from the spectral data. The pre-processing techniques are demonstrated in Figure 18 and 19. Figure 18 shows the second derivative of the spectra (Savitzky-Golay algorithm, window size 31), where spectral bands are resolved. The minima in the second derivative refers to band maxima in the original spectra. The effects of EMSC is illustrated in Figure 19, where the multiplicative effect in the spectra is to a large extend corrected.

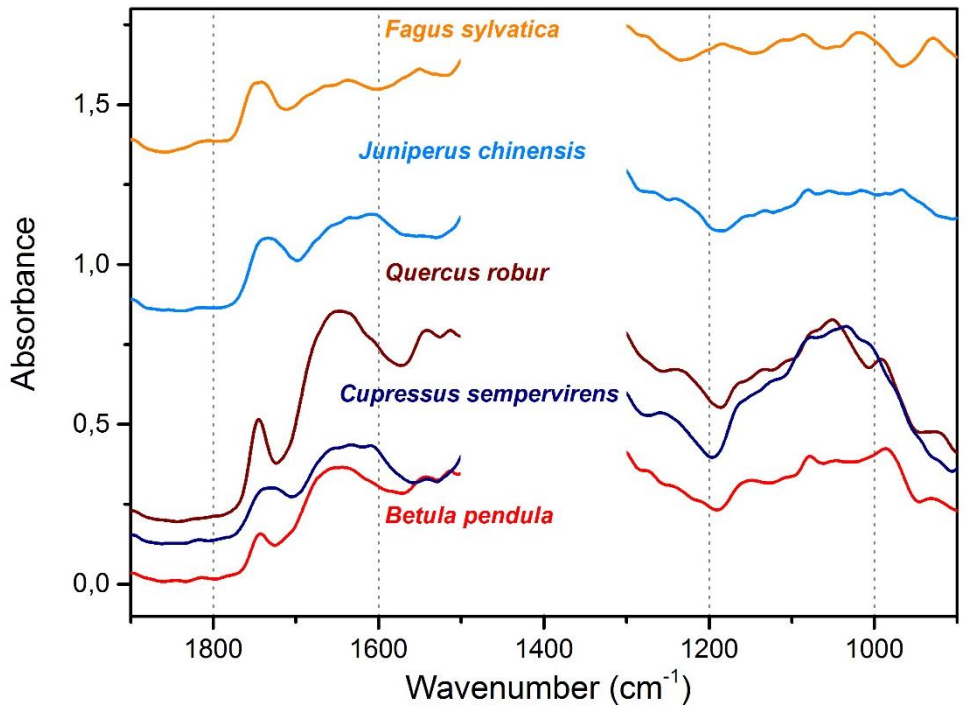


Figure 17: Raw spectra

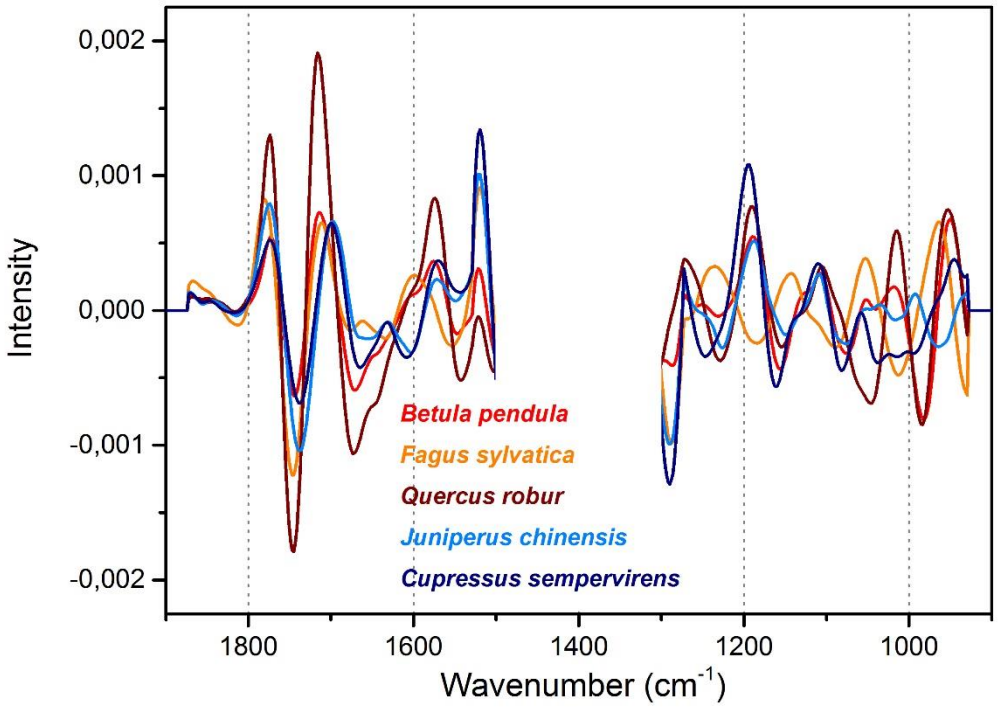
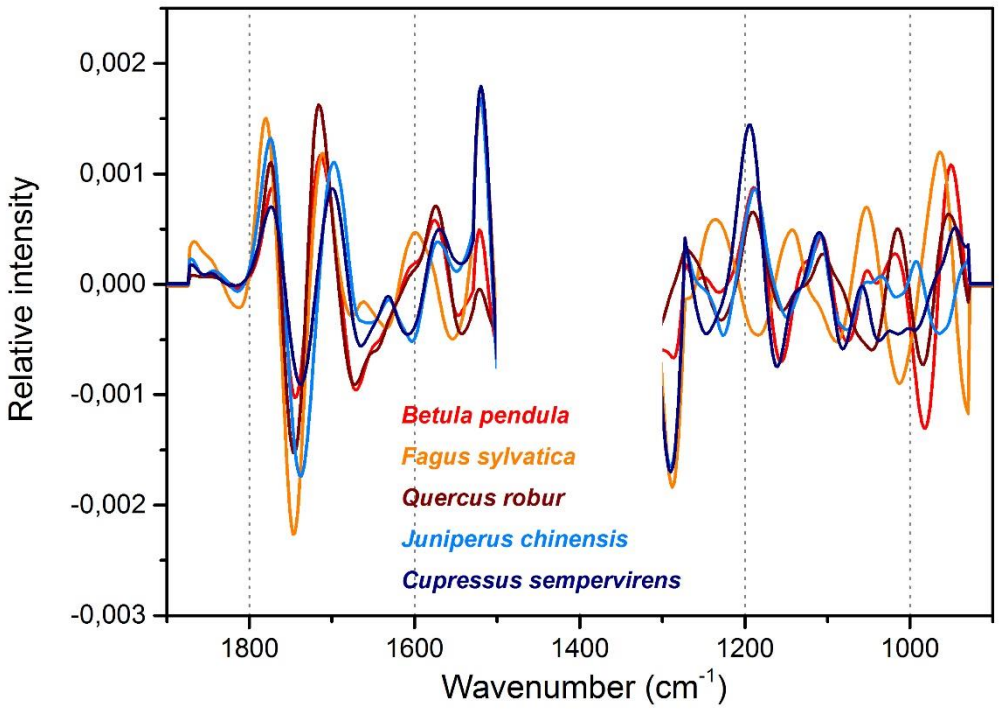


Figure 18: Second derivative of spectra, calculated by Savitzky-Golay algorithm



Figur 19: EMSC corrected spectra

6.4 Identification of species

6.4.1 Chosen spectra, original data

20 spectra of each of the five species was chosen. Figure 18 shows the PCA plot of 20 of the in-focus ("good") pollen samples of each species. Bad spectra were removed prior to PCA by visual inspection of the spectra. Figure 16 illustrates some examples of "good" and "bad" spectra of pollen from silver birch (*Betula pendula*). The "bad" spectra origins from pollen grains that were out of focus during measurement, or where the spatial orientation of the grain was not optimal. The spectral data was smoothed by the Savitzky-Golay algorithm with a window size of 13 points and pre-processed by EMSC. The plot demonstrates clear differentiation between the different pollen types. The Pinales are separated from the rest, and there is also a separation between Fagus and the other two Fagales species. The separation is mainly related to ratios of the main constituent chemicals: lipids and carbohydrates, and lipids/proteins and carbohydrates. This can be observed from the PCs loading plot in Figure 21 and 22. Figure 21 shows that the variation alongside PC1 axis in Figure 20 is based on the ratio of both lipids (characterized by the peak at 1750 cm^{-1}) and proteins (characterized by peaks at 1650 (amide I) and 1550 cm^{-1}), and carbohydrates (characterized by peaks in the 1050-950 cm^{-1}region). This variation is responsible for spectral separation of Pinales from Fagales samples. Figure

22 shows that the variation alongside PC2 axis in Figure 20 is predominantly based on the ratio of lipids (peak at 1750 cm^{-1}) and carbohydrates (peaks in the 1050-950 cm^{-1}region). This variation is responsible for spectral separation of pollen species with higher relative content of lipids (Fagus and Juniperus), from other three species that have smaller relative content of lipids [35].

