Chapter 8

Fourier Transform Infrared and Two-Dimensional Correlation Spectroscopy for Substance Analysis

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Additional information is available at the end of the chapter

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Abstract

The development of Fourier transform infrared (FTIR) has had widened its scope of perspective application on different types of substances in terms of technique of material analysis and identification. The tri-step infrared analysis has shown its powerful application in the analysis and interpretation of spectra from pure compound, fraction, raw material, natural product and complex mixture.

Keywords: Fourier transform infrared, pure compound, fraction, raw material, natural product, complex mixture

1. Introduction

1.1. Types of infrared spectrometer: dispersive and Fourier transform

Dispersive spectrometer has been described as a traditional way in the transformation of Fourier transform infrared (FTIR) spectrometer [1]. The basic function of dispersive spectrometer using diffraction gratings or prisms is to disperse the radiations with wave numbers at several positions. The moving of the gratings is the key that allows the radiations with wave numbers over a short interval of irradiation into the detector. Normally, the spectrum of a sample is created by the ratio between the beam passing through the sample and the reference or background. In comparison, Fourier transform infrared (FTIR) spectrometer is more sensitive and accurate in detecting and determining the higher signal-to-noise ratio within a short period of time. The principle of FTIR is generation of interferogram from the interferometer of the radiation produced by the source. Detecting the signal of radiations with different wave numbers by the Fourier transform to determine frequency domain instead of time domain will enhance the spectrum. In this way, the performance of modern

FTIR is advantageous compared with dispersive spectrometer. FTIR also simplifies the complex algorithm into presentable data and is user-friendly.

The development of numerous sampling accessories, such as attenuated total reflection (ATR), sample cell with different window material for liquid sample, 2DIR sample cell etc., widens the utility of FTIR for multi-sample type analysis. Therefore, the origin of the sample material has no barrier for FTIR, albeit the different objectives of the investigation. In fact, FTIR has been recognised as a rapid, direct and non-destructive analytical method. The challenge confronting FTIR is the interpretation of the qualitative or quantitative spectral data from different direction of view.

Factors that influence the frequency vibration mode of a polyatoms molecule included concentration, thermal, time and chemical reaction. This so-called perturbation is an additional input manipulating the vibration mode of the functional group. Typically dominating the motion of the molecules in the normal mode are only one or few groups which vibrate relatively. The establishment of two dimensional correlation spectroscopy via appropriate perturbation on mid-infrared could be used to enhance the detail of infrared spectrum interpretation.

2. Fourier transform infrared spectrum and 2DIR correlation spectroscopy for pure compound analysis

This is the conventional and most widely used spectroscopy in functional group determination. For a known pure compound, each peak of spectrum will identify the main functional group within the specific range of wave number. The matching of peaks in spectrum of unknown and known compound is commonly used as a complimentary analytical method besides LCMS and NMR. Hence, such evidences of compound structure configuration are reliable. Software library incorporated into the system plays a main role in authentication of the compounds. **Figure 1** showed the one dimensional Fourier transform infrared spectrum of delphinidin-3-*O*-sambubioside in the range of 4000–400 cm^{-1}.

The 2DIR correlation spectroscopy on pure compound needs to be presented in the form of synchronous and asynchronous spectra [2]. Synchronous spectrum is developed by combination and accumulation of two spectra of the same substance scanned under perturbation. The most commonly used perturbation is the thermal perturbation which supplies the heat at a range of temperature such as 50–120°C, and this supposedly provokes the vibration mode of the relevant bonds. The 2DIR spectra of pure compound, which represents the active sites of different bonding in the molecule, reacts to the heat simultaneously. The region of peak detected on the diagonal positively formed is named as autopeak. It is possible for the autopeak to increase or decrease during the thermal supply phase. The abundance part of 2DIR spectrum is the crosspeaks that are not scattered on the diagonal. They are either positive or negative, depending on the combination of increased or decreased spectral peak from both axes. In term of correlation, the correlation square

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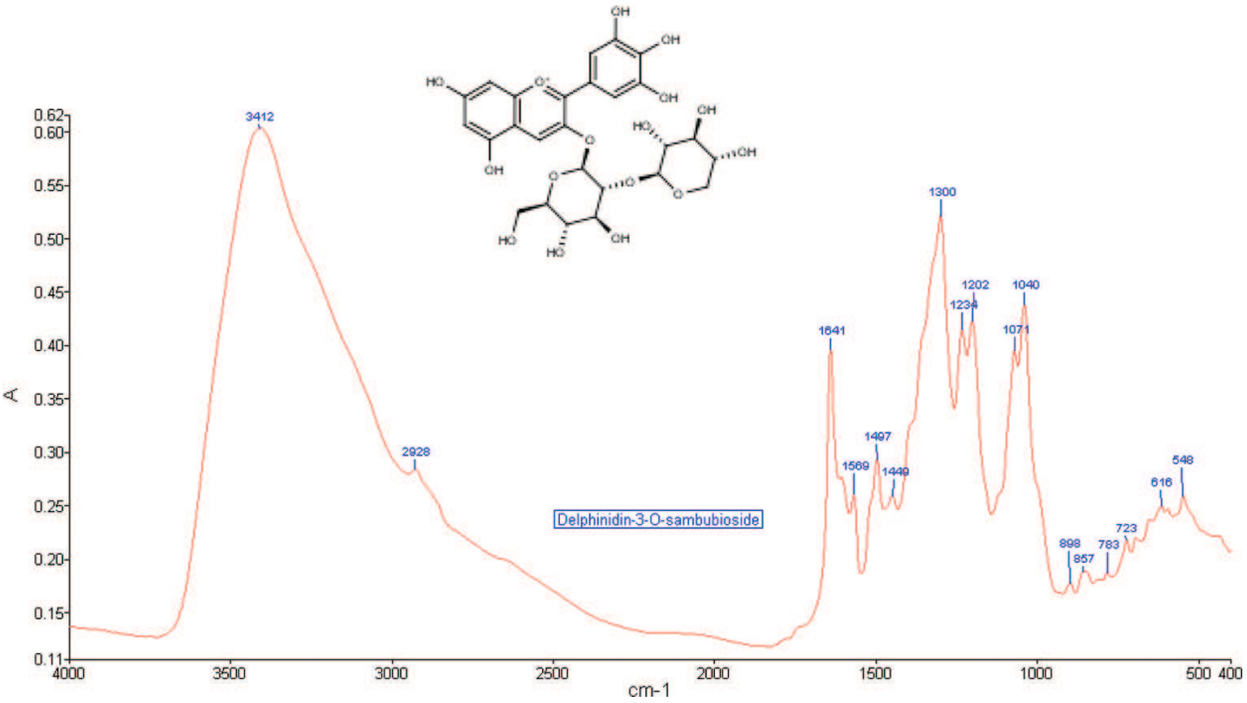

