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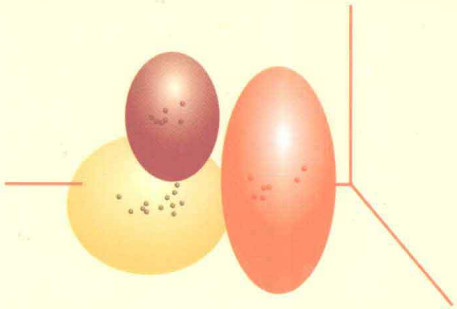
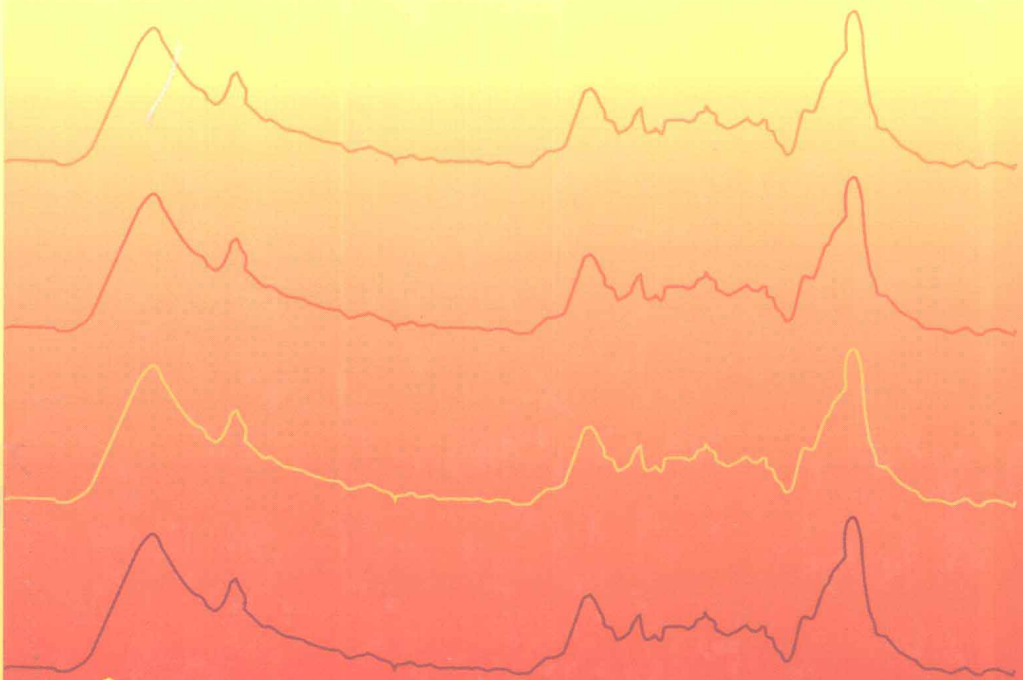


Infrared Spectroscopy for Complex Mixtures

Applications in Food and Traditional Chinese Medicine



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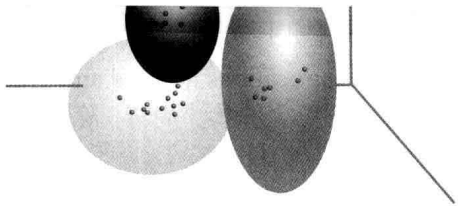

