Abstract

Pollen grains are one of the major causes of respiratory allergies. We briefly review the role of aerobiological monitoring centers in providing information about airborne pollen concentration for helping allergic patients to reduce exposition to allergens and to start appropriate drug treatments. Spatial and temporal resolution of this information should be increased. However, the effort required by the technique currently used to identify and count the airborne pollen grains hinders this improvement. Therefore, innovative classification approaches were investigated. In particular, we studied the feasibility of methodologies developed in the spectroscopic and biomolecular field, with the aim at providing rapid, accurate and possibly automated airborne
pollen concentration measurements. In this chapter the state of the art in this field is outlined as well as our obtained results; we discuss both the proof of principle of the applicability of such techniques for pollen quantification and, from a more practical point of view, the feasibility of implementing them in aerobiological centers as routine identification tools. Possible future improvements of developed techniques to solve current weaknesses are also examined.

1 Introduction

1.1 Health impact of allergenic pollen

The number of persons who experience allergic diseases like rhinitis, conjunctivitis and asthma in response to pollen of wind-pollinating plants [1-2] is continuously increasing. In these cases, allergy is an abnormal response of the immune system to non-infectious particles interacting with the mucous membranes of the eyes and the upper respiratory tract. According to a report by the World Health Organization (WHO), allergy is one of the most widespread disease, whose prevalence in the world population is around 20% [3]; in the European Community countries between 8% and 35% of young adults show sensitization to pollen allergens [4].

The development of allergic diseases is due to a complex interaction of heredity and environment. The so-called hygiene hypothesis [5] suggests that their rising prevalence is related to lacking exposure to microorganisms in early life, because of higher standards of cleanliness. Consequently, the immune system development would drift to an increased susceptibility and overreaction to environmental stimuli, such as pollen, resulting in allergy-promoting responses. Subsequent research studies put in perspective this hypothesis: evidences were found that “early infections do not seem to protect against allergic diseases”, while early germ exposure reduces the occurrence of allergic diseases later in life [6]. On the other hand, respiratory allergy is less frequent in people heavily exposed to orofecal and foodborne microbes [7].

The onset of allergic diseases is a complex process, therefore there are probably more factors and mechanisms to consider, and surely to discover. As an example, poor outdoor air quality has been associated with the increasing trend in the respiratory allergy prevalence; this seems evident in urban populations when compared with rural ones [8]. Exposure to air pollution would increase the allergic inflammatory response [9-10] and have microscopic effects on pollen grains structure and their expression of allergenic proteins, therefore increasing the pollen allergy potential
Additionally, climate changes can increase the risk of allergic sensitization by influencing the pollination calendar; this can lead to an increase of the seasonal amount of airborne pollen. Climate changes can also contribute to the introduction of non-endemic plant species, having in some cases high allergic potential (as an example ragweed) [11]. In Europe, the associated sensitization to pollen allergens in children is likely to have doubled during the last three decades [13].

Early diagnosis and proper medical treatment are fundamental not to compromise the quality of life in allergic sufferers and also to reduce significant social and health care costs. The correct management also prevents worsening of the disease. To a better control of respiratory allergies, more and more patients integrate the use of medication with proper changes of their habits and plan of their activities, based on current pollen concentration information. For allergic patients it is important to know the trends in pollen counts to take some measures to prevent or reduce symptoms: to formulate and apply a plan of pharmacologic therapy management in a timely manner, to limit outdoor exposure when pollen counts are high, to use air filters at home, work and in the car, to plan vacation accordingly to pollen forecast of the location that will be visited.

1.2 Pollen monitoring: characteristics and limits

The types and amounts of pollen grains dispersed in the atmosphere at any given moment in a given area depend on vegetational [14-15] and meteo-climatic factors [16-18]. The first ones are sufficiently stable, as they include the vegetational composition of the area and the associated flowering times of the various species, the types of pollination and the pollen grain morphology. On the contrary, meteo-climatic factors are changeable and their influence on variations in pollen counts, before and during the blossoming period, are hardly predictable. It is therefore extremely important to establish pollen counting networks for monitoring airborne pollen in a systematic and constant way, in order to provide reliable information about its composition, abundance and period of presence. Data gathered over several seasons are also fundamental to elaborate reliable pollen forecasts. Therefore, a large number of monitoring stations has been set up across Europe since last decades of the 20th century.