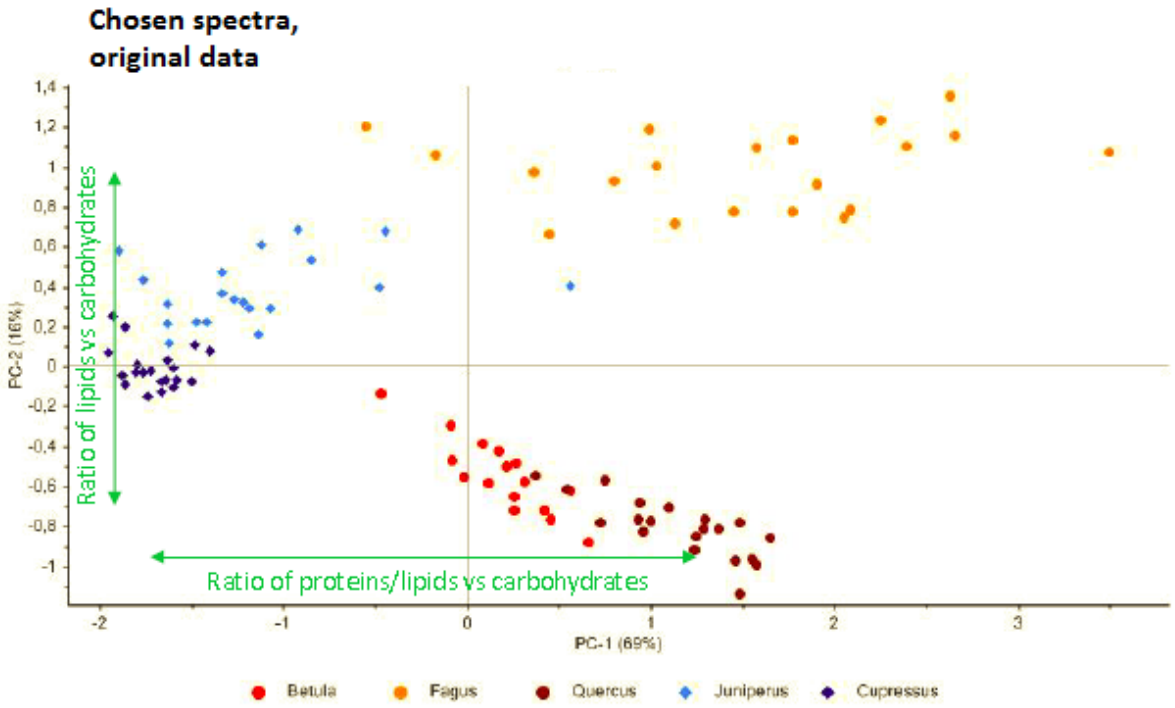
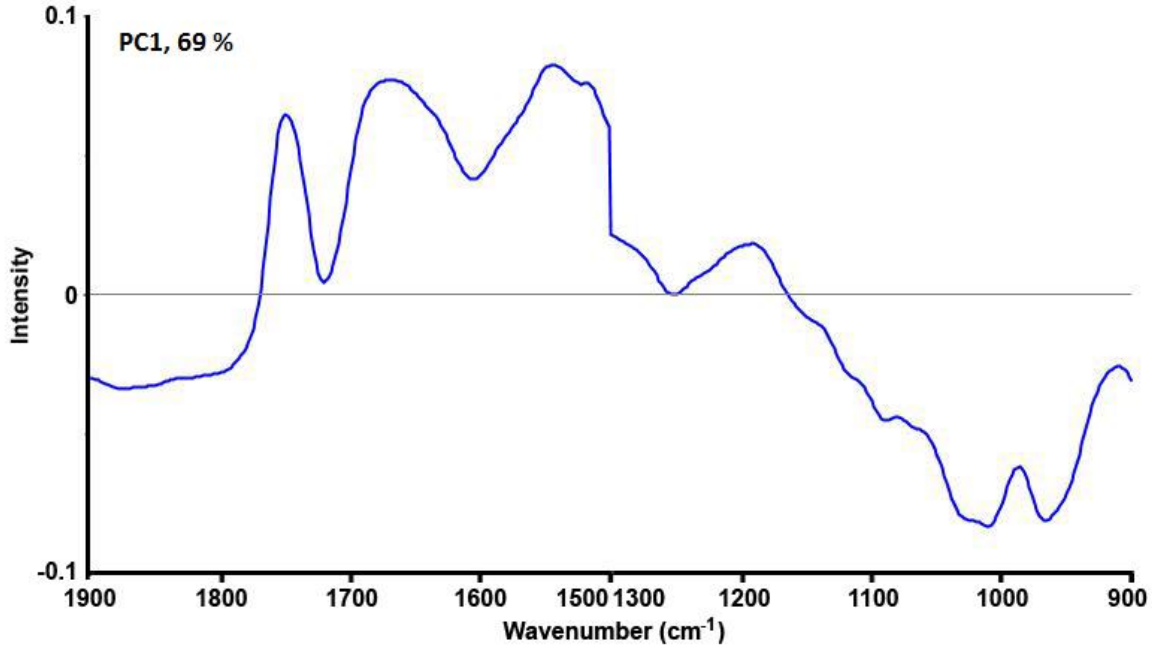
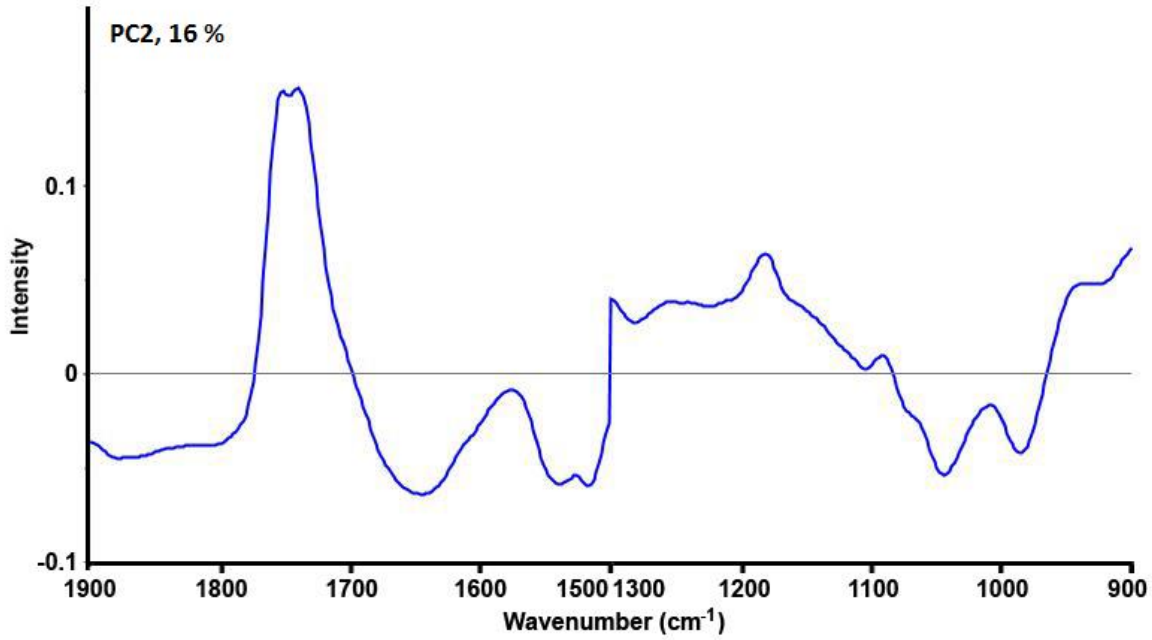


Figure 20: PCA plot of all five study species, 20 pollen samples per species. Spectra are smoothed and pre-processed by EMSC. Percentage variance of the two first PCs are: 69 %, 16 %. Color description: deep blue: Cupressus, light blue: Juniperus, red: Betula, yellow: Fagus, and brown: Quercus. Species of the beech order are represented by dots and the pine order species by diamonds.



Figur 21: Loading plot for PC1 (69 %) for PCA plot of chosen, original spectra (20 pollen samples of each type)



Figur 22: Loading plot for PC2 (16 %) for PCA plot of chosen, original spectra (20 pollen samples of each type)

6.4.2 Chosen spectra, second derivative data

In the PCA plot shown in Figure 23, the second derivative was applied to the spectral data by means of the Savitzky-Golay algorithm, and the spectra were pre-processed by EMSC. The separation of the different pollen types is close to perfect. When comparing Figure 23 with Figure 20, the effects of converting the spectral data into second derivatives is obvious: the separation follows the same pattern

as in the PCA plot of the original data (Figure 20) (although inverted), only enhanced. This is due to previously mentioned reasons, that derivatives emphasize band widths, positions, and separations while simultaneously reducing or eliminating baseline and background effects From the loading plot of PC1 and PC2, Figure 24 and 25, a similar pattern as in Figure 21 and 22 can be observed. Vibrations are present around 1745 cm^{-1}, associated with lipids, at 1550 cm^{-1} with the proteins, and in the carbohydrate-region 1200-900 cm^{-1}.

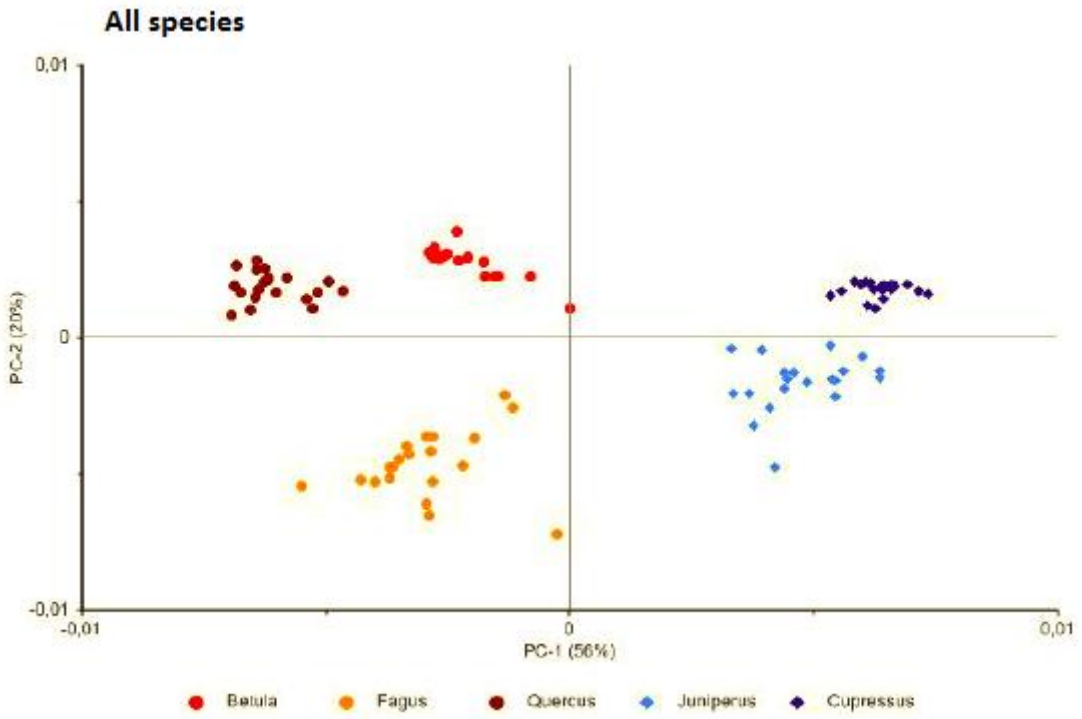


Figure 23: PCA plot of all five study species, 20 pollen samples from each type. Spectra are pre-processed by second derivative and EMSC. Percentage variance of the four first PCs are: 56 %, 20 %, 8 % and 4 %. Color description: deep blue: Cupressus, light blue: Juniperus, red: Betula, yellow: Fagus, and brown: Quercus. Species of the beech order are represented by dots, and the pine order species by diamonds.

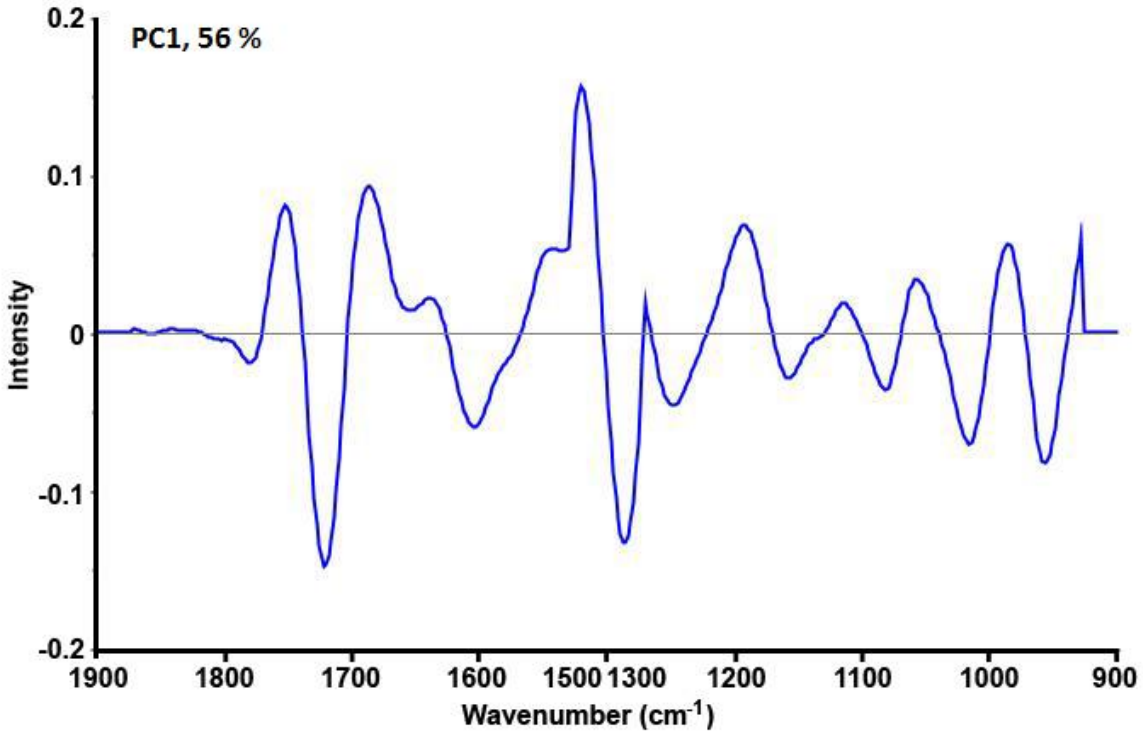


Figure 24: Loading plots for PC1 (56 %) for chosen spectra (20 of each species), second derivative data