Figure 1. The 1D FTIR spectrum of the pure compound delphinidin-3-O-sambubioside in the range of 4000–400 cm^{-1}. The appearance of Absorbance is transferred from 51.2% of transmission with the threshold of one. Most of the peaks are sharp and in various intensities depending on the absorption of each functional group under the wave number in the range of mid-infrared. The peak at 3412 cm^{-1} is assigned to –OH group. Methyl group could be determined by peak 2928 cm^{-1}. These two peaks are common and normally appear in all the organic material. The range from 1700 cm^{-1} downward is the specific and important range for different characteristic of each compound., e.g. peak 1641 cm^{-1} refers to amide I for bonding C-O, etc.

existing on certain autopeaks and crosspeaks is the best way to interpret the correlation circumstances during the perturbation. The asynchronous spectrum was created on region not related to synchronous spectrum. The Noda's rules [3] are abided during the interpretation data process.

Figure 2 showed the synchronous and asynchronous spectra of delphinidin-3-*O*-sambubioside in the range of 1700–700 cm^{-1}.

2.1. Important usage of FTIR and 2DIR correlation spectroscopy in pure compound

2.1.1. Qualification analysis for special functional group in the compound

Pure compound has the capacity to show the whole corresponded bond taking part in the infrared transmission. The vibration mode of the whole molecule will be determined under the specific assignment. Pure compound normally shows many sharp peaks compared to compound with low purity. This concept is ideal for qualification and the respective peaks are used to identify each component in the molecule itself. In some way, the selective range of particular peak could be used in quantification under the Beer's Law using the spectrum Quant software [4].

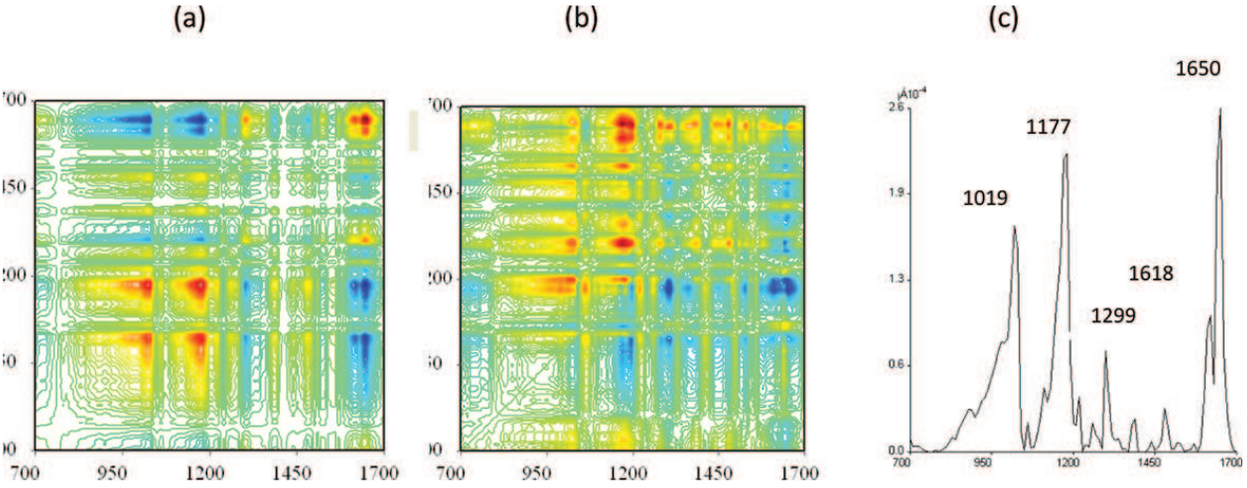

Figure 2. The 2DIR spectra of delphinidin-3-O-sambubioside in the range of 1700–700 cm^{-1}. (a) Synchronous spectrum. The correlation square is created from negative crosspeak at (1177, 1650), autopeak 1650 cm^{-1}, negative crosspeak (1650, 1177) and autopeak 1177 cm^{-1}. There is another bigger correlation square which is created from negative crosspeak (1019, 1650), autopeak 1649 cm^{-1}, negative crosspeak (1650, 1019) and autopeak 1019 cm^{-1}. A smaller correlation square is created from four red areas. They are positive crosspeak (1019, 1177), autopeak 1177 cm^{-1}, positive crosspeak (1177, 1019) and autopeak 1019 cm^{-1}. (b) Asynchronous spectrum. The positive crosspeak at (1177, 1650) determines the sequence action against perturbation of the area at 1650 cm^{-1} first reacted than area at 1177 cm^{-1}. The similar scenario for the bigger correlation square where the peak area at 1650 cm^{-1} reacted first than area at 1019 cm^{-1}. When compared with the area at 1019 cm^{-1} and 1177 cm^{-1}, 1019 cm^{-1} is reacted first than 1177 cm^{-1}. The sequence of decrease reacted to the thermal perturbation in The series is 1650 cm^{-1}, 1019 cm^{-1}, 1177 cm^{-1}. (c) The autopeak spectrum of the 2DIR.

2.1.2. Identification of the functional groups present in unknown pure compound

In identification of new compound by micro-fingerprinting via spectroscopy, this robust technique can be used to obtain useful information in chemical analysis.