In Italy, since 1985 the pollen bulletin and forecast have been issued by the Italian Network for the Aerobiologic Monitoring (R.I.M.A.®), coordinated by the Italian Association of Aerobiology (AIA)\(^1\)

\(^1\) http://www.ilpolline.it/
The network is composed of about 80 monitoring sites, providing information on the concentration of airborne pollen belonging to 36 families and 15 genera. They follow a standardized protocol for pollen sampling and treatment, which has become the standard UNI 11108 in 2004. The network is part of the European Aeroallergen Network - European Pollen Information (EAN-EPI)\(^2\). Twenty-five European countries are integrated in it and information are gathered from more than 400 pollen counting stations all over Europe [20]. EPI coordinates national networks and manages to make uniform their services by fixing common standards for the sampling, the pollen preparation techniques, the identification and the interpretation of the pollen counts data.

For conventional analysis [14,21], an Hirst-type volumetric pollen trap [22] is used with an air flow of 10 liters of air per minute, i.e. the mean respiratory capacity of a human being. Inside the trap a silicon-coated (sticky and transparent) strip rotates at constant velocity (2mm per hour), therefore allowing to recover the different hours and days of monitoring. The pollen and other airborne particles impact onto the adhesive surface. This is removed after a definite period of sampling and cutted in 24-hour portions, which are mounted on microscopic slides and analyzed in the laboratory. Pollen grains are identified and counted under an optical microscope. A daily average pollen concentration is calculated, expressed as the number of pollen grains of a certain plant taxon per cubic meters of air. These data are rarely available each day, while usually the bulletins are issued weekly (in the first days of the succeeding week). In any case, the published data do not represent real-time counts determined at the time of the report [23].

The pollen grain identification is done manually by highly skilled operators; it is a laborious and time-consuming task. Transmitted light microscopy with brightfield illumination is used to investigate the morphology of the grains. The relevant parameters are size, shape, polarity, symmetry, aperture, surface and inner structures. The identification is carried out by comparing these structural features with those of reference pollen. Examples of pollen grain images at different magnification are reported in Figures 1 and 2. Because of different flowering times, on a single microscope slide up to some thousands of pollen grains and several different pollen taxa can be analyzed. Although it is a commonly accepted rule to read only a small portion of the slide (the Italian standard UNI 11108:2004 recommends an analysis of at least 20% of the area), this activity keeps the trained personnel busy for an average of 16 hours a week, depending on the season and the skill of the operator.

\(^2\) http://www.polleninfo.org
For respiratory allergy sufferers, the pollen monitoring approach reveals some critical limitations. The identification and counting task is highly demanding in terms of time, people training and, consequently, money. This hinders a higher spatial and temporal resolution of the information, its real-time diffusion [23], and the possibility of increasing the sampling volume for more accurate measurements of low airborne pollen concentrations [23,24]. In addition, the pollen identification result is in part resting on the effectiveness of personal reasoning schemes [25]; therefore the data reproducibility is partially affected by the individual skill of the qualified personnel [23]. The related drawbacks are that more rapid, detailed, precise and accurate information about the exposure of population to allergenic pollen is currently not achievable: (i) the real-time availability of the bulletins would improve their effectiveness; (ii) increasing the count frequency would allow to follow the fluctuations of pollen concentration occurring during the day and point out to allergic patients the hours of higher stress; (iii) increasing the number of monitoring stations would better represent the pollen variability related to phytogeographical and vegetational characteristics of the interested area; (iv) considering larger counts would allow to improve the accuracy of measurements in case of low concentrations of pollen having high-allergy potential; (v) higher data quality is related to higher reproducibility, reduced uncertainty in concentration measurements, higher sensitivity and specificity in the identification of the different pollen taxa.