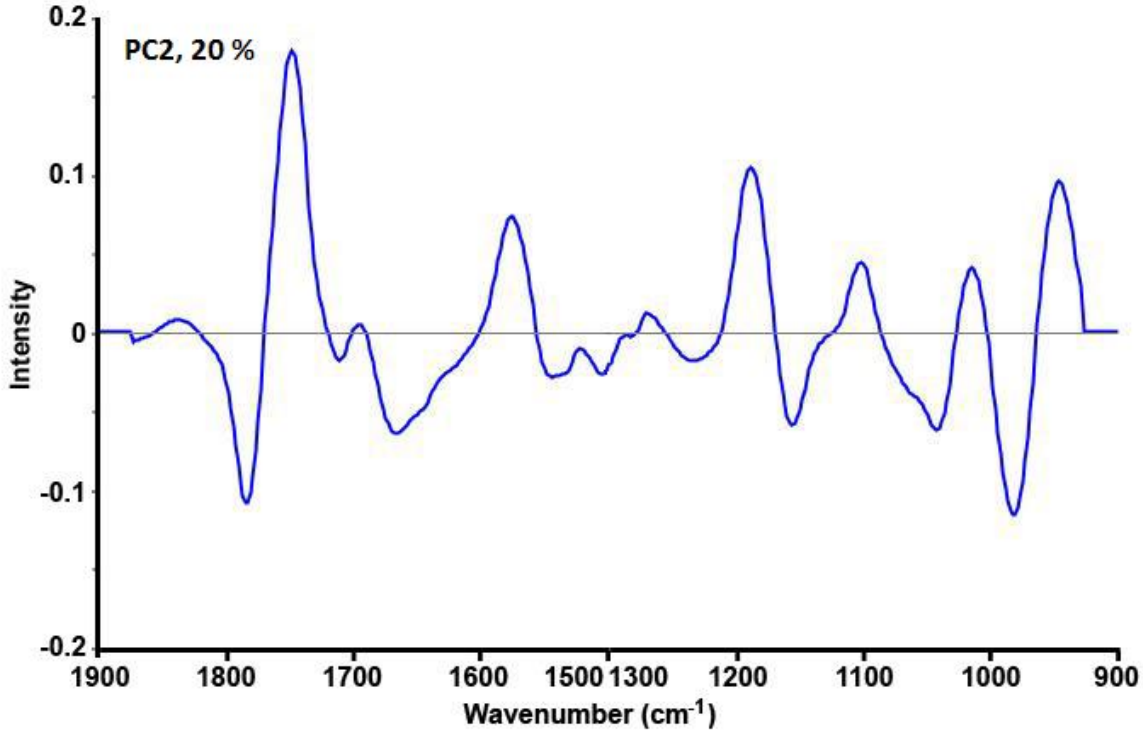


Figure 25: Loading plot for PC2 (20 %) for chosen spectra (20 of each species), second derivative data

The PCA plot in Figure 26 is of the same spectra as in Figure 23, but showing PC3 and PC4. Also in this plot, the clustering of different pollen species is clear, although the *Fagus* spectra are quite

dispersed (yellow dots). The loading plot of PC3, Figure 27, does not show any strong trends, while in PC4, shown in Figure 28, there seem to be some variations in the carbohydrate region, 1200-900 cm^{-1}.

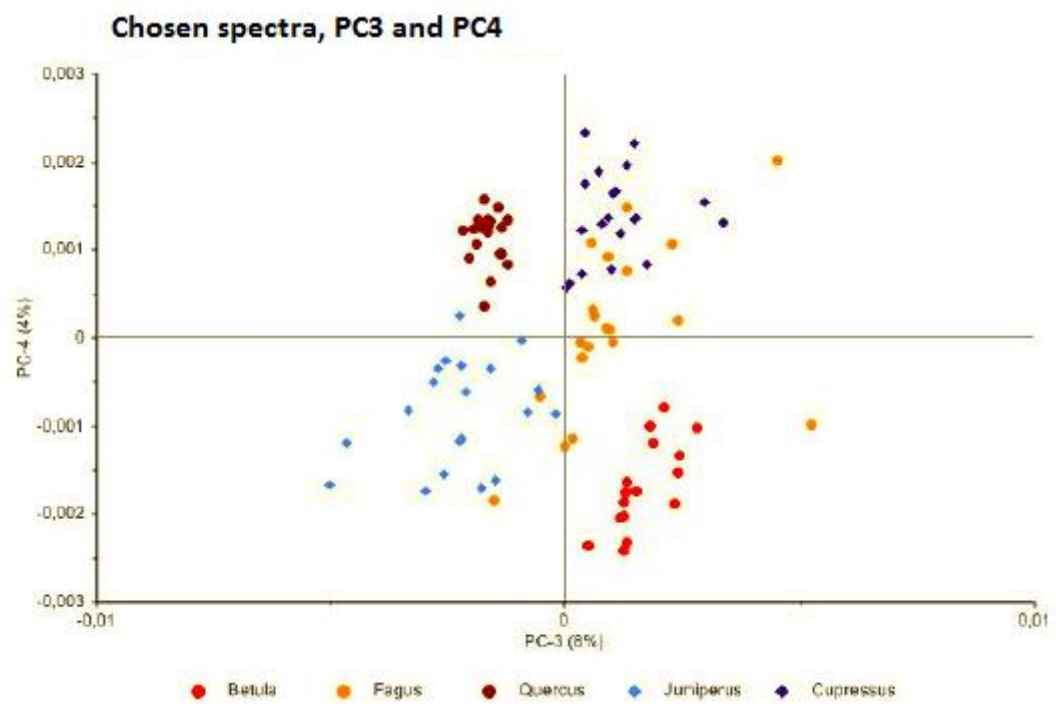
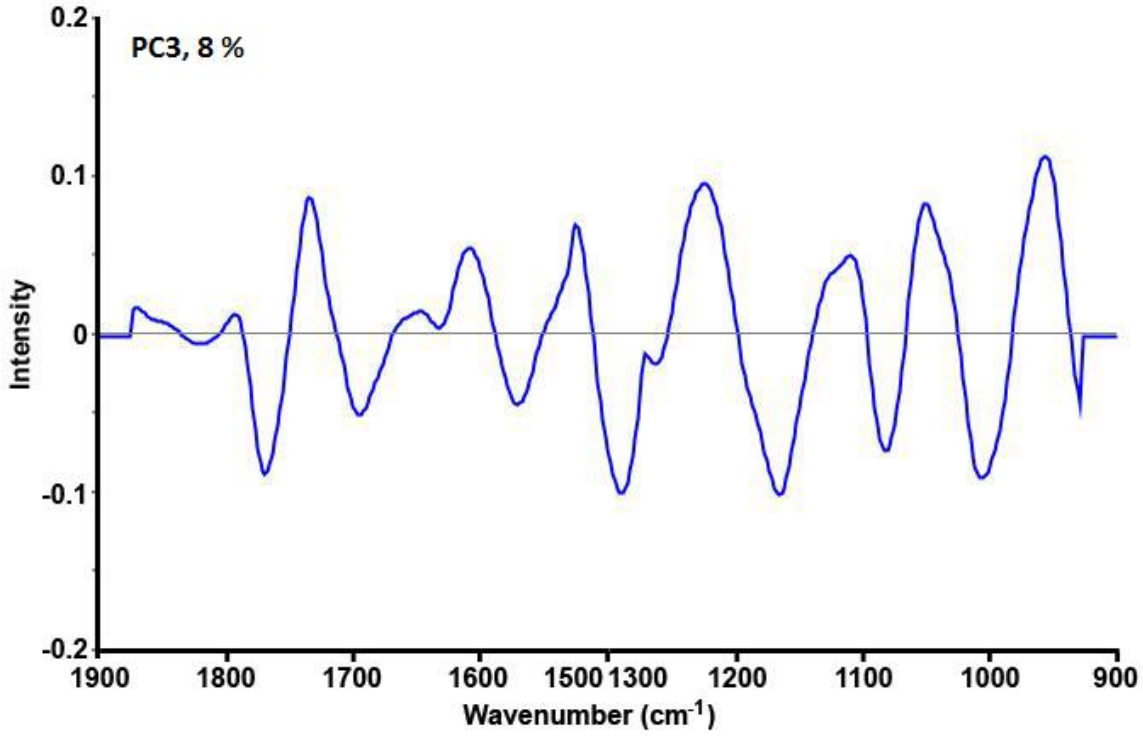
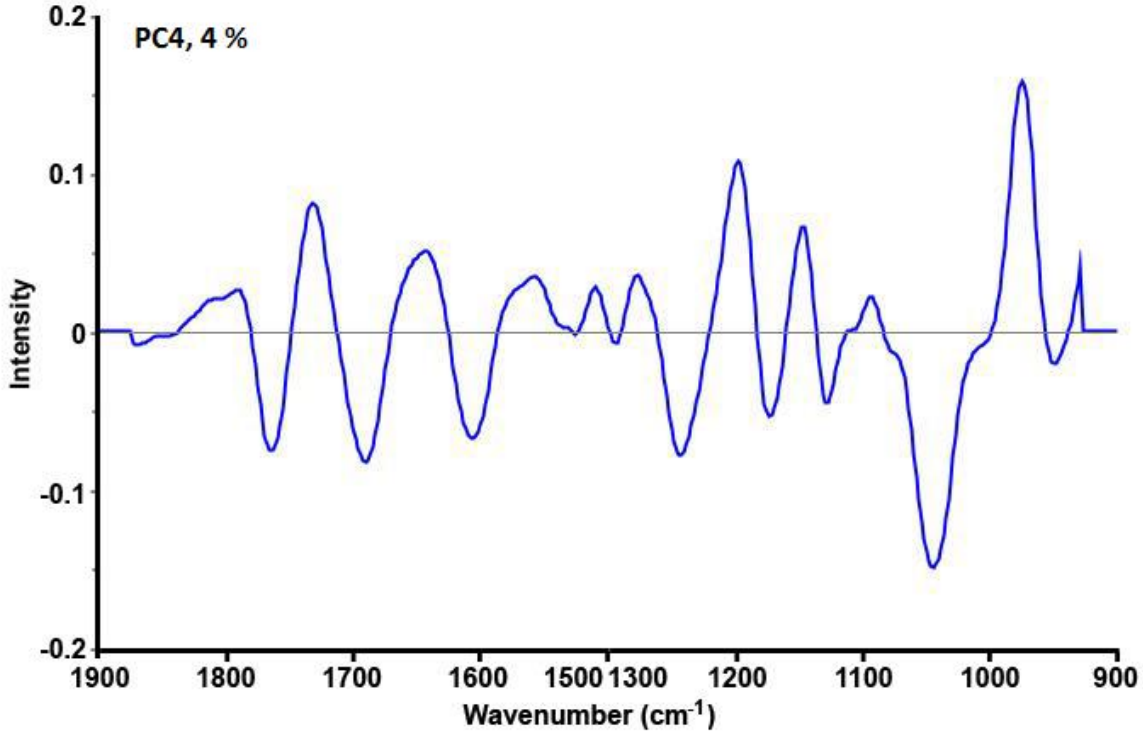


Figure 26: PCA plot of all five species, 20 pollen spectra per species. Conducted by PC3 (8 %) and PC4 (4 %). Color description: deep blue: Cupressus, light blue: Juniperus, red: Betula, yellow: Fagus, and brown: Quercus. Species of the beech order are represented by dots, and the pine order species by diamonds.