2.1.3. Correlation of main functioning group

Determination of correlation of main functioning group under autopeaks at diagonal line indicates another important interpretation that the bondings in the molecule react positively with mid-infrared. In addition, creating the correlation square among the autopeaks and the crosspeaks for pure compound is advantageous using 2DIR correlation spectroscopy interpretation of inter-molecule.

2.1.4. Quantification study for the sample

The innovative Quant software specific for quantification under mid-infrared is another scope of investigation. The standard compound with different concentration is the conventional method for plotting the standard graph. The new version of Quant software has either the single peak or the range of wave number chosen for the quantification compatible under the standard graph. The single peak is chosen for crude extract spectrum as the way to eliminate the interruption of enormous overlapping vibration mode from uncertain components, while the range of wave number is the best tool for purified compound.

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3. Fourier transform infrared spectrum and 2DIR correlation spectroscopy for fractions analysis

Fraction is the intermediate stage of extraction and is situated between crude extract and pure compound. This level eliminates most of the debris, fibre and primary metabolites, and exportation of secondary metabolomics of therapeutic value.

The FTIR spectroscopy analyses with fractions are dependent on the condition of the material. There are two methods on the solid form of fraction, i.e. making a disc with powder KBr, or directly scan with ATR. However, the consideration must be taken on the coverage of wave number using different method. The first method will cover the whole range of wave number, while ATR only scans until 650 cm^{-1}. The KBr method is able to detect more peaks compared to ATR method. Although ATR has its limitations, it is the best choice since in forming the original spectrum, and it is devoid of any problem in transmission percentage.

Liquid fraction is analysed using sample cell with different window material. The exploration of aqueous solution is restricted to any KBr matrix since the O-H bond affects the range around 3600 cm^{-1}. The CaF_2 matrix is appropriate for solution containing water, though the transmission is limited to below 900 cm^{-1}. BaF_2 has clearer transmission for acidic solution from 800 to 400 cm^{-1}.

The differentiation spectra by another calculation in second derivative has been recognised and proven to be the most proper derivative to correct nonlinear baseline anomalies. The

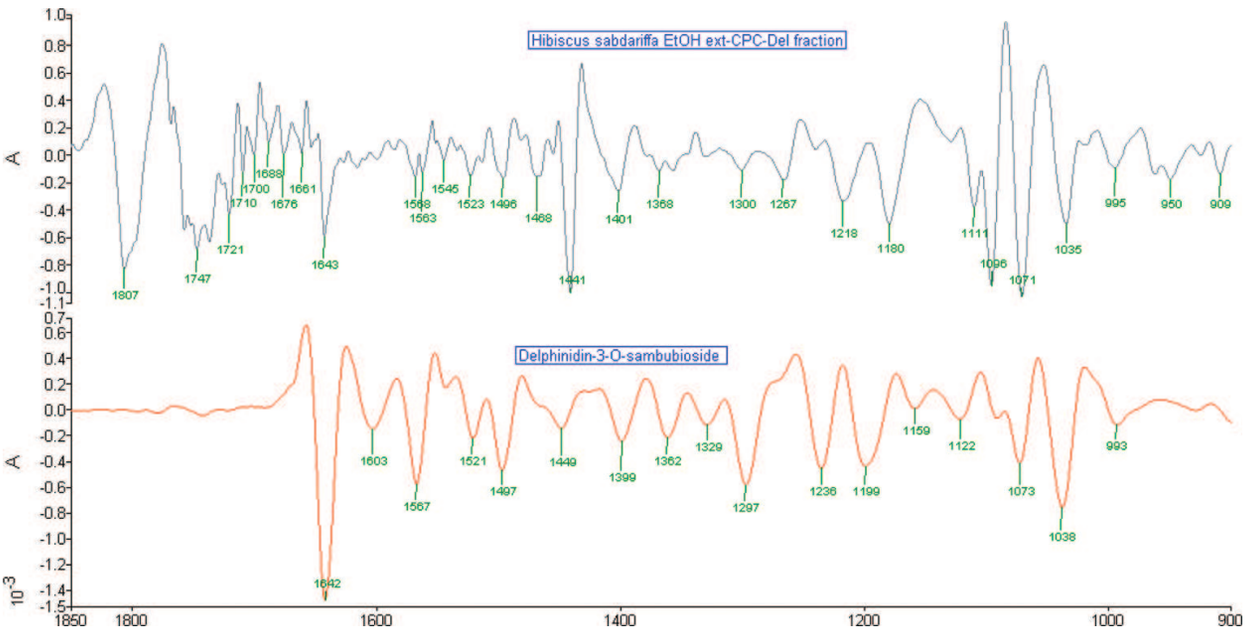

Figure 3. Second derivative spectra of CPC delphinidin fraction of *H. sabdariffa* ethanol extract in the range of 1850–900 cm^{-1}. There are 10 base peaks which are compatible when matching the peaks from fraction and the pure compound. The correlation between the fraction and the compound is 0.52. The base peak of the fraction within 1807 and 1643 cm^{-1} does not appear in the spectrum of delphinidin-3-*O*-sambubioside, since these could be the natural substances of *H. sabdariffa*.

compression of peaks at a single point could be expressed in wider wave number with second derivative. This is crucial as a fraction may consist of more than one compound. The problem of overlapping in 1D FTIR spectrum is caused by similar stretching of vibration mode from different compounds or numerous identical peaks from isomers. It is possible that peak forming in 1D FTIR is due to the combination of closer transmission and clumping together as one. Therefore, the reading on second derivative spectrum is indicative of several aspects initially based on the condition of the fraction.

Figure 3 showed the second derivative spectrum of *Hibiscus sabdariffa* L ethanol fraction purified by HPLC preparative in the range of 1850–900 cm^{-1}.