For all the above considerations, the development of an automated or semi-automated system for pollen grain identification and counting is highly opportune. This was already pointed out by Stillman and Flenley [25], who stressed the need to automate both sample preparation and analysis.

The approaches reporting on the automation of one or more pollen identification steps can be distinguished into three major types: (i) the application of microscopy together with image processing and pattern recognition techniques, in line with the manual analysis; pollen grain identification by less conventional approaches, exploiting (ii) the chemical characterization of taxa by different spectroscopic techniques and (iii) the application of biomolecular analysis methods to the identification of taxon specific DNA sequences.

Next sections take a deeper look into such different approaches.

2 The automation approaches
2.1 Microscopy and pattern recognition techniques

An automated optical pollen counting system should reduce as much as possible the need of manual interventions. Neither specialized sample preparations nor uncommon or expensive laboratory equipment should be required [26]. In addition, a modular design could allow to substitute/upgrade or add components. These characteristics may promote the spread of the system and extend its use to non-expert people. Apart from the already automatic gathering of pollen samples, all other steps of the current monitoring process should be automated and integrated into a single system: (i) the preparation of microscope slides; (ii) the location of pollen grains on digital images captured from a slide (i.e. the object recognition step typical of digital image analysis, called in the rest of this chapter “pollen grain recognition”); (iii) the application of image processing and/or pattern recognition techniques to extract informative features from the images and to use them in a supervised machine learning study, performing the classification of the recognized pollen grains into different taxonomic categories (i.e. the supervised classification step typical of machine learning theory, called in the rest of this chapter “pollen grain classification”). To accomplish step (ii) and (iii) a robotic optical microscope, a digital camera for image acquisition and a computer for image analysis and pollen classification are necessary.

The first research works about automated pollen identification were done around 1990 using scanning electron microscope (SEM) images [27-28 and reference therein]. High classification rates were obtained, but the approach required expensive microscope equipments and specific sample preparations. Therefore, the technique was judged difficult to fully automate and not particularly suitable for routine monitoring [26]. In addition, these works did not tackle the problem of pollen grain recognition. In 1997, first works based on conventional light microscopy were discussed [29-30 and references therein] and from then on different studies exploiting this optical technique have been proposed in the literature. None of them accomplished all the steps (i)-(iii) for a fully automatic system. However they often presented really innovative solutions for some of these tasks.

Supervised classification techniques learn a set of classification rules from well characterized training instances (therefore the supervised characteristic) and can consequently predict the class membership of unknown cases. In this chapter, the considered supervised methods are Multilayered Perceptrons (Artificial Neural Networks), Support Vector Machine, Instance-based learning (KNN/K-Nearest Neighbour algorithm) and statistical learning algorithms like the naïve Bayes classifier. Unsupervised machine learning techniques are based on exploratory methods, requiring no a priori knowledge about the samples. In this chapter, Hierarchical cluster analysis (HCA) is considered, that groups data from the computation of their dendrogram.
France et al. [26] proposed the first method for automating both the process of pollen recognition and classification using a neural network. Therefore, they moved the first step toward a fully automated procedure exploiting 2D images. However, they used a small dataset that did not represent accurately pollen grain variability and only three different pollen taxa were considered. In addition, the pollen density on the slides was sufficiently low to avoid grain clumps and grain occlusions by dust or other particles, which are instead quite common in routine air sample images.

The semi-automated system developed for the European project ASTHMA [31-32] worked on a sequence of 2D images from different focus planes obtained from pollen slides dyed with fuchsin. Interestingly, the features extracted for the classification were consistent with those of palynological knowledge. In fact, they included global measures on 2D images (like apertures, size, shape, etc) and a few 3D characteristics, like pore structure, reticulum and cytoplasm (the sequence of 2D images allowed to carry out the classification with 3D features). The best achieved classification rate was 77% by using a simple neighbor classifier together with a leave-one-out cross validation method on an image reference database of 350 pollen grains of 30 different pollen taxa. The classification discussion is though limited to “some preliminary results relating to a few allergenic pollen types, like Urticaceae or Poecaceae, or some groups of pollen types, like reticulate groups”. In addition, only images without other particles on the grains were used.