Figur 27: Loading plots for PC3 (8 %) for chosen, second derivative spectra (20 spectra of each pollen type)



Figur 28: Loading plots for PC4 (4 %) for chosen, second derivative spectra (20 spectra of each pollen type)

6.4.3 All spectra, original data

The PCA plot of all spectra is shown in Figure 29. The spectra have been smoothed by Savitzky-Golay algorithm with a window size of 11 points, and pre-processed by EMSC. In this plot, the saturation region 1500-1300 cm^{-1} is not cut out. This is because several of the spectra are out of focus and the saturation region by far accounts for the strongest signal (as shown in the PC1 loading plot, Figure 30). Without this region, the spectra out of focus could not be normalized by EMSC due to too high spectral variation between the "good" (in-focus) spectra and "bad" (out-of-focus) spectra Among the "good" spectra, clustering is apparent, with the largest separation between the birch and pine order (light and deep blue diamonds vs. red, brown and yellow dots). The PCA plot in Figure 29 is conducted from PC2 and PC3 as PC1 mainly separated the "good" from the "bad" spectra. From the loading plots of PC2 and PC3, Figure 31 and 32, peaks due to lipids, proteins and carbohydrates can be observed, in addition to the peaks in the saturation region.

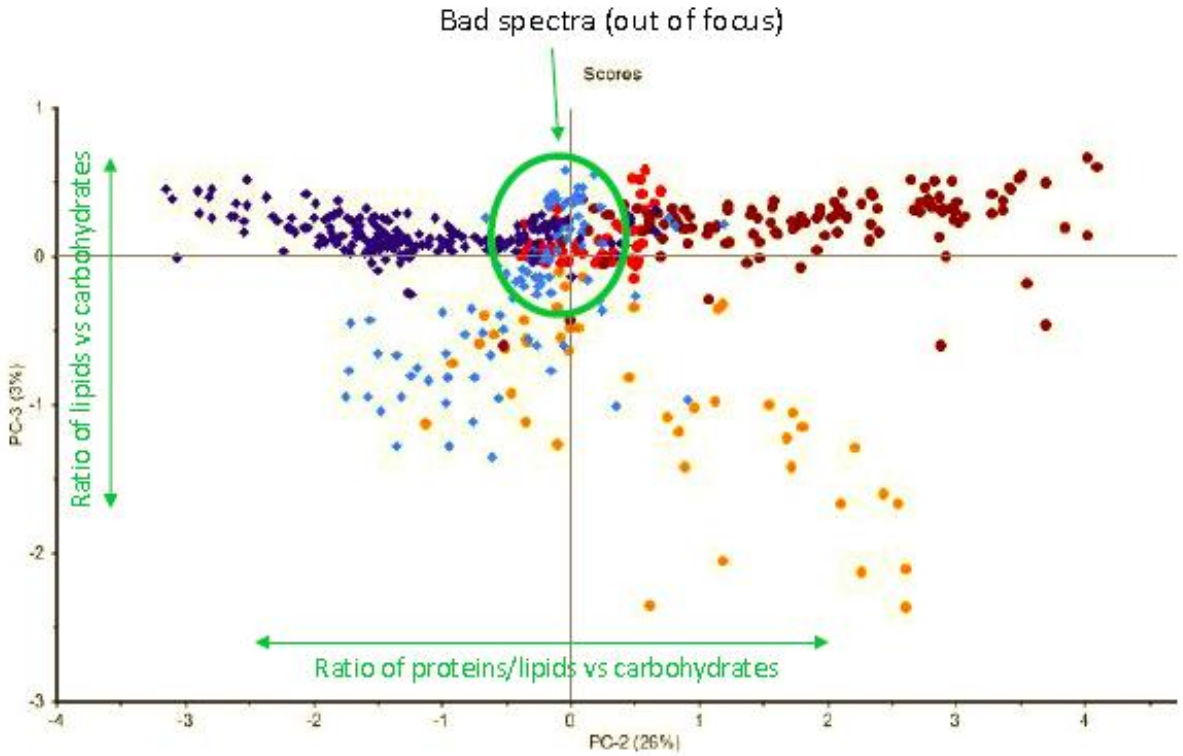


Figure 29: PCA plot of all species, all data, PC2 and PC3. Spectra are smoothed and pre-processed by EMSC. Percentage variance of the three first PCs are: 66 %, 26 % and 3 % Color description: deep blue: Cupressus, light blue: Juniperus, red: Betula, yellow: Fagus, and brown: Quercus. Species of the beech order are represented by dots and the pine order species by diamonds.

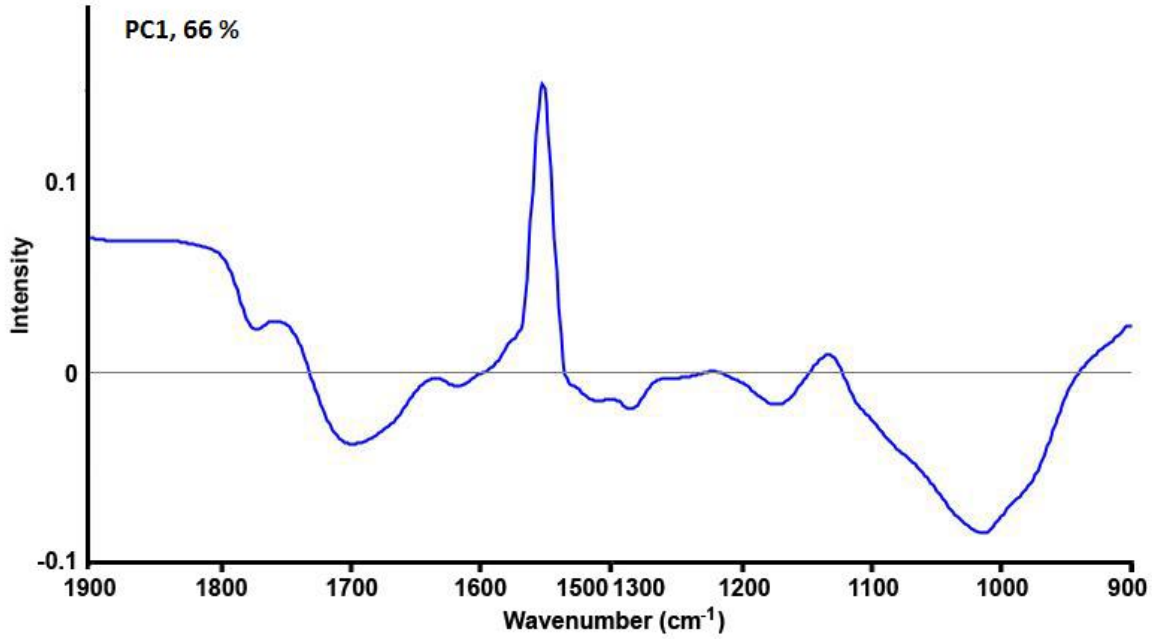


Figure 30: Loading plot of PC1 (66 %), all spectra.

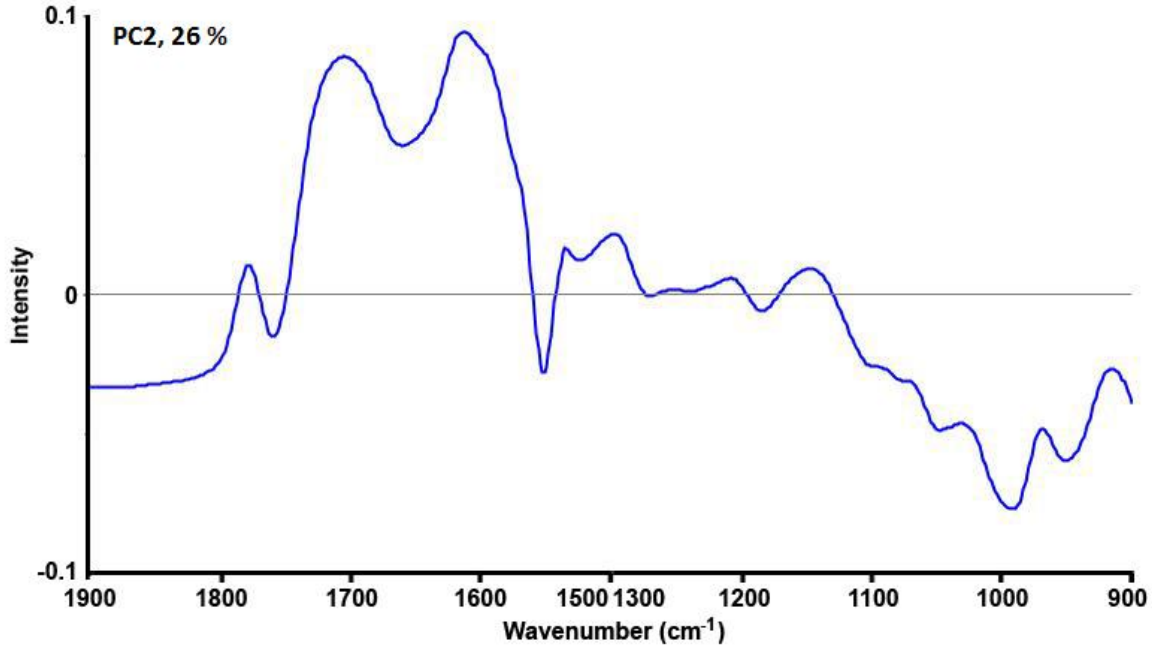


Figure 31: Loading plots of PC2 (26 %) all spectra

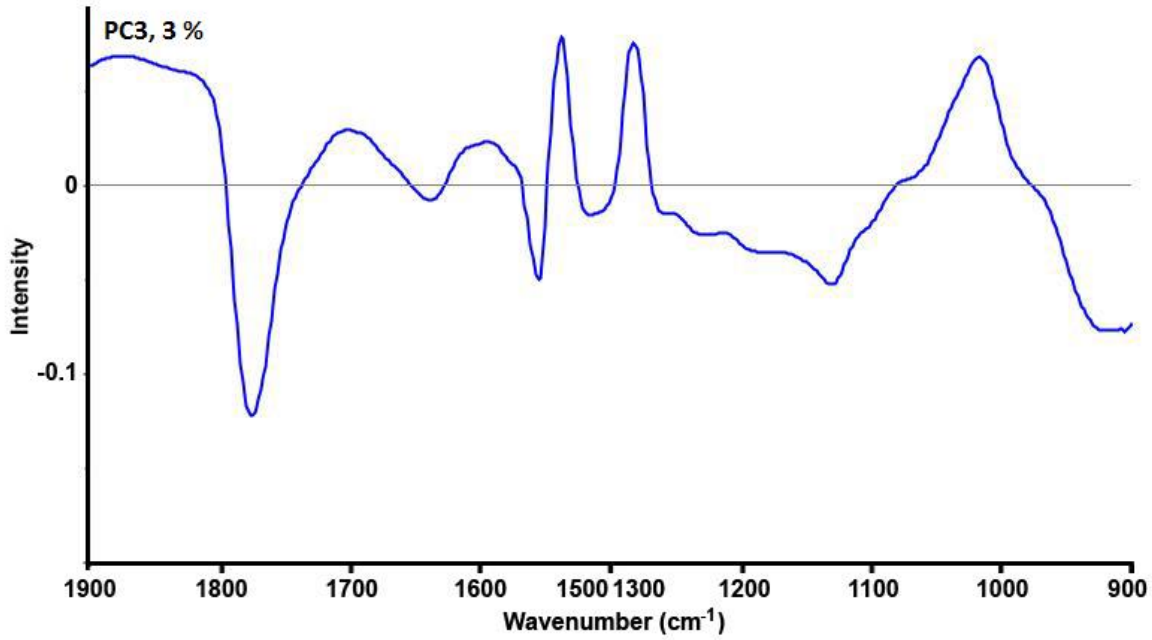


Figure 32: Loading plot of PC3 (3 %), all spectra

7. Results: Economical aspects

7.1 Service description

PollenID will primarily be a service business that offers pollen analysis. Figure 33 illustrates the PollenID services, the areas in need of further development and the system put into context. The FTIR spectrometer is stationed in Ås, so the analyses are performed there. The central should be operated by one technician. For the pollen traps, another type of adhesive strip (polyethylene-based) has to be used, as the one used today (polyester-based) is not transparent in the infrared. The new foil will be similar to the one used for measurements in this study. Changing the foil in the collectors is not a big operation, as contemporary studies include daily and weekly changes. The pollen samples are sent to Ås by A-mail, just as the procedure is today, only to another address. After analysis by FTIR spectroscopy and PCA, the results will be sent to the customer, or posted on the pollen forecast webpage. The details about how the information will be provided has to be discussed further, but there are several opportunities. Possibilities are E-mail, regular mail, webpage (the pollen forecast is already online), newspapers and smartphone applications.