3.1. Important usage of FTIR and 2DIR correlation spectroscopy in fractions

3.1.1. Investigation of the quality of extraction and isolation

The spectrum of a fraction could be different when compared with the pure compound. A fraction is actually the specific range of peaks chosen from the crude extract chromatogram. Technical experience of the operator will help to determine the nature of the fraction as a single or mixed compound. The pattern of the fraction can be authenticated as macro-fingerprint of the identified material. In fact, each fraction has a specific spectral pattern depending on the quality of extraction. A sample may exhibit different pattern of spectrum when different method of extraction is implemented. For example (**Figure 4**), the pattern of *H. sabdariffa* raw material spectrum showed 20% dissimilarity with *H. sabdariffa* ethanol crude extract when both spectral relatively correlated with spectrum of residue. The fraction of *H. sabdariffa* spectrum showed content of anthocyanins at the peak 1071 cm^{-1} and the pattern is completely different from the anthocyanin pure compound. Hence, the spectrum of material can be used to estimate the quality of extraction and purification. Assignment of peak in spectrum is an alternative method for identification of the main compounds in the fraction isolated from HPLC preparative. The spectrum of fraction has the higher percentage of similarity with pure compound compared with extract. The quality of extraction from the raw material and compound can be clearly discerned monitored by spectroscopy.

3.1.2. Detection of enriched compound

Most of the herbal medicinal products in the market are in the form of fraction. The possibility of the presence of enrich compound added in these products can be detected by FTIR and 2DIR. The concentration of certain active compounds in standard extract is the guide to the formula. Examining this kind of distinctive criteria could be carried out by quantification using spectrum Quant. The plotting of the straight line with various dosage of standard compound is typically prophetic on the targeted compound concentration in the fraction. Normally, the specific spectrum peak has to be determined accordingly because there are procedures for peak selection instead of using the whole spectrum.

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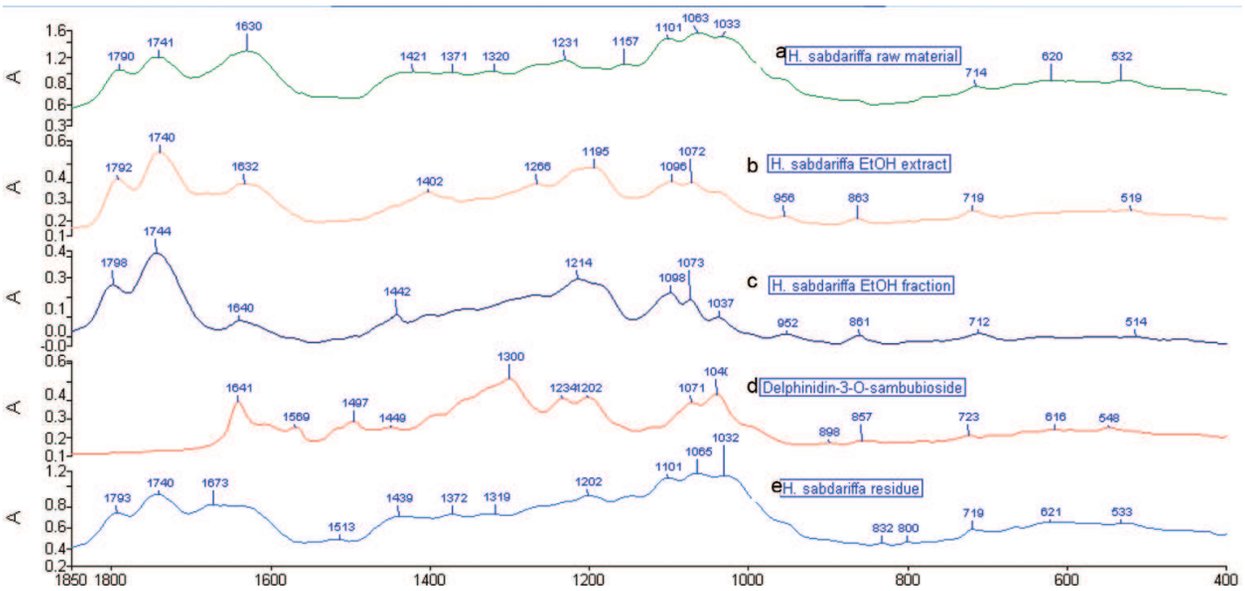

Figure 4. FTIR spectra of *H. sabdariffa* sample material in the range of 1850–400 cm^{-1} from different level of extraction (a) *H. sabdariffa* raw material. The whole spectrum is divided into three areas and represented most of the primary metabolism. The first three peaks started from peak 1790 cm^{-1} and is unique for this plant. Peak 1741 cm^{-1}, assigned for C-O bonding, normally refers to ester components, while peak 1630 cm^{-1} refers to amide I. There is no amide II bonding, since the range from 1500 to 400 cm^{-1} consisted of the bonding for fatty acid and carbohydrate. (b) *H. sabdariffa* ethanol extract. The first three peaks are still maintained except peaks in the range of 1500–1000 cm^{-1} are replaced by few new peaks. Intensity of peak 1632 cm^{-1} is reduced and peak 956 cm^{-1} and 863 cm^{-1} appear and are not found in raw material spectrum. (c) *H. sabdariffa* Centrifugal Partition Chromatography (CPC) delphinidin fraction. The curve between the peak 1214 cm^{-1} and 1098 cm^{-1} is getting wider. The intensity of peak 1640 cm^{-1} and 1037 cm^{-1} is getting lower compared to the raw. But the peak 1073 cm^{-1} and 1037 cm^{-1} which matched with delphenidin-3-*O*-sambubioside are sharp in appearance. (d) Delphenidin-3-O-sambubioside pure compound. There are only three peaks that matched with *H. sabdariffa* fraction and two with *H. sabdariffa* ethanol extract. (e) The *H. sabdariffa* residue. The peak 1640 cm^{-1} is lost and 89% of the spectrum correlated with raw, 69% correlated with extract, and 47% correlated with fraction. Only 11% of the content is extracted from the raw material.