Some studies were focused on more representative and challenging sets of air sample images. In [33], an airborne pollen dataset containing 3686 pollen grains belonging to 27 plant species was considered. The acquired images showed high and low density of particles, big objects inside and even bubbles of glue: in the recognition process, which was therefore particularly critical, a sensitivity of 93.9% was achieved, but the precision was 8.9%, (i.e. a high number of false-positive recognitions). A Bayesian classifier was applied to the automatically detected spots including only the eight most numerous pollen classes and the false alarms (i.e. the false positive identifications), and a classification rate of 64.9% was achieved. Although the classifier correctly discarded most of the non-pollen particles (i.e. the false alarms were not wrongly classified into one of the pollen classes), the results obtained in the recognition and classification steps pointed out that a reliable image dataset and a significant number of pollen taxa seriously complicate the automation process. In [34], color and shape features were exploited to recognize 65 pollen grains in 17 image stacks from successive focal planes, obtaining a sensitivity of 86% and an improvement of the precision, equal to 61%, with respect to [33], but also in this case a manual intervention would still be needed. In addition, the described approach (limited to the automation of the only pollen grain recognition step) presented a large computational complexity that did not reduce significantly the
time when compared with that required for a single conventional analysis session.

Rodriguez-Damian et al. [35] tackled the interesting problem of discriminating pollen grains of 3 species of the same Urticaceae family, which are morphologically similar and are not distinguished by the traditional approach, where these pollens are normally classified to the family level. Palynologists may distinguish the species of the family Urticaceae using SEM but not with a conventional light microscope. In [35], grains of manual prepared samples, without clumps or occlusions, were first located by the Hough transform and their silhouettes were extracted. Shape and texture features were then computed and used for the classification task, in which different classifiers and feature selection methods were tested. The best achieved classification rate was 89%, outperforming palynologists’ results. The authors acknowledged that improved performances could be obtained using 3D images and that results should be further validate on a larger independent testing set.

The work described in [23] requires a specific comment. To our knowledge these authors only presented a fully automated pollen counting system, which integrates pollen sampling, microscope sample preparation, image analysis and pollen grain classification. It has been developed since 2003 within the OMNIBUSS project (Online Monitoring of Airborne Allergenic Particles). It can work “as a standalone system for field measurements providing hourly online pollen data”. The technical solution developed (in particular the advanced microscope equipment) and the economic requirements moved this system away from the requisites of common and inexpensive laboratory equipments previously discussed. However, this approach was remarkable. They used grey-scale based invariant features on 3D volumetric data and a Support Vector Machine (SVM) to carry out grain classification. Only preliminary tests were reported in [36], based on automatically prepared samples from the on-line monitor. Pollen grains belonging to 3 different genera (Alnus, Corylus and Taxus) were added to aerosol samples collected in a (winter) period of low pollen concentration. The automated classification exploited an external reference database and achieved a recognition rate and precision equal to 91%. The authors planned to extend the reference database to represent at least 20 different pollen taxa and to provide a consequent and necessary final validation of their system. They also planned to enter production in limited numbers in years 2007-2010. Meanwhile, in [36] they presented an improvement of the segmentation of pollen grains in the acquired digital images, developed for this pollen-monitor system.

Finally, some tests about a working system “AutoStage” that automated the recognition and classification steps and worked with different sample preparations were presented in [24,37 and references therein]. A multi-layer perceptron classifier exploited shape and gray-value based
texture features. The whole system accomplished the desirable low cost requirement.

2.2 Alternative approaches: classifying pollen by spectroscopic techniques

The approaches described in this section focus on the biochemical characterization of the pollen grains by use of vibrational spectroscopies and microspectroscopies (Raman and FTIR – Fourier transform infrared) as rapid and non destructive techniques for structural and molecular characterization of a biological sample, also in conjunction with multivariate statistical analyses, as first proposed in 1991 by Naumann et al. [38].