In this study, the feasibility of performing analysis of single pollen grains by FTIR was examined. The methodology of an automated system needs further research, as it is now done manually. The positions of pollen grains on adhesive foil were chosen by hand, focusing was done manually, and liquid nitrogen had to be poured every 4th hour. The spectra were then downloaded from the measurement system (spectrometer with dedicated PC), and the PCA analysis was performed on a different computer. In the final product, every operation will be automated. There will be one system where the input is the pollen sample, and the output is the pollen analysis.

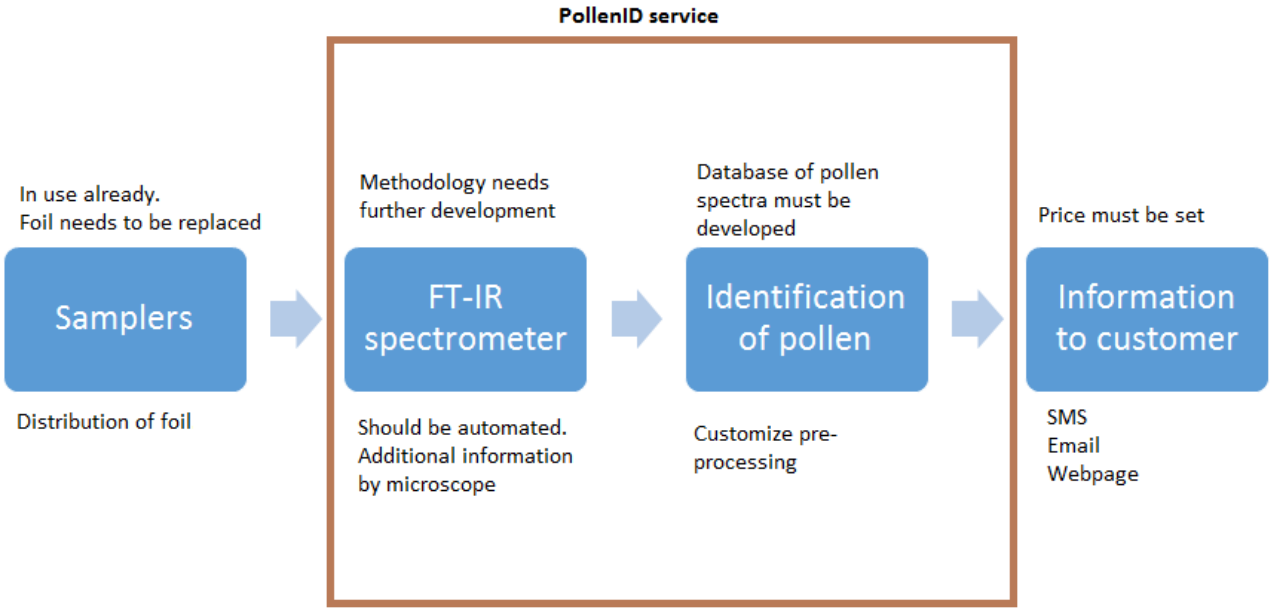


Figure 33: An overview of PollenIDs services, and further development needed.

7.2 Effects of allergy and pollen forecasting

7.2.1 Socio-economic effects related to allergy

According to Helserådet and the European White Paper (1997), the costs related to allergies is approximately 10 000 NOK per person per year [1]. About one million Norwegians suffer from allergies, so Norway's expenses amounts to 10 billion NOK per year, most of which are associated associated with allergic rhinitis. A Danish study from 2009 determined that pollen allergies cost the Danish community 7500 NOK per year per person [2]. These expenses include medicine costs, consultations, hospital care and sick leave. Assuming a population of 146 million patients affected by allergic rhinitis in Europe[2], the expenses are in total 1100 billion NOK per year. As the number of persons with allergies is increasing, the expenses will rise accordingly the next few years. In Denmark, the number of allergy sufferers is estimated to increase by 800 000 by 2020 [2]. Extrapolating to Europe, this gives an increase of 100 million allergists in total in Europe by 2020. Consequently, the expenses will rise by approximately 750 billion NOK per year by 2020.

Allergies do not only affect the economy. In addition to the physical symptoms, allergies can cause mental health problems. Impaired sleep due to nasal congestion is one of the major complications of allergic rhinitis [4]. This might lead to fatigue, irritation and work and learning impairment. Students

[2] 20 % of Europe's' population

with allergic rhinitis achieve on an average lower grades than their non-allergic colleagues for the exams taken during pollination season [2]. According to Meltzer (2001), Productivity at work is impaired with 35 to 40 % [41]. Meltzer also pinpoints that children might experience other issues related to allergic rhinitis than adults, experiencing emotional disturbance and feelings of isolation from an inability to participate in certain activities during the pollen season, such as playing on the grass or camping trips. This might lead to frustration, sadness, and anger [41]. Overall, it is shown that the quality of life in general is lower during the pollen season for patients with allergic rhinitis. In particular, difficulties with breathing, sleeping and sex, and the overall inability of performing everyday activities reduces the quality of life for the patients [2].

7.3 Effects and side effects of allergy medications

According to local pharmacies in ÅS, the most common pollen allergy medicines are: Zyrtec, Citrizin, Livostin and Aerius, which are all antihistamines [42]. This is in accordance with information provided by NAAF [22]. Common side effects of antihistamines are dry mouth, drowsiness, headaches, nausea and dizziness [42]. However, first generation antihistamines (FGAs) caused the most severe side effects, while the second generation antihistamines (SGAs) do not cause significant side effects [1, 5]. There is no difference in symptom relieving effect noted between FGAs and SGAs [5]. FGAs have been reported to cause drowsiness in 25 % of patients [5], and can, in some cases, affect driving to a larger degree than alcohol [5]. A research conducted in 1993 by Vuurman et al. demonstrates that the school performance of children treated with SGAs was almost as good as the performance of non-allergic children, while those treated with FGAs did worse than the placebo group [5]. FGAs have been evaluated to cause serious impairment of both cognitive and psychomotor functions, while SGAs cause mild impairment in comparison. However, when taken together (as recommended by Helserådet), a mild sedation effect might occur [5]. The sedating effect of SGAs also varies between the different medications, and might be stronger if higher doses than recommended are consumed [5].

A survey conducted in 2013 by Norfakta Markedsanalyse on 293 Norwegian doctors [43], states that doctors estimate that 7 out of 10 patients with allergic rhinitis experience adequate effects from the medication provided. In addition, 6 out of 10 use a combination of medicines. On the other hand, professor and head of department at Sørlandet hospital, Sverre Steinsvåg, does not agree. He mentions an Italian research which concludes that half of the patients with moderate to serious symptoms were still troubled by symptoms despite appropriate treatment [43].

7.3.1 Necessity of pollen forecasting

Many allergy medicines should be consumed before the pollen season to give optimal effect, making is necessary to keep track of the pollen concentration in the air. During the pollen season, allergy patients may be able to regulate their dosage according to the pollen prevalence [2]. For highly allergic persons special measures are needed, such as staying indoors or leaving areas when the pollen concentration of certain pollen types are peaking. Thus, it would be valuable to have detailed information about the start and the end of the season for specific pollen types. This could delay or otherwise reduce the intake of pollen medicines, subsequently reducing their unpleasant side effects and allergy symptoms.

A survey conducted by Markets- og Mediainstituttet (MMI) in 1998 concludes that more than 50 % of the 1000 allergy sufferers asked find the pollen forecast useful or very useful [2]. One out of five used the pollen forecast for medicine dosages, and seven out of ten checked the forecast more than three times a week. Since access to information on the internet is more available now than in 1998, a reasonable assumption would be that even more people make use of the pollen forecast today.

The survey (MMI 1998) also found that one out of five allergists reported less absence from work as a response to checking the pollen forecast. Extrapolating this number onto today's population of five million, this signifies 200 000 individuals benefiting from increased productivity today in Norway. However, it is important to highlight that the pollen forecast is guiding, and it is practically impossible to follow it blindly.

7.3.2 Accuracy of the pollen forecast

There are several uncertainties associated with the pollen forecast. Firstly, the success rate of pollen identification by human palynologists should be questioned, but there is little information to be obtained on this topic. Dell'Anna *et al.* (2009), mention a success rate of ~80 % estimated in their aerobiological monitoring network [13]. Secondly, it is challenging to create a model that will accurately predict the upcoming pollen concentration. According to Eggen *et al.* (2013), pollen forecasting is challenging due to gaps in current knowledge about the dynamics of pollen production and dispersal, and the complexity related to how pollen impacts human health [44]. Further, Eggen states that "pollen counts alone are unlikely to give an accurate indication of health risks for allergy or asthma sufferers, as pollen potency can vary widely" [44]. Usually the pollen forecast predicts the pollen concentration for the next day, and occasionally a few days ahead, based on the pollen concentration in the air yesterday, or previous days. Research by Smith & Emberlin (2005) examined

the possibility of constructing a medium-range (7 days) pollen forecast for grass pollen in north London [45]. The models were constructed on basis of grass pollen and meteorological data from 1990 to 1999, and tested on data from 2000 and 2002. The accuracy of the models were 67 % in 2000, and 47 % in 2002 [45].

7.4 Industry analysis

The «pollen monitoring market» is hardly a market at all. The pollen count and analysis is done manually, at scientific institutions. So far, this market is completely non-commercial, meaning that no one takes out profit from pollen forecasting, as it is too expensive for private companies to buy pollen analyses, and the service is practically not available for private customers. The method of pollen analysis is in principle the same worldwide.

7.4.1 Samplers

Sampling is the first step for making the pollen forecast. There are a few different samplers on the market, but the most common is the Hirst volumetric spore sampler from Burkard Scientific (commonly referred to as the Burkard sampler) [46]. The sampler draws in air at 10 l/min through a narrow slit, and the pollen grains are stuck to a glass plate with a sticky surface that moves past the slit at 2 mm/h. The glass plate has to be changed every 24 h, and will then contain a 48 mm long trace where any point will represent the pollen concentration in the air at that hour. Alternatively, a rotating drum covered by adhesive tape can be used. The drum performs a full rotation in seven days, and can therefore be changed only once a week. The sampling efficiency of the sampler ranges from 68 % to 85 % [46]. The Burkard sampler costs 2550 GPB [47]. Figure 34 shows the Burkard sampler with additional equipment.

Figure 34: The Burkard sampler ***A:*** *The exterior of the Burkard sampler [48],* ***B:*** *The equipment following the sampler: Extra drums, double sided tape, Melinex tape, 1 kg Gelvatol, carrying box, flow meter (from the datasheet of the Burkard sampler).*

German Bluestone Technology GmbH developed another pollen sampler: The Personal Pollen Sampler[49]. As revealed in the name, this sampler is a smaller, more practical option for individual sampling. The sampler comes in two editions: the wearable and the portable. The wearable can be attached to a belt etc., and weighs only 92 g. The portable edition is slightly bigger (130 g), and includes a touch screen for user interaction, to track allergy symptoms etc. Both samplers feature a GPS-tracking system, so it is possible to know where the pollen was encountered. They also log the temperature, pressure and relative humidity. According to Bluestones webpage [49], the sampling efficiency matches the Burkard samplers'. Photos of the samplers proved difficult to obtain. When visited a month later (30.10.14), the webpage only feature one type of sampler, and describes it as a prototype.