3.1.3. Investigation of adulterant in commercial products with similar molecular weight but different structural configuration

The differences could be the pattern of spectrum or the absence or the presence of strangle peak in 1D FTIR. Second derivative is an alternative to detail the differences, but the most powerful is the 2DIR correlation spectroscopy. Tri-step macro-fingerprint infrared method is also able to enhance the discrimination and distinguish the real product.

3.1.4. Determination of halal and non-halal food

Since in the fraction or extract, the main components can be easily mixed up with other ingredients in food, the challenge to separate halal and non-halal food through spectroscopy is choosing the proper range of wave number f or comparison. **Figure 5** showed the example of determination of halal food with 2DIR.

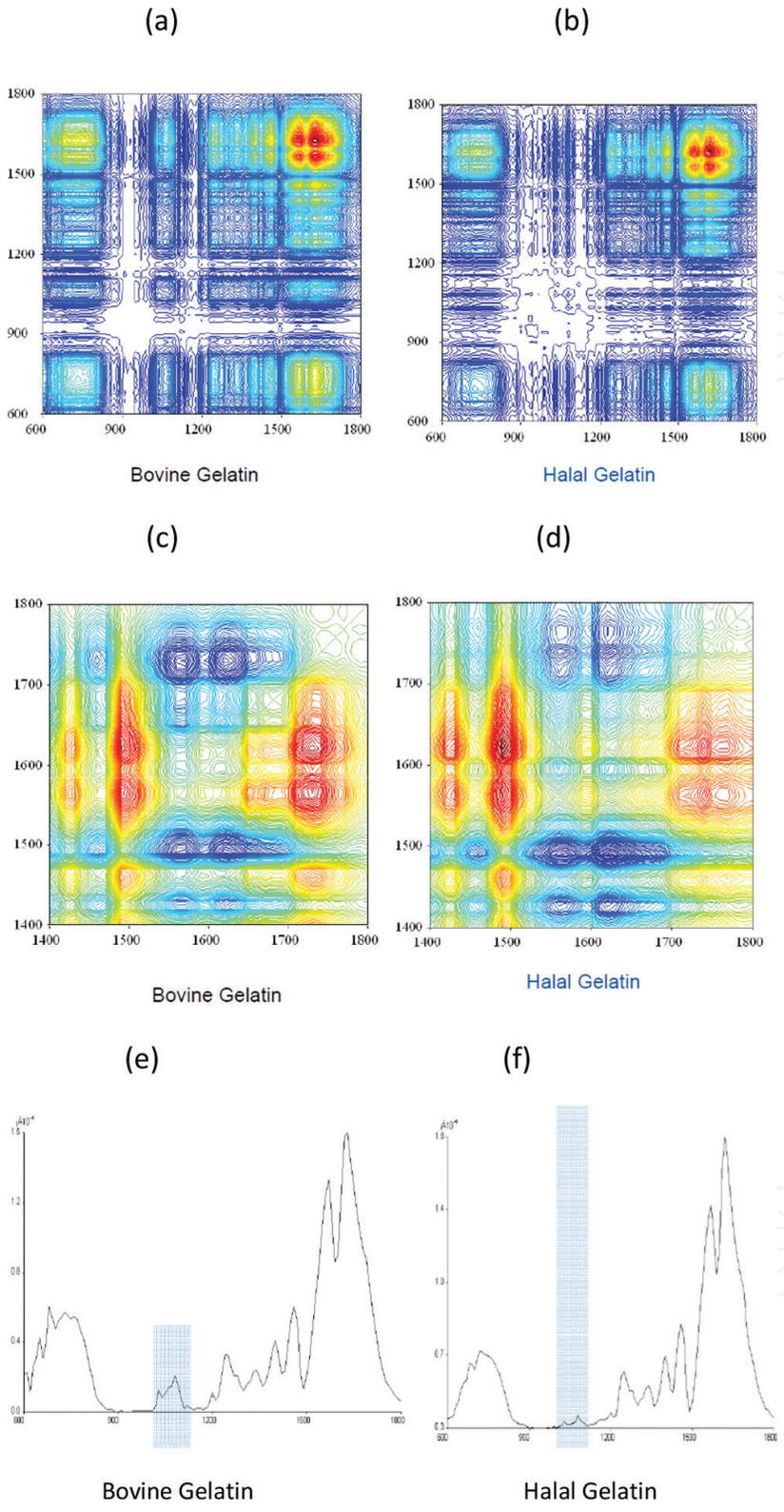

Figure 5. Comparison of bovine gelatin and halal gelatin using 2DIR. (a) Synchronous spectrum of bovine gelatin in the range of 1800–600 cm^{-1}. The higher intensity in the area of 1100–1000 cm^{-1} compared to spectrum of halal gelatin. (b) Synchronous spectrum of halal gelatin in the range of 1800–600 cm^{-1}. (c) Asynchronous spectrum of bovine gelatin in the range 1800–1400 cm^{-1}. The differences with halal spectrum are the intensity of the crosspeaks at (1480, 1625), (1572, 1733) and (1620, 1733) is more intense. (d) Asynchronous spectrum of halal gelatin in the range of 1800–1400 cm^{-1}. (e) The autopeak spectrum of bovine gelatin in the range of 1800–600 cm^{-1}. (f) The autopeak spectrum of halal gelatin in the range of 1800–600 cm^{-1}.

3.1.5. Elaborate the correlation of the main compounds with 2DIR in the fraction

The fraction spectrum profile of the pure compound usually shows the quality of extraction. The majority of the debris, precipitate and high fibre content has been discarded. The elaboration on the main compound that reacted and correlated in the overall profile of the spectrum could be clearly shown by 2DIR correlation spectroscopy. The exploration of the main compounds in the fraction increases the degree of the correlation and less problematic compared with crude extract.

4. Fourier transform infrared spectrum and 2DIR correlation spectroscopy for raw material analysis

Application of the new method of FTIR and 2DIR on raw material is still raising doubt due to the severely overlapping of peak making interpretation difficult. The detected peak could be due to more than one compound with similar assignment. Methods must be developed to ensure the assignment is interpreted correctly. In real life, raw material can be analysed using FTIR and 2DIR which will indirectly reduce the time for sample preparation. It is convenient in term of preparation and conserves the majority of the raw content.