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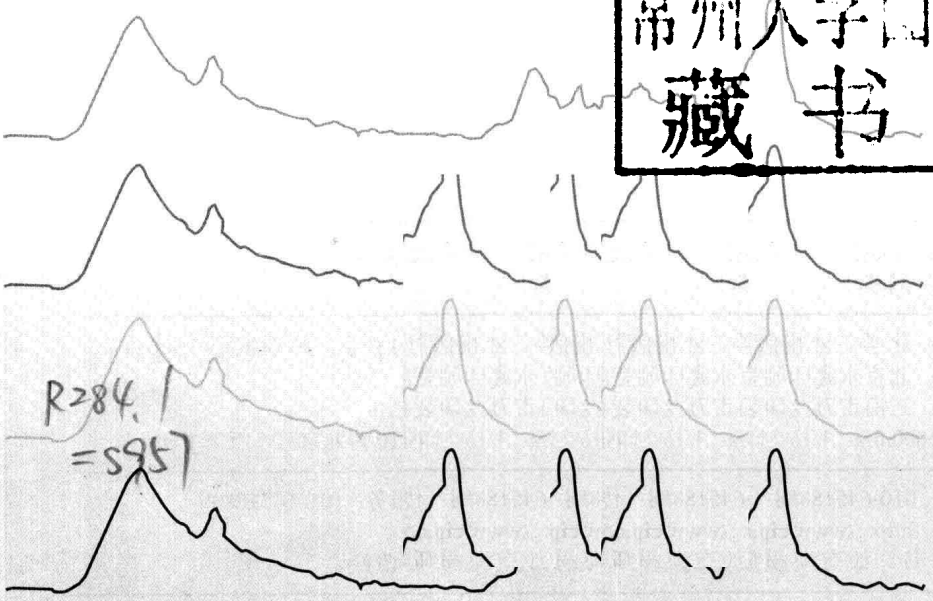

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Preface and Acknowledgements

It is generally not safe to draw a conclusion from incomplete data, which is right for analytical chemistry. More information concerning unknown samples is available, more accurate assessments can be acquired. For complex mixtures, such as pharmaceuticals, foodstuff, materials, biological samples and so forth, which the daily analytical chemistry copes with, it usually requires demanding analytical methods and costs a large number of labor, time and money to obtained complete information concerning every component. On the other hand, mixture samples may be incorrectly determined if only information concerning several components is provided by simple analytical procedures and the other components are not taken into account. Is there a compromise between laborious methods required for complete information and easy methods leading to inaccurate conclusions in mixture analysis?

Practically, the required information concerning each component in mixtures may be very diverse, according to particular sample types and purposes of analysis procedures. To get accurate assessments on complex mixtures, detailed qualitative and quantitative information concerning some critical components is usually necessary, while simple information concerning other subordinate components is often enough. Therefore, complex mixtures should be analyzed by gradual procedures. That is to say, for a complex mixture sample, a general view of total components should be obtained before the carefully detection for several specific components, and one component should be qualitatively identified before the quantitative analysis. If there are spatial or temporal variations in the mixture sample, a global observation should precede the study on regions of interest. In brief, complex mixtures have to be analyzed following the principle of 'fractional from holistic, qualitative before quantitative', which not only ensure enough information for accurate conclusions but also avoid laborious work for redundant information. A perfect mixture analysis methodology should be able to tell how to obtain appropriate information required for accurate assessments on specific mixture samples within affordable cost of time, money and labor. It is important to point out that components in mixtures are usually so complex that no analytical technique can provide enough information by itself. The combination of various techniques is very necessary for mixture analysis.

Why are we going to discuss the infrared spectroscopy in mixture analysis? Obviously, there are a number of advantages for infrared spectroscopy to be employed in complex mixture analysis. First of all, most compounds have fingerprint-like infrared spectral features, which makes infrared spectroscopy a versatile label-free analytical technique. A first hint of a mixture sample with no or little prior knowledge, as well as guidelines for further investigations, can be obtained from its infrared spectrum, which simultaneously contains information concerning total components. Fingerprint-like features make infrared spectroscopy not only suitable to profile the whole mixture sample but also be able to detect specific components. Secondly, with the help of computer and chemometrics techniques, both qualitative and quantitative information, such as the molecular structures and concentrations of compounds, can be acquired by infrared spectroscopy. Furthermore, the spatial and temporal scales of mixture samples studied by infrared spectroscopy can vary in a wide range, from ordinary dimensions to micro samples on the order of nanogram in weight or micron in size and variations within picoseconds. In addition, diverse sampling techniques ensure infrared spectra of mixture samples in any forms can be measured simply, quickly and non-destructively. Application of infrared spectroscopy costs little and causes no harm to the environment. In summary, infrared spectroscopy is able to meet various challenges in mixture analysis, from general observations of total components to detailed qualitative and quantitative detections of specific compounds, from accurate and fast measurements of homogeneous and stable samples to advanced investigations with high spatial and temporal resolutions. However, it is difficult for infrared spectroscopy to detect components with very low contents in mixtures, which can be complemented by other analytical techniques such as chromatography and mass spectrometry, as well as some separation and concentration procedures. For complex mixture analysis, the combination of infrared spectroscopy and other analytical techniques must be an important trend in future.