Inside the Personal Pollen Sampler, there is an exchangeable cassette (each cassette can be used for two full days) containing an adhesive strip [49]. This strip has the same function as the tape in the Burkard sampler, and can be used for analysis. Bluestone offers an analysis service, where the strips will be analyzed microscopically (manually), and the data made available on the customer's account in the Personal Pollen system. The cost of this service depends on the type of the analysis. In an e-mail,

Torsten Sehlinger, one of the directors of Bluestone Technology, explains; “The customer can choose the time resolution, the pollen to be quantified, if spores shall be counted, the priority of the analysis, and the quality of the counting (e.g. full area, 50 %, 10 %)”. The prices range accordingly, from 4 EUR to 28 EUR. The most common order is all pollen types at 10 % quality, with 2-hour resolution and 10 days delivery. This service cost 13 EUR. The sampler itself cost 400 EUR. There is no information available about the accuracy of the analysis method, but as it is performed with optical microscope, the same accuracy as for the pollen forecast is assumed (~80 %) [13].

7.4.2 Pollen monitoring in Norway

The pollen count in Norway is done by one pollen analyst and one assistant, at NTNU (Norwegian University of Science and Technology) in Trondheim [50]. There are 12 pollen collectors in Norway, located in Kirkenes, Tromsø, Bodø, Trondheim, Ørsta, Førde, Bergen, Geilo, Stavanger, Kristiansand, Lillehammer and Oslo [23]. The positions of the pollen traps are shown in Figure 35. The pollen concentration in the air changes considerably over short distances [8], so between these locations the pollen forecast will be inaccurate, or simply completely wrong. For instance, the pollen forecast for Eastern Norway (Østlandet), virtually only applies for Oslo. One reason for the big differences is the gardens and parks in the bigger cities, with imported plants not found in more rural parts of Norway.

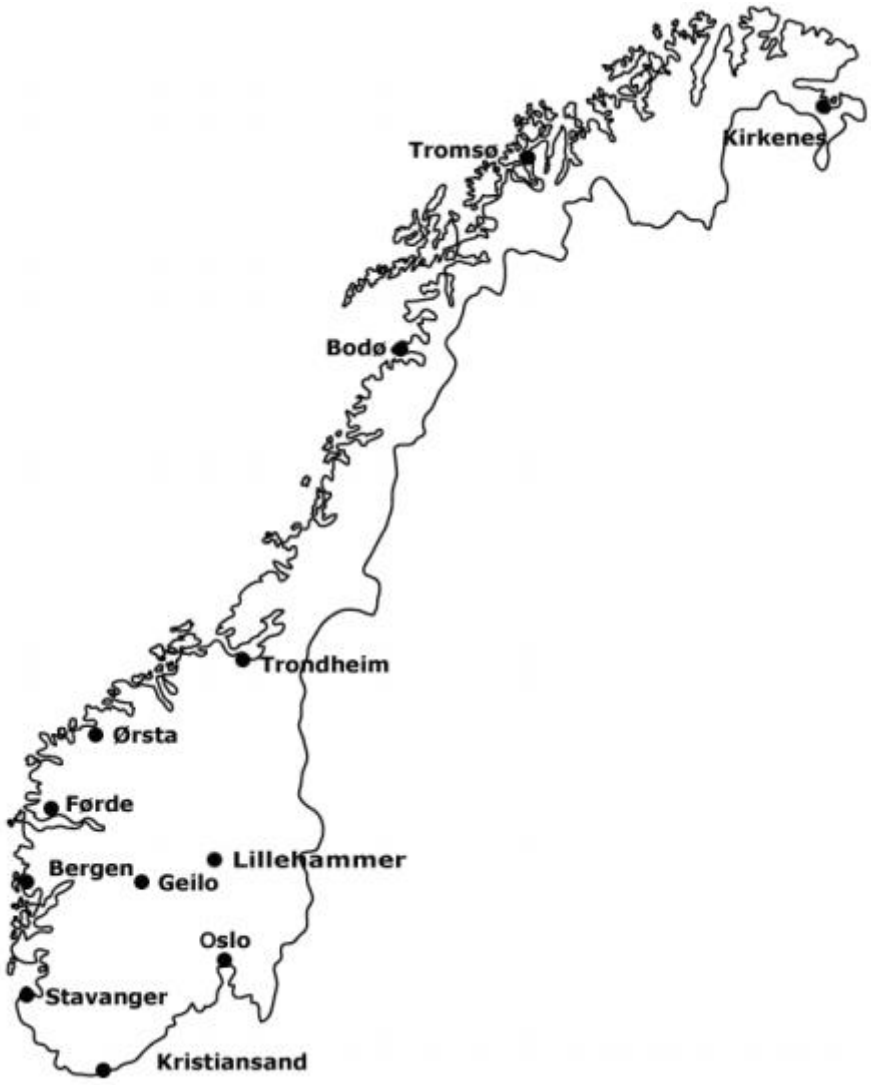


Figure 35: Positions of pollen samplers in Norway. From [23]

Every day the tape inside the collector, where the pollen grains are stuck, is sent for analysis at NTNU in Trondheim by A-mail [51]. In high season, the analysis of one sample takes about one hour, but due to different conditions (e.g. the weather), not all of the samplers will be full every day [52]. Approximately 10 hours of work per day can be assumed. During high season, a few assistants are hired to facilitate the pollen counters. Still there is not always enough time to analyze all the samples, according to pollen analyst Hallvard Ramfjord. Therefore, the pollen forecast is based on not only the pollen count, but also on historic data and the general experience from previous years.

7.4.3 The need for a pollen database

As commented by Holt & Bennett (2014), the performance of a pollen counter, human or machine, depends on two key aspects: finding the pollen grains and identify them [9]. However, when

performed by a human palynologist, the analysis might suffer some inadequacies due to human error. Subjectivity will be present in the analysis to some extent, although the palynologists do their utmost to ensure that they are consistent [9]. Further, there might also be disagreements between palynologists, as human analysts might suffer from fatigue and over-familiarity with their samples. According to Holt &Bennett, it has been shown that machine systems are always more consistent than human palynologists when repeatability has been assessed [9].

As mentioned in section 2.8, "Criteria for automated pollen analysis", other areas in need of improvement is speed, finer resolution and determination, in addition to objectivity. An essential tool to achieve this is to assemble a database that could be shared electronically, such that all pollen monitoring systems would base their identification on the same reference data. Today, human pollen counters use references such as books and reference slides [9], which cannot easily be shared. Automated system could in addition constantly update the database, and store information about the identified pollen grains. However, when based on morphology, the database may contain some errors, as it would be based on human recognition.

In addition to the before mentioned problems, the pollen monitoring industry is about to encounter another difficulty: the lack of qualified personnel. Rather few people specialize in palynology, and most of them are engaged in other industries, including increased demand from geology industrial sector. In sum, automation in this sector is certainly needed. Today there is one attempt of an automated pollen monitoring station, which is in use at four locations in Germany and Switzerland: the BAA500 developed by Hund GmbH in Germany.

7.4.4 An automated pollen monitor – Hund in Germany

The only instrument developed for automatic pollen analysis is BAA500, developed by Helmut Hund GmbH in Germany. The Hund system is based on morphological analysis of pollen grains by optical microscopy. Morphological features are obtained by image analysis and the identification is done on the basis of these morphological properties [53]. The whole process is automated, from sampling to counting. For the analysis, both a 3D-printer and a digital camera with a microscope are used. 70 pictures are taken of each sample, and a computer system determines the pollen taxa and count. The results can then be transmitted to the weather station at a given time interval, e.g. every hour. BAA500 can analyze 8 to 24 samples a day, at 90 % accuracy. However, this claim is doubtful as there is no scientific documentation approving this accuracy. Only the six most common allergenic pollen types can be identified: Hazel (*Corylus*), Alder (*Alnus*), Birch (*Betula*), Grass without Rye (*Poaceae*),

Mugwort (*Artemisia*), Ragweed (*Ambrosia*) [54]. BAA500 also recognizes six types of non-allergenic pollen. Figure 36 shows the exterior of BAA500 in A, and B the interior.

Figure 36: The pollen monitor BAA500. ***A:*** *Exterior, from [55],* ***B:*** *Interior, from [56]*

BAA500 seems to satisfy all the basic needs when it comes to pollen analysis, but where Hund differs from PollenID is in the matter of service. Hund mainly distributes BAA500 as an entire system, it is a large and expensive instrument, which makes it less available. It is, however, possible to send in a pollen sample, but this seems to be an exception. According to Dr. Jörg Haus from Hund it is "also possible to prepare one or more sample plates and to insert it into the machine via a dedicated port." [57] When asked how to manage this, he replies, "As we use special sample carriers for our pollen monitoring system, every other carrier would be problematic to employ", and suggest that they send one of the carriers designed for the system to prepare, heat and apply the pollen grains, and send back to Germany. He does not state how much time the analysis will take, or how much it will cost. Therefore, it can be assumed that the instrument is designed primarily to analyze the pollen in its immediate surroundings, and this service will not be offered on a larger scale. The price of BAA500 varies, but Dr. Jörg Haus writes that it "will be more than 100.000 EUR".

BAA500 is currently in use four at four different sites in Germany and Switzerland: München, Payerne, Wetzlar and Aachen. There is not much information about the stations in operation, but their

findings can be viewed at Helmut Hunds webpages. Figure 37 shows the concentration of grass pollen in München in May 2014.

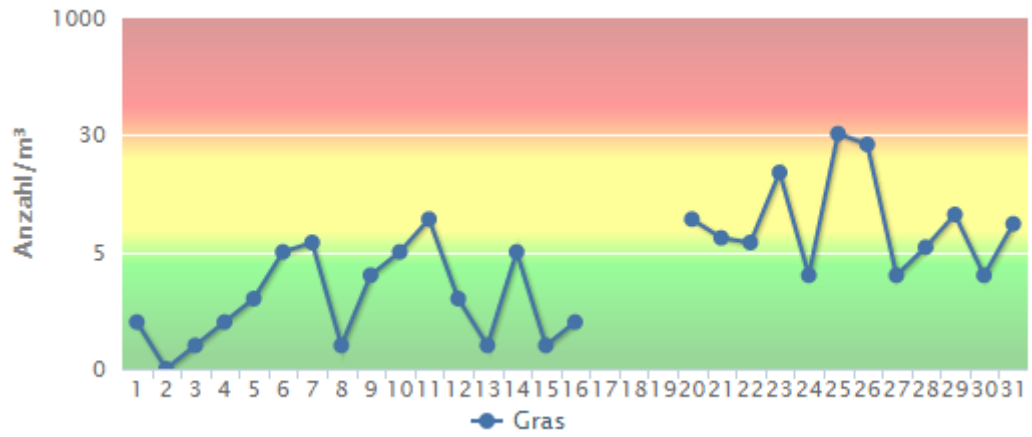


Figure 37: Concentration of grass pollen, München May 2014. From [58]

In sum, Helmut Hund GmbH seems to offer the technology of an accurate and reasonably fast pollen count, but lacks the practical implementation to a commercial market. Large institutions may consider acquiring BAA500 to make the pollen forecast, but it would limit the geographical area of the forecast, as BAA500 primarily counts the pollen grains where it is situated. Buying instruments for more sites is rather expensive, at least for private customers and smaller firms. With a service-based method, like PollenID, more sites will be covered at a much smaller cost. However, the results of the analyses are possibly acquired faster with the stationary instrument.