Traditional Chinese medicines are the primer study model as raw material in the dried form using FTIR and 2DIR [5]. The difficulties, besides the problem of overlapping assignment, also include searching the main compounds which may coincide with the raw material spectrum. This kind of matching is reliable if the sufficient literature background on the raw material exists. Despite interruption of the 1D FTIR spectrum on raw material by mixture of compounds, the spectrum can be confirmed when 60% or more transmission is achieved with KBr. The second derivative is mandatory for the raw material spectrum. Modification on the derivative of spectrum shows that second derivative is the most appropriate for exploring the spectrum since the diagram is easily interpreted. In addition, the 2DIR correlation spectroscopy is another step to enhance the detail of the spectrum for detailed interpretation. Therefore, the tri-step analysis combine method, which involves stepwise progression from the superficial to high level of analysis, is advantageous for raw material using the mid-infrared spectroscopy analysis.

4.1. The advantages of FTIR and 2DIR for raw material

4.1.1. Reduction in sample preparation steps

Raw material from natural product has to be dried completely and pulverised before making disc with KBr. In FTIR and 2DIR analysis, the sample preparation process is simple. Since the presence of moisture in the environment affects the FTIR processing method, it is necessary to use dehumidifier to maintain the dryness of the environment.

4.1.2. Rapid

By simplifying complicated preparation steps, analysis of raw material is rapid and direct. The analysis of the spectrum with air as background could be performed immediately after the infrared scanning. The pattern of each raw material will possess footage for the identifica-

tion. Each raw material will be able to show the first image of its content under the scanning. The fingerprint of the original raw material will further confirm the material profile.

4.1.3. No-destructive method and conserve the material information

A raw material conserves most of the natural contents except when contaminated with pollutants. The vulnerable step in the raw material processing is grinding, sufficiently drying and sieving with at least 200 mesh of sieve. However, these are considered less destructive when compared with further extraction on the raw material. In fact, the raw material can be tested under ATR which does not require a disc. The interferon beam has a glimpse of refection on the sample with diamond platform and produce spectrum in the range of 4000–650 cm^{-1}. Comparison of the *H. sabdariffa* raw material spectrum performed with KBr and ATR, respectively, is shown in **Figure 6**.

4.1.4. Interpretation of poly-nutrient in crude

When the whole profile of the raw material is presented with spectra especially in 2DIR, the correlation square generated under the perturbation is based on the active compounds responding concurrently. Such response is associated with a variety of compounds found in the raw extract. Information derived from such interpretation is vital as it provides a holistic picture of the reaction. **Figure 7** showed the correlation square formed between flavonoids peak and carbohydrate peak in *H. sabdariffa* raw material 2DIR synchronous spectrum.

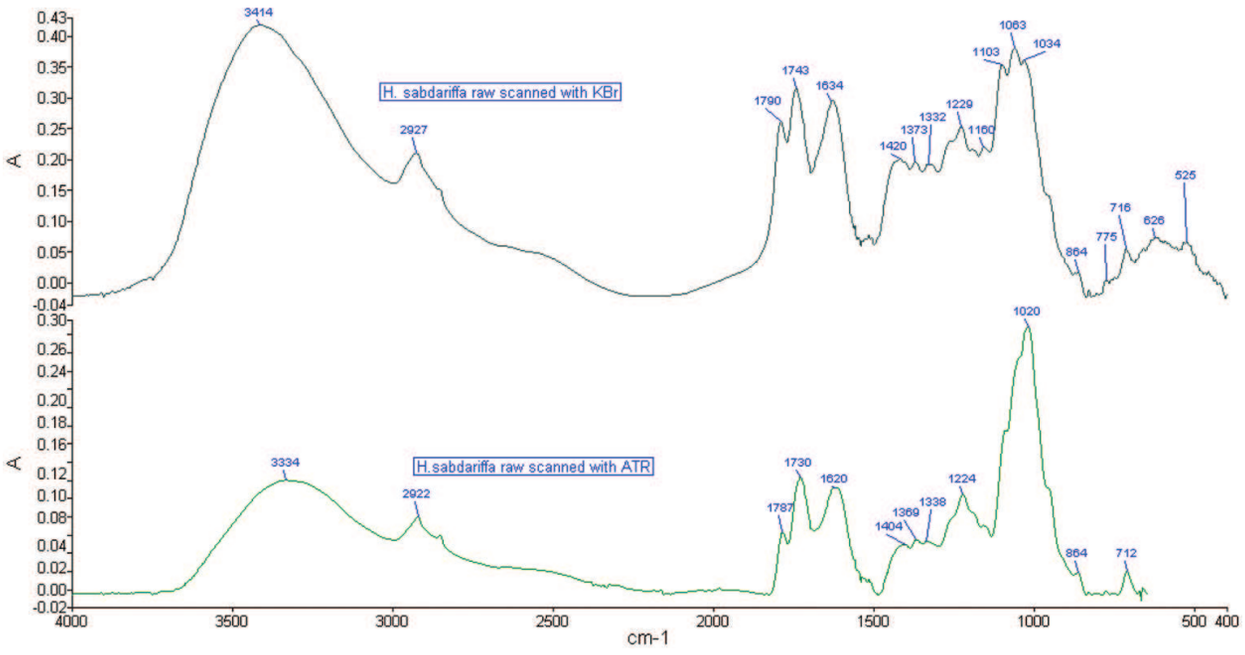

Figure 6. 1D FTIR spectra of *H. sabdariffa* raw material scanned by two analysis methods. First spectrum is produced by mixing the Kbr powder with *H. sabdariffa* raw power dried sample in the ratio 200:1. The range of the spectrum is maximised from 4000 to 400 cm^{-1}. Second spectrum is background with air and the raw material was placed on a diamond platform under ATR method. The range of spectrum is achieved till 650 cm^{-1}, while the spectrum from 650 cm^{-1} to 400 cm^{-1} is lost. More number of peaks is observed under KBr method. However, ATR method is simpler since no disc is needed.