Raman and FTIR spectra, particularly in the mid-infrared, allow to determine the global composition of a given biological sample in one single experiment, providing highly specific “fingerprints” which include complementary information on functional groups or bonds in the biochemical components such as proteins, lipids, nucleic acids and carbohydrates. FTIR and Raman are complementary techniques in that different selection rules apply for absorption (FTIR) and scattering (Raman) by a molecule. FTIR spectroscopy and microspectroscopy (obtained by coupling a spectrometer to a vis-IR microscope, as shown in Figure 3) are particularly easy-to-use, rapid and versatile. Depending on the optical properties of the samples, many experimental techniques can be utilized, such as transmission, diffuse or specular reflectance, and attenuated total reflection (ATR) [39]. Using conventional sources, micro-FTIR in the mid-infrared allows to reach down to 10-20 µm spatial resolution, limited by the signal to noise ratio. On the other side, the micro-Raman technique has a lower speed and demands relatively complicated instrumentation, but is suitable for highly space resolved investigations (~ µm), provided that fluorescence effects are avoided with an appropriate choice of the laser excitation wavelength.

In pollen monitoring studies, information provided by these techniques is a complex chemical fingerprint used for supervised (and sometimes unsupervised) classification of pollen grains of different taxa, obtained by applying multivariate data analysis techniques. In the spectroscopic field, studies for the development of a fully automated monitoring system are probably premature; published works preferably focused on the possibility and limits of these spectra in characterizing pollen grains, and only seldom discussed about a possible practical implementation of their results.

As to Raman spectroscopy, Laucks et al. [40] showed the possibility of analyzing pollen particles directly from the atmosphere by trapping them in an electrodynamic balance chamber coupled
with a Raman spectrometer. This approach discarded the problem of water solubility of flavonoids present in [41]. In addition, the single pollen grain trapping avoided the sample preparation for Raman measurements and would therefore represent a possible solution for a future system automation. However, an appropriate charging method and a well controlled method for removing unwanted trapped particles were not completely achieved. In addition, the examined pollen grains gave rise to fluorescence that even after oxygen- or photo-bleaching (at 514 nm) did not allow to sufficiently distinguish among spectra of different pollen taxa. The authors also found that using a near-infrared (e.g. at 780 nm) laser source could reduce the fluorescence background and allow an increase of intensity of spectral structures in the 400-1600 cm\(^{-1}\) range, where the spectra appeared to be identifiable. However neither an automated classification approach was developed, nor this study involved a sufficiently representative set of pollen taxa.

Boyan-Goitia et al. [42] analyzed single pollen particles by Raman microscopy, requiring a minimal sample preparation by fixing pollen on an aluminum slide with an ethanol drop. Because of the fluorescence background and a strong carotenoid signal common to all spectra, only weak spectral intensity differences characterized the examined taxa and in particular pollen grains belonging to different species of the same family. No unique attributions of chemical fingerprints were in this case possible. The taxa considered in this work were absolutely not representative of a seasonal aerosol sample. On the other hand, the pollen grains were freshly harvested, as the authors (to our knowledge for the first time) observed significant variation of the Raman signal with pollen aging. Fresh pollen use is also unavoidable in the development of an alternative monitoring approach.

Ivleva et al. [43] used Raman microscopy, therefore obtaining spectra from single grains, and applied unsupervised multivariate analysis to cluster four different freeze-dried pollen taxa. As remarkable characteristics of this research, an aim was the actual shortening of the time needed for the pollen identification, necessary for a practical implementation of the approach. In addition, the Raman spectra, excited with a He-Ne Raman laser, showed a good signal to noise ratio and spectral resolution, as compared with previous works. Consequently, in the 400-1700 cm\(^{-1}\) range they exhibited spectral features, corresponding to characteristic pollen grain components (like carotenoids, proteins, nucleic acids, sugars and lipids), able to discriminate among the four different pollen taxa. Once more, to assess the feasibility of this approach for a routine monitoring to be performed in the aerobiological networks, a larger dataset of different pollen taxa should be considered.