7.4.5 Key success factors

A new service in the market should meet some criteria. For the pollen monitoring market, the following three criteria was determined:

Measurement accuracy – the ability to classify the pollen grains correctly
Efficiency – complete the pollen count at a given time
Cost-efficiency – provide the service at a small cost

In other words: the new method should have an equal or higher accuracy rate than manual work; complete the pollen count in shorter time as human palynologists and also the pollen monitor BAA500, at a smaller cost. More concretely, a new system should provide an analysis at 80 % accuracy, completed in less than one hour. The cost per analysis is not available neither for the conventional method, or from Hund GmbH. However, the analysis price from Bluestone (28 EUR) and the price mentioned by Stillman & Flenley (50-200 USD (1995 currency)) can be used as guiding.

7.5 Customer profile

It appears that the only customer interested in pollen analysis in Norway today, is the Norwegian Asthma and Allergy Association (NAAF). This seems also to be the case for most other countries – the state provides the pollen forecast, which is usually done by researchers at scientific institutions [15, 24]. However, there seem to be an interest in better technologies for pollen analysis. According to Bo A. Gleditsch from NAAF "more efficient systems/technologies for pollen analysis are obviously of interest" [59].

Besides NAAF, other possible customers might be:

- Municipalities. Providing local pollen forecasts will help the allergy sufferers in that geographical area, which might cause a reduction in expenses related to sick leaves (see section 7.3.1 "Necessity of pollen forecasting").
- Hospitals. The welfare of the patients is of great concern in hospitals. Allergic patients might benefit from knowledge of the pollen concentration, so they can be properly medicated
- Sport and recreational areas. A local pollen forecast could improve the experience of both performers and audience, as they can be medicated before the sporting event.
- Private persons. Combined with Bluestones Personal Pollen Sampler, it is possible to customize individual pollen forecasts. This may be an option for extremely allergic (or curious) persons. Another option is to make an application for smart phones, and charge a small fee per year.

Obviously, there is no commercial market for pollen analysis today; it has to be created. The target groups do currently not see the need for such a service. Even though the expenses due to allergies amount of billions of NOK, and there is an opportunity of reducing them, the target groups are on tight budgets, and the willingness to prioritize pollen analysis seems low. As an example, the district doctor of Ås, Sidsel Storhaug, simply replied «No interest» when asked about the need for local pollen analysis in Ås [60].

With sufficient marketing, and more information about the expenses related to pollen allergies and how accurate pollen forecasting might reduce these costs, there may be a possibility to create customers from the above mentioned target groups. In addition, if the method is proven more efficient and accurate than the existing, NAAF might be interested.

An informal market research indicate that there might be an interest for a pollen application. NAAF provides an app based on the current pollen forecast, that costs 7 NOK per year. About half of the persons asked replied that they would definitively be interested in buying a pollen app if they knew it would be more accurate and detailed than the existing one, or if it was recommended by friends. The pollen app from NAAF has been downloaded by 500+ Android users, which means something between 500 and 1000 persons. On the other hand, the free pollen app, developed by developer Håkon Nilsen, has more than 10 000 downloads and a better rating. There is no information on what kind of technology this app is based on, but since there are only 12 pollen samplers in Norway that are counted, the free pollen app is probably based on the data from NAAF. As the general knowledge about pollen analysis is limited, claiming a better method of pollen identification might be a weak argument in this context.

7.6 Cost and price estimates

With current settings, the FTIR microscope measures one sample in one hour. Within one hour, 120 pollen samples (pollen grains) are measured, as one pollen sample takes approximately 30 seconds to measure with 64 scans. The measurement time might be further reduced to 5-10 seconds per pollen sample by reducing the number of scans per pollen. To what extend this is effecting the discrimination ability for different pollen species future studies need to show. Currently the pollen positions are selected manually, a procedure which in a future application can easily be replaced by an automatic camera-controlled system. Assuming some time between the measurements, for setting the positions, re-filling liquid nitrogen etc. at least 60 analyses per day can be feasible in the future. More samples can be prepared at the same time, the positions set, and the instrument will measure automatically. As the pollen season is only 6 months a year [16], we can assume a capacity of 10 920 samples analyzed a year (if the instrument is running 7 days a week for 26 weeks).

7.6.1 Cost estimate

Table 2 shows the estimated costs for PollenID for one year. All costs are in NOK.

Table 2: The costs for PollenID per year

Labor costs	1 000 000
Depreciation	385 000
Maintenance	10 000
Materials	5 000
Rental costs	50 000
Total	**1 900 000**

This estimate is based on the following assumptions:

- Labor costs: one person with a bachelor in technical engineering operates the instrument, and sends out the information.
- Depreciation: economic lifetime for the FTIR microscope is 6 years.
- Maintenance: the instrument does not require much maintenance
- Materials: liquid nitrogen and other possible materials
- Rental costs: the rental costs include both working space and instrument
- No acquisition cost, as the instrument is already paid for.

The cost per unit (sample) at maximum capacity will therefore be: 1 900 000/10 920 = 174 NOK

7.6.2 Price estimate

The existing provider of pollen analyses in Norway, NAAF, does not charge anything per sample. However, there is a cost connected with the analyses (labor cost, transportation, maintenance etc.) of 2.5 million NOK per year [59], which is more than the costs estimated for PollenID. The only price available is the analysis price from Bluestone, which is 28 euro for the most precise analysis. Since Bluestone and PollenID will not work in the same geographical market, this price will not have a big impact on sales, but could be used as guiding. However, the analysis from Bluestone is of poorer quality than the one from PollenID, and the value can therefore be assumed lower. Stillman & Flenley states that the price for one sample analysis is between 50 and 200 USD [8]. The price is given in 1995 currency, and would correspond to 77 to 307 current USD[3]. In conclusion, there are not sufficient guiding prices in the market, and we have to consider the production costs and the market demand to set a price.

As the market for pollen analysis is not exactly commercial today, it might be necessary to create demand. Possible customers, such as municipalities, hospitals and sport arenas, have managed without their own, customized pollen forecast so far, and might not see the use for the service. Therefore, it can be argued that a penetrating price strategy should be chosen, and the service provided at a low price. Based on the calculated cost per unit of 174 NOK, the suggestion for a price is NOK 200 per analysis, adding some profit to the product.

[3] According to http://futureboy.homeip.net/fsp/dollar.fsp?quantity=200&currency=dollars&fromYear=1995 (9.12.14)

7.6.3 BEP-calculations

In the case of PollenID the fixed costs are the labor costs and the depreciation. The variable cost is NOK 1.4 (15000 NOK annual costs/10920 analyses), and the price per sample is set to NOK 200. According to equation 10, the break-even quantity is therefore:

$$\frac{1\ 885\ 000}{(200 - 1.4)} = 9492\ units$$

given a total fix cost of 1 885 000 NOK. This gives 9 492 analyses per year, which is within the capacity of the instrument.

Setting the price per sample at 300 NOK gives a break-even quantity of 6 313 units per year:

$$\frac{1\ 885\ 000}{(300 - 1.4)} = 6\ 313\ units$$

NAAF needs 2 148 analyses per year (12 samples per day, seven days a week for 26 weeks). Basing the price on this demand and adding some for other customers, a quantity of 3 000 samples per year would be reasonable. Then the break-even price would be 630 NOK:

$$\frac{1\ 885\ 000}{3000} + 1.4 = 630\ NOK$$

7.7 SWOT-analysis

The SWOT-analysis provides an overview of the company's internal strengths and weaknesses, and external opportunities and threats. Table 3 shows the SWOT-analysis for PollenID.

Table 3: SWOT analysis for PollenID

Strengths	**Weaknesses**
Provides analyses quickly Does not require specialized education Is capable of covering a larger geographical area Avoids scattering More accurate than existing method Easily implied to existing samplers	Not fully developed Not completely automated
Opportunities	**Threats**
Few competitors Prevalence of allergies is increasing, and the existing pollen forecast is inaccurate	No interest/customers Difficulties with "creating a market" as no commercial service of this sort exists today

Possibility of expansion (both geographically and in product mix) Could easily adapt to indoor air monitoring	Only useful six months a year Uncertainties connected with the effects of pollen forecasting

The SWOT-analysis illustrates that there are several strengths connected with the technical aspects (such as speed, simplicity, accuracy), but serious threats associated with the market (no interest).

Part III: Discussion and conclusion

8. Discussion

Short summary

8.1 Technical aspects

The method of using FTIR spectroscopy for identification of different pollen types has been proven successful in various studies [12, 13, 35]. However, most attempts measured multigrain pollen samples. Only Dell'Anna *et al.* (2009) measured single grain pollen, with varying results, most probably due to problems with scattering effects [13]. In this study scattering was avoided by embedding the pollen grains in paraffin between two layers of polyethylene foil. The single grains could therefore be measured without further treatment. The potential for the analysis of single pollen grains is obvious. This is a great achievement compared to previous studies where bulk samples of pollen where successfully used for identification [6, 12, 13]. The identification of bulk samples is not suitable for air monitoring, since for the bulk analysis a large amount of pollen samples of the same pollen type is required. This is only feasible when pollen are directly collected from trees.

This study also shows that differentiation of pollen taxa is possible by using FTIR spectroscopy although parts of the IR-spectra were removed due to saturation regions from paraffin and polyethylene foil. These regions are small relative to the complete spectra. Therefore, sufficient information about the chemical composition of the pollen grains was obtained, such that PCA could be carried out, and differentiation between the pollen types was possible. The pollen types could be separated by order (pine/beech), and species. This is especially apparent in Figure 23, the PCA plot of the second derivative and EMSC corrected data of 20 chosen spectra of each taxa. Similar clustering patterns as in Figure 23 are visible in the PCA plot of the original data of 20 chosen spectra (Figure 20), and in the plot conducted by PC3 and PC4 (Figure 26). Clear grouping tendencies are even visible in PCA plots of the whole dataset (Figure 29), without cutting the saturation region and calculating the second derivative. This demonstrates that biochemical differences between the pollen species are clearly caught by the new sampling technique, allowing the clear distinction of different pollen types.

The loading plots of the PCs show that the main signals in the PCA are due to lipids, proteins and carbohydrates. The peak at 1750 cm^{-1} visible in most loading plots, corresponds to the C=O stretch associated with lipids [35]. Strong signals are visible in the carbohydrate region, 1200-900 cm^{-1}, and in some loading plots (Figure 31 and 21), vibrations at 1650 cm^{-1} and 1550 cm^{-1}, associated with proteins, are apparent. This is in accordance with knowledge of the main discriminative chemical components in pollen [35].

Scattering is an important issue in spectroscopy, as it can distort the spectra [14]. By embedding the pollen grains in materials with approximately the same refractive index, the problem of Mie-scattering is avoided. It is therefore not necessary to apply complex algorithms with limited success rates to the spectra; the spectra can be used for PCA after standard pre-processing. This both simplifies the process and makes it more robust.

A crucial issue FTIR microscopy of pollen is the focusing of the microscope optics. Currently it has to be performed manually. For a routine analysis, an automation is needed. The "out of focus spectra" were removed before the final PCA (Figure 18), to see how well the method worked if all the spectra were focused. The other possibility was to measure the pollen again with different focusing, but this was not done due to time limitations. Bruker system is able to perform automated focusing, however comprehensive testing of this instrumental option was out of scope of this study. In addition, the Bruker spectrometer is capable of changing the aperture size, such that even the largest pollen grains can be measured (in this study aperture size was fixed at 25×25 μm), which is not possible with the optical microscope. Therefore, removing the poorly focused spectra was appropriate, as this will not be an issue when the method is fully developed and an autofocus system is implemented.

The reproducibility of the measurements is not adequately documented, as most of the pollen types were only measured once due to time limitations. Only *Betula pendula* (silver birch) was recorded twice at two different dates. The two *betula*-measurements are not distinguishable by the spectra, which indicates good reproducibility. Concerning the other measurements, some dispersion is noticeable. This could be because all pollen grains are slightly different, or it could be the result of an experimental error. In addition, the inexplicable pattern of the loading plot of PC3 of the second derivative PCA (Figure 27) might be caused by fringes. These two issues will be subject to further experiments.