Above viewpoints are summarized from our researches on the analysis and quality control of foodstuff and traditional Chinese medicine (TCM) by infrared spectroscopy in the past years. Basing on abundant research achievements, we have introduced the term of 'macro-fingerprints' to underline infrared spectral features of complex mixtures, which are very different from those of pure compounds, and founded the theory of 'multilevel infrared spectral macro-fingerprints analysis' to guideline the application of infrared spectroscopy in complex mixture analysis. After the publication of more than two hundred articles in English and in Chinese and two books in Chinese, we are now writing this book in the hope of presenting the theory

and methods of infrared spectroscopy for complex mixture analysis in an organized and clear way. Chapter 1 of this book is a brief introduction of infrared spectroscopy, in order to help readers unfamiliar with this technique to better understand following chapters. Chapter 2 presents our thinking about principles for mixture analysis. Moreover, the theory of 'multilevel infrared spectral macro-fingerprints analysis' is introduced in this chapter, as well as a summary of difficulties, methods and advantages concerning the application of infrared spectroscopy in mixture analysis, especially for foodstuff and TCM. Various techniques for the interpretation of infrared spectra of complex mixtures are classified into three groups, which are named as 'tri-step identification', 'macro-interpretation' and 'intelligent analysis', are separately elucidated in Chapter 3, 4 and 5. The last three chapters, i.e., Chapter 6, 7 and 8, present some typical examples, which are all selected from our researches, for the application of infrared spectroscopy in investigation and quality control of TCM, including raw materials, intermediate and formulated products. By this book, we wish that the important role of infrared spectroscopy in mixture analysis can be shown to a certain extent, while some thinking and methods may be helpful to the investigation and quality control of foodstuff, TCM and other similar complex mixtures.

We are indebted to a great many people who contributed in many ways to these researches and the publication of this book. For their great contributions, we are especially grateful to the members of our research group in Tsinghua University. The graduate students and postdoctoral members are mentioned here: Bian Wei-Dong, Du De-Guo, Zuo Lin, Huang Hao, Hua Rui, Li Ying-Ming, Yu Lu, Zhan Da-Qi, Wu Jing, Qin Zhu, Liu Hong-Xia, Wu Yan-Wen, Zhang Yan-Ling, Lei Yu and Tu Ya. We are also grateful to many undergraduate students who contributed to these researches but not mentioned here.

A large number of our researches are completed with the participation of many scholars, students and other peoples from universities, institutes, manufacturers and governmental agencies. The number of participators is so great that we cannot mention everyone here, and there are also some people who contributed in ways that we may not exactly remember. We are indebted to all these people for their very important work.

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Contents

Chapter 1
Introduction to Infrared Spectroscopy

1.1 Introduction

Infrared spectroscopy (IR) has been one of the most important analytical techniques to elucidate the structures of unknown molecules in the past decades. However, this is only a small part of the applications of the IR techniques nowadays, while nuclear magnetic resonance (NMR), mass spectrometry (MS), X-ray diffraction (XRD) and some other methods are more common. With the innovations enabled by the Fourier transform techniques applied to infrared spectrometers, more and more applications are becoming available for modern IR techniques. Compared with the previous dispersive spectrometers, Fourier transform infrared (FTIR) spectrometers are able to collect a spectrum of a sample with much higher signal-to-noise ratio (SNR) and better wavenumber accuracy in a shorter time, which in turn makes it possible to measure the infrared spectra of samples in nanometers or processes lasting femtoseconds. Various sampling accessories, such as attenuated total reflection (ATR), have been developed and different kinds of samples can be measured directly and quickly. As it should be, computers make the spectral interpretation simpler and more versatile, e.g., to identify an unknown sample by searching its spectrum in the spectral libraries to find a match. As a label-free and fingerprint-like analytical technique, infrared spectroscopy is increasingly used for the analysis of complex mixtures, such as biological samples and foods. Combined with chemometrics techniques, both qualitative and quantitative information can be obtained from the infrared spectra of the mixture samples. [1-3]