A significant contribution to the chemical classification of pollen grains by Raman microspectroscopy was given by [44]. The authors collected spectra from 15 different plant
species related in some cases at the genus and family level. Five to ten samples were acquired from each pollen taxon (totally 91 spectra), to study not only the inter-species but also the intra-species variations. The complete Raman signature (380-1700 cm-1) was used to group the spectra by hierarchical cluster analysis. Results showed that the spectra of different individuals of the same species formed separate clusters. Therefore, the distinction by micro-Raman of pollen grains belonging to different species (in some cases within the same genus) was possible. In addition, phylogenetic groups were investigated, and the hierarchical clustering allowed to identify species belonging to the same genera and genera belonging to the same families. This approach showed potential in the development of a monitoring system based on Raman chemical fingerprints. However, the requirement for a fast, automated system was in part hindered by the need of 1 hour of laser irradiation on each sample to deplete its carotenoid content. The reason of this pre-treatment was that the signal of carotenoids was present in the spectra of each pollen taxa and was often stronger than any other molecular signal; it could therefore compromise the classification results.

In the literature, many studies described the application of FTIR spectroscopy to the chemical characterization of different types of biological samples. However, to our knowledge, [45-47] are the only papers discussing FTIR spectroscopy and microspectroscopy applications for pollen grain identification. Pappas et al. [45] first compared spectra of dried pollen from 20 different plants, collected in different regions of Greece. They demonstrated the existence of a peculiar spectral signature, unique for every pollen taxon, in the 1500-800 cm-1 range. The authors built a reference library of the spectra of the studied taxa. The chemical fingerprint allowed to distinguish among different pollen species by comparing unknown spectra with those of the reference repository. In particular, it was shown the FTIR spectroscopy capability of identifying different plant species of the same genus (Citrus and Cistus). FTIR spectroscopy does not require sample pretreatments; it allows to identify pollen grains in a non-destructive, simple and fast manner. However in [45] the samples contained a high number of grains of a single plant species, while airborne pollen grains of a single taxon are normally sampled mixed to other pollen taxa and can be present in limited quantities. Therefore further investigation is needed for a possible application of FTIR spectroscopy to routine airborne polling counting. An improvement in this sense is represented by the work of Gottardini et al. [46] that demonstrated the power of this technique in discriminating two different pollen taxa in a single very unbalanced mixture. Results should be validated on a larger representative dataset of allergenic pollen mixtures with different mixing proportions.
3 The CARPOL project

The authors of this chapter were involved in the biennial Project CARPOL, founded in 2006 by Cassa di Risparmio di Trento e Rovereto Bank Group. CARPOL aimed at exploring different innovative techniques for the rapid and possibly automated classification of airborne pollen. The project focused on two distinct fields of investigation: FTIR microspectroscopy and molecular biology. Results of both investigations were deeply discussed in [47] and [48].

3.1 FTIR microspectroscopy

Pollen samples of 11 plant taxa of 7 different families, typically monitored in our region (Trentino, Italy) during the flowering season, were collected. In one case (Cupressus arizonica, Cupressus sempervirens), two species belonged to the same genus. Thanks to the microscopic equipment (see Figure 4), five distinct pollen grains for each taxon were used to acquire five mid-infrared spectra in transmission mode. In this way we studied both the intra- and the inter-species variability. It is important noting that the classification of single grain-spectra automatically implies the grain counting and, straightaway, the estimation of the corresponding pollen concentration. Therefore, concerning the real applicability of alternative techniques to the monitoring currently performed in dedicated aerobiological stations, FT-IR microspectroscopy appears particularly promising. We achieved a classification rate of 84% by using a simple supervised KNN classifier together with a leave-one-out cross validation method, outperforming the “manual” classification accuracy. Therefore FTIR microspectroscopy was demonstrated to be feasible for pollen classification to the species level. When thinking about a possible implementation of these results into an aerobiological monitoring network, two limitations emerged: (i) for two out of 11 taxa the classifier performances were unsatisfactory. The reason of this result was that the different chemical pollen grain variability of different taxa was not adequately represented by the considered reference library. Therefore, increasing the number of training spectra would reduce the classification error; (ii) the spectra acquisition was a time-consuming step, which partially hindered the possibility of a rapid monitoring process. More details on this study can be found in Dell’Anna et al. [47].