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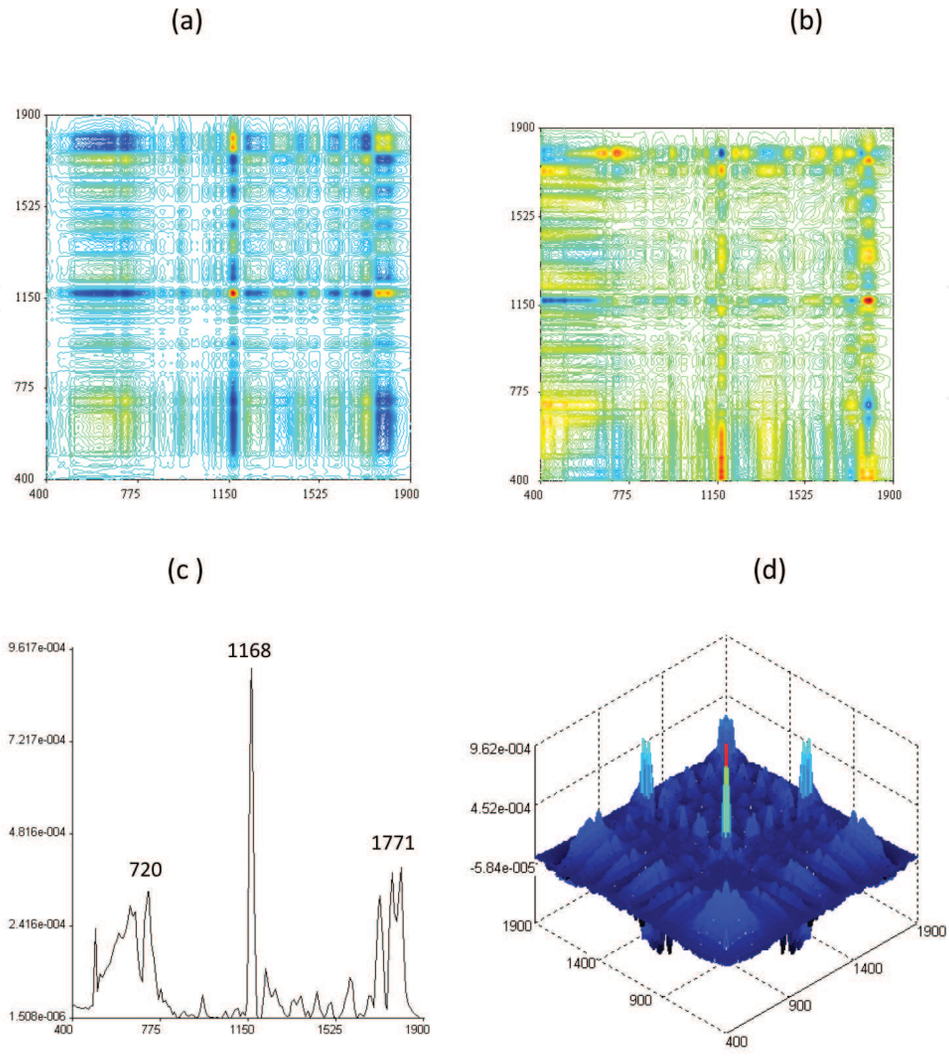

Figure 7. 2DIR spectra of *H. sabdariffa* water extract with Trifluoroacetic acid (TFA) in the range of 1900–400 cm^{-1} from Selangor, Malaysia. (a) Synchronous spectrum. Three correlation squares are formed which are correlated with the C-O bond from ester group with carbohydrate group. They are negative crosspeak (720, 1771), 1771 cm^{-1}, negative crosspeak (1771, 720) and 720 cm^{-1}; positive crosspeak (1168, 1771), 1771 cm^{-1}, positive crosspeak (1771, 1168) and 1168 cm^{-1}; negative crosspeak (720, 1168), 1168 cm^{-1}, negative crosspeak (1168, 720) and 720 cm^{-1}. The spectrum indicates the holistic view of perturbation reaction of the different types of compound in the water crude extract. (b) Asynchronous spectrum. The sequence of reacted decrease as determined by Noda's rules under the thermal perturbation between 30 and 120°C with 10°C interval degree, i.e. 1171 cm^{-1}, 1168 cm^{-1}, 720 cm^{-1}. (c) Autopeak graph. (d) 3D spectrum. The colour in dark blue illustrates the negative peak facing downward from plane.

4.1.5. Standardised method for authentication of a raw material derived from natural product

The origin of the raw material in certain commercial medicinal products is difficult to be authenticated especially when they are presented as powder. Such products could be a portion of plant or other types of organism. Screening of the powder using KBr or ATR method will elucidate its chemical profile as the spectrum will confirm the origin as well as the purity. The compendium and monograph of herbal plants should include the spectrum as one of the methods in the authentication of medicinal products.

5. Fourier transform infrared spectrum and 2DIR correlation spectroscopy for natural product analysis

Analysis of natural product with FTIR and 2DIR have to confront with the obstacles coming from the natural texture of the subjects, since their contents consist of water in living cells found leaf, fruit, stem, flower, seed or fruiting body, stalk and sclerotium from medicinal mushroom. The spectroscopy analysis of natural product needs to avoid the interrupting H_2O background and preserve the nature of the features. Therefore, the analysis must apply the correct method chosen as well as appropriate spectrum interpretation on natural product.

There are three choices of FTIR spectrum scanning for natural product. Firstly, mixture with KBr if the natural product is completely dried and without the need for further drying process, such as seed, some rhizome or hash portion of fruiting body. Secondly, ATR is the best choice if the sample is sensitive to the environment conditions when they are being removed or still contain water and mucous secretions. Even though there is a slight refection on the sample surface through the diamond platform, the spectrum created is still sufficient to represent the actual profile when using air as a background. In comparison, the juice of the natural product containing water can be analysed using liquid sample cell with calcium fluoride window. The third method is the FTIR imaging attached to a microscope with a spectroscopy system. The combination enriches the scope of analysis and is more powerful and user-friendly.