As a simple, quick, direct and non-destructive analytical method, infrared spectroscopy can be used to measure either pure compounds or complex mixtures, can be used for either qualitative or quantitative analysis, and can be used either to study the spatial distribution of the components or to track changes over time, which gives this technique wide applicability both in the laboratory and in the factory. Besides the structural elucidation and identification of pure compounds, infrared spectroscopy seems likely to play a much more dominant role in the qualitative and quantitative analysis of mixtures, biological and biomedical samples, micro-samples and hyperspectral imaging as well as the investigation of dynamic processes and interfacial phenomena. [4-8]

1.2 Principle of Infrared Spectra

1.2.1 Generation of Molecular Spectra

Light is electromagnetic radiation, which can be considered to be two mutually perpendicular electric and magnetic fields being propagated as a sine wave. Therefore, two parameters, the wavelength (λ) and the frequency (ν), can be used to describe the feature of the light. The wavelength is the distance between two adjacent peaks of the electro- or magneto- wave and the frequency is the number of cycles per second. The relationship between the wavelength and the frequency is

$$c = \lambda \cdot \nu \tag{1.1}$$

where the c is the velocity of light, which is a constant value of $2.997\ 925 \times 10^8$ m·s^{-1} for all light in a vacuum.

Electromagnetic radiation can be divided into different wavelength regions, which are classified as radiowave, microwave, infrared, visible, ultraviolet, X-ray and γ-ray. The infrared region includes radiation with wavelength between 0.78 μm and 1000 μm and these can be further divided as middle infrared spectroscopy (mid-IR, MIR), near infrared spectroscopy (near-IR, NIR) and far infrared spectroscopy (far-IR, FIR). The MIR region lies at wavelengths range from 2.5 to 25 μm, containing most fundamental absorptions of the molecular vibrations, which is usually mentioned as IR for short. Besides the wavelength, another unit which is used more popular for the infrared radiation is the wavenumber ($\tilde{\nu}$), which is the reciprocal of wavelength expressed in centimeters or given by

$$\tilde{\nu}(\text{cm}^{-1}) = \frac{10^4}{\lambda(\mu\text{m})} \tag{1.2}$$

In quantum theories, light can be regarded as a stream of photons for which the energy (E) is

$$E = h\nu = hc\tilde{\nu} = \frac{hc}{\lambda} \tag{1.3}$$

where the h is Planck constant with the value of 6.626×10^{-34} J·s.

There may be reflection, refraction, scattering, transmission and absorption when radiation is irradiated on the molecules. Molecules can absorb or emit photons and transfer into states with higher or lower energy. According to quantum mechanics, molecules must exist in some quantized discrete energy levels. Therefore, only photons with energy values equal to the differences between the initial and the final energy levels can be absorbed or emitted by the molecules, which can be expressed as

$$\Delta E = E_{final} - E_{initial} = h\nu \tag{1.4}$$

Some photons with specific frequencies are absorbed and the others remain when radiations with particular wavelength ranges are irradiated onto some molecules. The absorption spectrum can be obtained by recording changes in the intensities of radiation passing before and after these molecules. Since the discrete energy levels are determined by the nuclear and electronic structures of the molecules, the spectrum contains the structural information of these molecules. These energy levels are not stationary but affected by some broadening factors and vary across certain intervals. Therefore, peaks with measurable widths appear in the spectrum, instead of infinite lines at some precise frequencies.

The energy of a molecule is considered as the sum of the translational energy (E_t), the rotational energy (E_r), the vibrational energy (E_v) and the electronic energy (E_e), as well as a constant energy (E_0), which can be expressed as

$$E_m = E_t + E_r + E_v + E_e + E_0 \tag{1.5}$$

The translational energy levels may be continuous, but the rotational, vibrational and electronic energy levels may only be discrete. Gaps between rotational energy levels correspond to the radiations lying in the microwave and far infrared regions. Gaps between valence electronic levels correspond to the visible and ultraviolet regions, while gaps between the inner-shell electronic levels may correspond to X-rays. Differences between the vibrational energy levels correspond to the infrared region and therefore infrared spectroscopy is a kind of the molecular vibrational spectroscopy. Transitions between particular energy levels give rise to various kinds of molecular spectroscopy. [9-11]

1.2.2 Diatomic Molecules

A diatomic molecule is usually considered as a harmonic oscillator model, in which two rigid balls are connected by a massless spring (as shown in Fig 1.1). The two balls represent atoms and the spring represents the covalent bond between the two atoms.