3.2 Molecular biology

We investigated for the first time the feasibility of a real-time polymerase chain reaction (PCR)
approach for airborne pollen grain identification and quantification. Eighteen allergenic pollen species were considered in this study, which well represent the main allergenic pollens of our region. For DNA isolation from pollen grains, a modified extraction protocol was set up; it works both on free grains and on pollen immobilized on a monitoring tape. A bioinformatics or literature analysis allowed to identify taxon-specific DNA sequences and to design ex novo taxon specific primer pairs and probes (see Figure 5), able to start the amplification of the sequences by PCR, allowing the identification of the considered pollen taxa. Quantification assays on pollen grains were successfully performed on some single species (Ostrya carpinifolia, Betula pendula and Parietaria officinalis) and in mixes, applying the quantitative real-time PCR. An exhaustive discussion of this approach is reported in Longhi et al. [48].

4 Conclusion

In this chapter we discussed the characteristics and limits of the conventional approach to seasonal airborne pollen monitoring. The benefits deriving from the development of a system for the automated pollen analysis have been deeply analyzed: they may ensure more spatially and timely detailed pollen information to allergic sufferers. The literature review highlighted that the research tried to automate the microscopic pollen counting as well as to base the identification on biochemical or biomolecular pollen fingerprints. In the microscopy and spectroscopy approaches the classification step was carried out by supervised and sometimes unsupervised machine learning methods, which are typically objective and time-effective.

As a general comment, no approach met all the requirements of a fully automated system, i.e. both the entire automation of the process (from sample capturing and preparation to pollen identification and counting) and the low cost and easiness of use of the equipment. Major part of the studies presented in this chapter was indeed devoted to propose solutions for the pollen grain recognition and pollen grain classification step; in this respect, their results should be often extended to the identification of a more representative number of pollen species, and a major attention should be devoted to the discrimination of pollen species flowering in the same period.

In the spectroscopic field, further advances may be obtained applying the results of new efforts to understand pollen biochemistry. A consequent effective selection of spectral features may improve unsupervised classification results or allow the application of different classification methods. Concerning FTIR microspectroscopy, our research work is now dedicated to speed up the acquisition of FTIR spectra by using a suitable detector to simultaneously acquire a high
number of spectra. We also intend to go on with the characterization of airborne pollen mixture with more realistic mixing proportions using FTIR spectroscopy.

Concerning the biomolecular approach, to further speed up the developed method, the use of the designed primer pairs and probes could be tested in multiplex reaction for a simultaneous detection and quantification of different pollen taxa.

An inexpensive and fully automated system, providing fast, accurate, and precise information on the complete airborne pollen load is probably still not realizable in the short term. However, a decision support system, able to identify a proportion of pollen grains to be analyzed and indicating doubt or not classifiable cases to the human expert, acting as a supervisor, would surely reduce the time analysis and contribute to increase objectivity and quality of the data.
Figure Captions

**Figure 1**: Sample for microscopic evaluation and counting of airborne pollens. (a) *Fraxinus ornus* L., (b) *Cupressus sempervirens* L. and (c) *Artemisia vulgaris* L. Olympus BX51 microscope. Magnification 40x.

**Figure 2**: Sample for microscopic evaluation and counting of airborne pollens. (a) Helianthus tuberosus, (b) Zea mays. Olympus microscope. Magnification 100x (Courtesy of Laboratorio Biologico, APPA Bolzano, Italy).

**Figure 3**: The Bruker Vertex 70 spectrometer coupled to a Hyperion 3000 vis/IR microscope, which was utilized for the mid-infrared measurements described in [47].

**Figure 4**: The photo shows how the microscope allows to view the sample and to choose the desired aperture. A FT-IR spectrum can then be acquired from the selected measuring area. In this way, the single-pollen grain spectra studied in [47] were collected. In this image, a single *Alnus glutinosa* pollen grain is centered inside a 25×25 μm² aperture.

**Figure 5**: Amplification of extracted DNA - primer designed on *Betula*, as described in [48].
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