This study is a proof of concept. Further development is needed in some areas, such as the automated focusing mentioned previously. The most demanding task is to create the database of different pollen spectra. In addition, a standardized quality test has to be developed, to reject non-pollen particles automatically before starting the measurements. As for now, the method seem promising, and the remaining tasks are time-consuming, but not unattainable.

Another advantage is that the FTIR technique can without any major instrumental modifications be combined with pollen identification by optical microscopy and image analysis, such as the BAA500 pollen monitor from Hund GmbH, since the infrared microscope is combined with an optical

microscope. This could further improve the accuracy and precision of the analysis. However, this requires further research.

8.2 The automated system

The pollen classification method described in thesis, has the potential to satisfy most of the needs addressed by Stillmann & Flenley (1996) and Holt & Bennett (2014):

- Volume: more sampling sites can easily be covered, and a considerably larger amount of pollen grains identified.
- Speed: with the settings used for the measurements discussed, one sample would take one hour to analyze. That speed matches the time consumed by a human pollen counter. With less scans per pollen sample, the analysis can be completed three or even six times quicker. However, the quality of the analysis cannot be guaranteed by this study, and needs further investigation.
- Objectivity: the 'personal equation' is completely eliminated as machines will not be subjective or suffer from fatigue. In addition, a classification algorithm, such as PCA, makes expertise on spectral interpretation unnecessary, so misinterpretation of spectra will not be a problem.
- Determination and resolution: according to previous studies [6, 12], determination on species level is possible with FTIR spectroscopy.
- Compatible with existing system: the paraffin/foil embedding used for measurement can easily be used in existing pollen traps, and transported the same way as today.

However, the accuracy of the method was not measured for this research. The same accuracy as in the study by Dell'Anna et al. (2009), 84 %, can be assumed since the methods are quite similar, with the exception of sample preparation. In fact, embedding the pollen in paraffin and polyethylene should result in higher success rates, as the problem of scattering is avoided.

The experiments considered are close to the real life scenario of pollen monitoring. When sampled outside in pollen traps, the grains will be more diffusely distributed, and nonpollen particles will also be apparent in the samples. These issues can easily be solved by above mentioned techniques. The other factors of the experiment will remain the same.

8.3 Socio-economic effects

There is an uncertainty associated with the effect of pollen forecasting on allergy patients' lives, and hence with the methods' influence on allergists' daily life. A survey conducted in 1998 by MMI, mentioned in Helserådets report [2], indicate that more than 50 % of pollen allergists find the pollen forecast useful or very useful, and that one out of five report less work absence as a result of checking the forecast. This actually indicates that for 80 % of allergy sufferers the pollen forecast has no influence on their work absence. It is not further stated by Helserådet what "useful" means in this context. There are numerous factors influencing a persons' life, and the pollen forecast can only be seen as guidance, and not followed blindly. In conclusion, it seems that an updated and more extensive research on the topic is needed to draw conclusions on the effects of pollen forecasting. Furthermore, Eggen *et al.* (2013) highlights the fact that forecasting the pollen concentration is challenging due to limited knowledge of pollen dispersal and impact on human health. Other issues associated with the well-being of allergy sufferers might be relevant to address first, such as diagnosing a larger amount of people and improving the medications.

The effect of allergy medicines is another topic connected with high uncertainty. Documented effects of the medications and statistics have proven difficult to obtain. The European White Paper [1], Helserådet [2] and NAAF [3] all mention different studies on the topic, but without references. Words such as "many", "some" and "several" are used about the allergy patients that experience good effects from the medications. One article in a Norwegian online medical magazine [43] mentions a survey conducted among 293 Norwegian doctors. The survey states that 7 out of 10 allergy sufferers experience an adequate effect from allergy medicines. However, the professor and doctor interviewed in the same article did not agree with its findings. The survey itself is not publicly available.

Based on the factors mentioned above, it can be assumed that not all allergy patients receive sufficient beneficial effects from allergy medicines. If the medication is of limited use for the allergy sufferer, so is timing the intake of medicine with the pollen forecast. The principle advice from most allergy associations and experts is allergen avoidance [1, 3]. In the case of pollen, this is practically impossible. Some people may have the possibility to go away for the whole or parts of the pollen season, but most people do not have that possibility. If the pollen forecast could provide information on the pollen concentration in the different parts of the city, the patient's daily life would become easier. However, such a detailed forecast is virtually impossible.

In conclusion, pollen forecasting seems to have some effect on the welfare of allergists, but it is uncertain to what extent. There is also uncertainty associated with the effects of allergy medications, and therefore with the pollen forecast. Pollen avoidance is difficult, but the forecast provides some

guidance. Some people might be able to go away for parts of the pollen season, or work from home. Overall, pollen forecasting might give some impact on the lives of allergy patients, but other issues may be more important.

8.4 Market and interest

If proven successful, there is a high possibility that NAAF could be interested in using PollenID's services instead of the conventional method for pollen analysis. On the other hand, expanding the market may prove difficult. The representatives asked from the target groups show limited interest about the service. This response might be due to budget restrictions, and that they do not find such a service necessary. The general knowledge about how the pollen forecast is made today is limited, and the need for improvement is not apparent to the public. With sufficient marketing, an interest might be created, but this heavily depends on a better documented effect of pollen forecasting on allergists' lives.

The SWOT analysis (table 3) implies more strengths than weaknesses, and the weaknesses mentioned can be resolved by further development of the methodology. Considering the opportunities and threats, the amount is approximately the same, but the threats are more crucial than the opportunities. If there is no interest for the product, or the pollen forecast is proven to have little or no effect on allergy sufferers' lives, providing pollen analyses is pointless. These topics need further investigation.

If there is an interest, and the needs are proven, the system could be sold as an all-inclusive package. That is, the pollen taxa database and computer program, customized for the area to which it is sold. The customer would need to acquire a FTIR spectrometer and pollen samplers. PollenID would only cover the software providing the pollen analysis. Today there is no market for selling the entire system, but it might be a possibility in the future.

Compared to the pollen monitor BAA500 from Hund GmbH (ref), it is likely that PollenID can provide more precise pollen analyses. The PollenID method is based on biochemical analysis instead of morphological features, which gives the advantages of a more successful classification as the chemical composition of species is approximately constant, as opposed to morphologic properties that can vary considerably. In addition, PollenID will use a database built on objective information. Furthermore, the BAA500 does not have the property of expanding the market. It is too expensive for small companies and private persons, and possibly an investment too extensive for hospitals and municipalities, that are on tight budgets and might not need a pollen forecast every day. However, the

Hund GmbH system holds one advantage over PollenID; the analysis will be completed sooner, as the sample does not have to be sent anywhere.

8.5 Cost and income aspects

The operating costs of PollenID were calculated to 1.9 million NOK per year, which is considerably less than the 2.5 million NOK budgeted by NAAF for the conventional method. However, this does not include the development costs, which need to be estimated. The whole system comes with a large investment cost (the FTIR spectrometer), which would be relevant if the system is to be sold as a whole.

For the price, a penetrating pricing strategy was chosen, considering the vague response in the market. A lower price might also make the analyses more available, and provide information to more allergy patients, which is in correspondence with the vision of PollenID. Consequently, the price was set to 200 NOK per sample, which is assumed to be a manageable price for customers such as municipalities and hospitals. At 200 NOK, the BEP-calculation indicates that 9 492 analyses have to be sold each year to break-even, which is within the capacity of the spectrometer (10 920 analyses/year). However, there might not be a market for such a large amount of analyses. NAAF, the only certain customer of pollen analysis, would request 2 184 analyses per year (12 pollen samples, every day for 26 weeks). In comparison, a price at 300 NOK might be a better option, which is still at the level of the analysis of Bluestone (and a reasonable price), and gives a break-even quantity of 6 313 analyses per year. If the break even quantity was set to 3000 units (analyses), to match the analyses needed by NAAF and some more, the break-even price would be 630 NOK, which again might restrict the market. Analysis of the price sensitivity was attempted during the research, but none of the representatives from the target groups had any suggestion to this.

The pollen forecast is only necessary six months per year, but in the remaining six months, there are other opportunities to use the system. With the same technology and method, both indoor air and molds and spores analyses can be provided, and the system could even be used for criminal investigations [61].

9. Conclusion

9.1 Technical aspects

The method of pollen analysis described in this thesis is a promising technique for quick and accurate pollen forecasts in the future. By embedding pollen grains in paraffin between two polyethylene foils, high-quality infrared microspectroscopic spectra of single pollen grains could be obtained. Multivariate analysis of the obtained spectra demonstrated that pollen samples could be clearly grouped based on their biochemical information that was caught by infrared spectroscopy. In addition, scattering was almost completely due to the fact that the paraffin film surrounding the pollen samples reduce the refractive index change on the pollen surface and thus remove Mie scatter effects. The method meets most of the criteria stated by Stillman & Flenley in 1996, and Holt & Bennett in 2014: 1.It has the possibility of covering more sampling sites and measure a larger amount of pollen grains in the same sample. 2. The analysis of one sample will cost considerably less than the price indicated by Stillman & Flenley (200 NOK vs. 50-200 USD). 3. The time needed per analysis matches the time used by human palynologists, and objectivity is retained. 4. In addition, the analysis method is compatible with the existing infrastructure in the field of pollen monitoring. The only change needed is the adhesive tape in the pollen samplers, which has to be replaced with polyethylene foil and paraffin, similar to those used in this study. However, further development is necessary to provide a fully automated system. See section 10, "Further work".

9.2 Economic aspects

The economical estimates show that the operating cost of PollenID amount to 1.9 million NOK, which is lower than the budgeted cost of the existing method (2.5 million NOK). However, development costs are not estimated in this study.

There is an uncertainty connected to the actual effect of pollen forecasting on the quality of life of allergy patients. The effect is not adequately documented, and other issues might be of higher importance. For instance, having more people diagnosed, and improving the quality of the medications, could potentially have a great impact on the well-being of allergy sufferers. Nevertheless, pollen forecasting is an important issue. Since the new method could provide a more precise pollen air monitoring, allergens may be avoided to a higher extend.

The Norwegian Asthma and Allergy Association may be interested in a new pollen monitoring method, if it proves more successful than the conventional method of pollen analysis. Other potential markets must be created, as there is no such service provided today, and the potential customers do not

seem to see the need for customized pollen analysis. Regardless of the extended market, the field of pollen monitoring and counting is in need of improvement and modernization, and FTIR spectroscopy in combination with multivariate analysis has shown potential for replacing the existing method of pollen analysis.

10. Further work

This study demonstrates that identification and separation of pollen species is possible by the use of FTIR spectroscopy and multivariate analysis. However, several areas need further development for the system to be completely automated. Firstly, an algorithm for rejecting non-pollen particles and setting the positions of the pollen grains that are to be measured is needed. Secondly, autofocusing has to be developed. Finally, a database of the infrared spectra of pollen grains (and possibly other airborne particles) has to be created. This is perhaps the most extensive work, but also the most important.

Concerning the measurements, reproducibility has to be assured. Further, the effect of reducing the number of scans per position (pollen grain) must be examined. In addition, further research on the paraffin and foil embedding should be performed, to determine the optimal materials. For this study, paraffin was transferred onto the foil by hand, which makes the equal distribution of paraffin difficult. Thus, it is expected that an industrially produced foil with a paraffin film will improve reproducibility even more. Besides, for the system to be implied in real life, a paraffin spread foil should be mass-produced to be available to those handling the pollen samplers.

Finally, a more extensive market research should be conducted. In this thesis, the possibilities of extending the market was examined, but further investigation is needed. The price sensitivity should be determined, and the interest for pollen analysis mapped in more detail.

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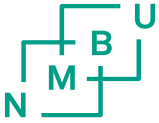

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