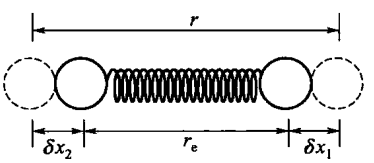

Fig. 1.1 Harmonic oscillator mode of a diatomic molecule

As a result of the interactions between the nuclei and the electrons, there should be an equilibrium internuclear distance (r_e) and the two nuclei vibrate slightly around their equilibrium positions. The frequency of the vibrations of the nuclei, which is called the classical vibrational frequency, is

$$\nu = \frac{1}{2\pi}\sqrt{\frac{k}{\mu}} \quad \text{or} \quad \tilde{\nu} = \frac{1}{2\pi c}\sqrt{\frac{k}{\mu}} \tag{1.6}$$

where k is the force constant of the covalent bond and μ is the reduced mass of the two nuclei which is

$$\mu = \frac{m_1 m_2}{m_1 + m_2} \tag{1.7}$$

Generally, a molecule will absorb photons with the same frequency of the vibrations if the dipole moment change during the motion and the electric field, at least a part, parallel the dipole moment.

With the concept of energy level, the vibrational energy of the diatomic molecule can be expressed as

$$E_\upsilon = \left(\upsilon + \frac{1}{2}\right) h\nu \tag{1.8}$$

where υ is the vibrational quantum number, which should be non-negative integer.

The molecule is in the ground state when υ is zero. Absorption of a photon with the corresponding frequency may lead to a transition of the molecule from the ground state to the first excited state where υ is 1. Molecules in an excited state where $\upsilon = n$ can be stimulated to a higher energy level where $\upsilon = n+1$ by absorption of photons or fall back to a lower energy level where $\upsilon = n-1$ by emission of photons. Almost all molecules stay in the ground state at room temperature as given by the Boltzmann distribution law. So the transition from the ground state to the first excited state should be the dominant process when the molecules absorb photons with specific frequencies. [9-11]

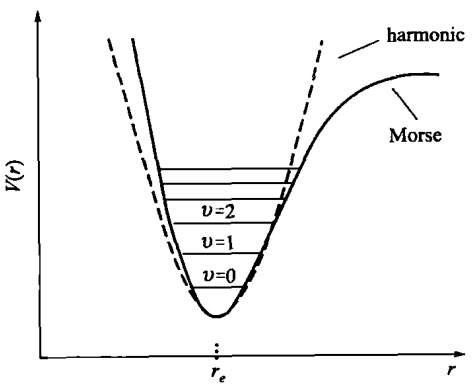

Fig. 1.2 Potential energy of a diatomic molecule

The harmonic oscillator model is not exactly correct for diatomic molecules. For example, the potential energy of a diatomic molecule should be zero when the distance between two nuclei becomes infinite, since the molecule would have dissociated into neutral atoms at this point. Some anharmonicity corrections are needed for the profile of the actual potential energy of the diatomic molecules and a good approximation is the Morse potential energy function (shown in Fig. 1.2). Therefore, the energy of the diatomic molecules should be

$$E_\upsilon = \left(\upsilon + \frac{1}{2}\right) h\nu_e - \left(\upsilon + \frac{1}{2}\right)^2 x_e h\nu_e \tag{1.9}$$

where x_e is an anharmonicity constant and ν_e is the classical vibrational frequency given by Eq. 1.6.

1.2.3 Polyatomic Molecules

Polyatomic molecules containing N atoms have $3N$ degrees of freedom, three of which represent the translation and another three (two for linear molecules) represent the rotation of the molecules. There remain $3N-6$ ($3N-5$ for linear molecules) degrees of freedom for the vibrational motions of the molecules. Linear combinations of the vibrational motions give $3N-6$ normal modes of vibration described by the normal coordinate. For each normal mode, all of the atoms make displacements at the same frequency with different amplitudes. The frequency for a normal mode is determined by the molecular structure and chemical environment. The vibrational energy of polyatomic molecules can be expressed as the sum of the energy of the normal modes

$$E = \sum_{k=1}^{3N-6} E_k = \sum_{k=1}^{3N-6} \left(\upsilon_k + \frac{1}{2} \right) h\nu_k \tag{1.10}$$

Only the normal modes during the vibrations for which the dipole moments of the molecules change are infrared active, which means that only infrared radiation with the same frequencies as the vibrations of these normal modes can be absorbed by the molecules. Different normal modes may have the same vibrational frequency, which are called degenerate. Therefore, the number of absorption peaks is usually less than the number of the normal modes.

A polyatomic molecule is at the ground state when all the vibrational quantum numbers are zero. Energy levels with the quantum number of 1 for one of the normal modes and zero for all others are called the fundamental levels, while levels with the quantum number is greater than 1 for one of the normal modes and all others are zero are called the overtone levels. Levels with the quantum numbers are nonzero for more than one normal mode are the combination levels. Transitions from the ground state to the fundamental levels correspond to the fundamental absorption peaks in the spectra. Transitions from the ground state to the overtone levels correspond to the overtone bands and transitions with the quantum numbers of more than one normal mode change correspond to the combination bands (as shown in Fig. 1.3). The absorption intensities of the overtone and combination bands are much weaker than those of the fundamental peaks. [9-11]

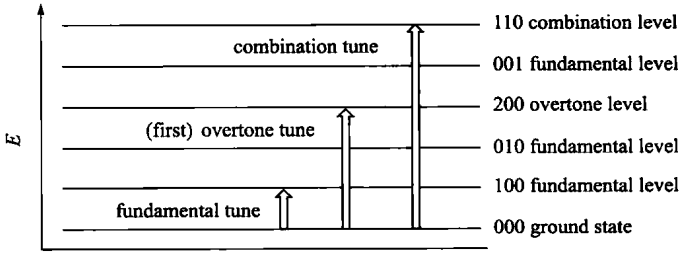

Fig. 1.3 Transitions between vibrational energy levels

1.3 Infrared Spectrometers

1.3.1 Dispersive and FTIR spectrometers

An infrared spectrum is obtained by recording the absorption intensity versus wavenumber (or wavelength) when a beam of infrared radiation passes through the analyte. Therefore, at least three important components are needed for an infrared spectrometer. There must be an infrared source to produce the radiation and a detector to measure the infrared radiation. Besides, there must be an element to distinguish radiations with different wavenumbers (or wavelengths).

According to the means of distinguishing radiation with different wavenumbers, infrared spectrometers may be classified as two kinds, dispersive and Fourier transform infrared (FTIR) spectrometers. In a dispersive spectrometer, diffraction gratings (or prisms for early stage spectrometers) are used to disperse the radiations with different wavenumbers at different positions. Rotating the gratings to make the radiations with wavenumbers over a small interval irradiate into the detector in turn, the spectrum of the sample is recorded by the ratio between the beam passing through the sample and that passing through the reference. In an FTIR spectrometer, an interferometer is used to generate an interferogram from the radiation produced by the source. The interference periods are different for radiations with different wavenumbers and so the total interferogram can be used to extract the signal of radiations with different wavenumbers by the Fourier transform.

Fig. 1.4 shows the main workflow of an FTIR spectrometer. The beam of infrared radiation from the source passes into the interferometer and generates a continuous interferogram. The interferogram passes through the sample is recorded by the detector and the single-beam spectrum of the sample is acquired by Fourier transform of the recorded interferogram. The transmission spectrum of the sample is the ratio of the single-beam spectrum of the sample to that of the reference (background).

Compared with dispersive instruments, Fourier transform spectrometers have three significant advantages: the Fellgett's (multiplex) advantage, the Jacquinot's (optical throughput) advantage and the Connes's (wavenumber precision) advantage. If an infrared spectrum from 4000 to 400 cm^{-1} with a 4 cm^{-1} resolution needed, there are 900 resolution elements to be measured. All the resolution elements can be measured simultaneously in 1 second by an FTIR spectrometer. For a dispersive spectrometer, the resolution elements should be measured one by one and it may take about 15 minutes to obtain a full spectrum. At the same time, the SNR of the spectrum measured by the dispersive spectrometer is only 1/30 of that of the spectrum measured by the FTIR spectrometer.