No doubt that human error is unavoidable when dealing with natural product analysis. The content(s) may have been lost during the sample processing, poor quality of extraction, physical deterioration and extended period of storage. The more the natural content of the subject model is preserved, the more accurate it can be analysed. These kinds of natural product are found in Malay, Chinese [6] and Ayurvedic medicine such as medicinal mushroom. Many remedies in these communities contain ready to use formulae of the natural products for therapy, e.g. use of turmeric product [7] for blood clotting. FTIR is one of the methods to study the key chemical contents in such treatment.

5.1. Analysis of natural product and raw material

5.1.1. Appropriate for quantification of chemical marker analysis

In order to determine the exact quantity of the chemical(s) involved in the treatment, the Spectrum Quant is especially effective for this purpose, using different concentrations of standards that will generate the range of spectra for the targeted compound. The other algorithms such as PCR+, PLS and Quant C can also be chosen depending on the respective objectives of the study. The results of analysing an unknown sample can be used to predict its concentration by comparing with the range of standards.

5.1.2. Exposure to various factors

The original natural product is the best type of sample for chemical fingerprint profiling, maturity or storage period determination. Time factors such as oxidation, exposure to air, etc., could affect the whole experiment. The sample of natural product analysed using FTIR and 2DIR is prevented from the detrimental effects of these factors.

5.1.3. Determination of different geographical origin of natural product using principal component analysis or SIMCA

Natural product especially the medicinal plants and mushroom of different geographical origin would have a variety of chemical contents with respect to the quality and the quantity. Mean spectral data from randomly chosen plants from a plantation and those from individual plants can be compared. This kind of statistical value could be plotted using a software such as Assured ID which will provide overall comparison of the similar type of natural product collected from different geographical locations. The differences are due to factors such as different types of soil, water, weather, pH of the soil, water, etc. These factors will influence the distance and the percentage of rejection of SIMCA created by three principle components. **Figure 8** showed the example of SIMCA result of *H. sabdariffa* [8, 9] sample from two different locations in Malaysia.

Generate Model Summary

Number of problem materials with extreme samples : 2
Number of overlapped materials : 0

Diagnostic Report

Inter Material Distances

Material	JHR (J1-J35)	P,P (S1-S35)
JHR (J1-J35)	-	3.06

Classification Performance Report

Material	% Recognition rate	% Rejection rate
JHR (J1-J35)	97(101/104)	100(105/105)
P,P (S1-S35)	100(105/105)	100(104/104)

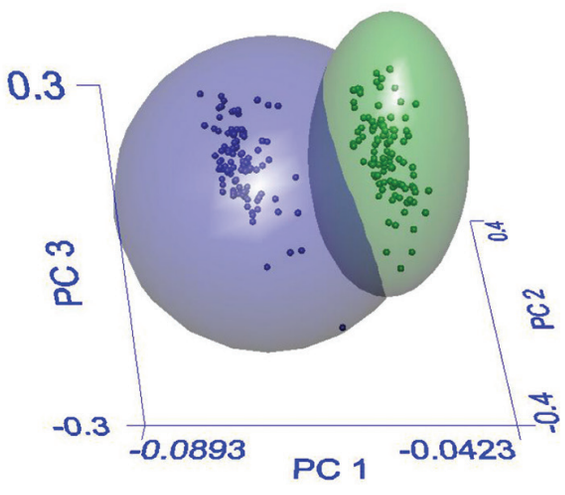

Figure 8. Statistic result checking with SIMCA on *H. sabdariffa* collected from two different locations in Malaysia. Thirty five plants were randomly selected from a plantation in Johor (JHR) and Penang (P.P) where there are separately 340 km away. The fruits from each plant were collected in different net bag. Thirty-five net bags of *H. sabdariffa* fruits from two locations were processed and produced the pulverised dry powder for the ATR spectroscopy method. Two locations of samples spectrum were grouped in the software Assure ID with triplicates. The result showed that there are some factors caused the samples from two locations was successfully discriminated with the inter-material distance 3.06. Two spheres sharp of diagrams created ever combined at a portion that joined them together under Principal Component (PC) 1, 2 and 3. The conclusion is the samples from two different locations are different in term of some factors such as water, weather, air, soil, but they still contain certain factors that they are having the same. The data produced can be used for further prediction for geographical origin of the unknown sample.

6. Fourier transform infrared spectrum and 2DIR correlation spectroscopy for complex mixture analysis

Today, use of combo medicinal contents has been reported to be more efficient compared to single compound when treating viral diseases. Research on the effects of combination of different substances has been reported *in vitro* and *in vivo*. This method is claimed to be

effective against mutating virus. Several medicinal products are known to kill selectively and prevent the spread of the infection virus. Many possibility of combination of medicinal products exist [10], e.g. raw and the fractions, fractions from different plants, fractions plus compound and raw with compounds. Identification of this kind of the contents in this type of combination is not a priority as long as the efficacy is enhanced and patient acceptance is good. On the other hand, analysis of the interaction of components in the combination is crucial to generate information for the design of new medicinal product in future. The 2DIR correlation spectroscopy plays a vital role in the combo medicine interpretation.

6.1. Difficulty in interpretation

Analysis of the complex mixture of samples required pre-knowledge of the main compounds involved. The addition of different types of standards is needed to determine the assignment of bond in 1D FTIR. There could be no actual matching of standards peak with the complex mixture because of the overlapping and interruption of the bonds from each other. Consequently, more standards or fractions need to be used for different scope of comparison. The additional library in the software could be helpful in this aspect. When the peaks have been confirmed, then the next step will be carried out as usual. The 2DIR with synchronous spectrum is the best option to investigate the interaction of mixture under the appropriate perturbation.

6.2. Level of sensitivity

It is often difficult to address the interaction of a mixture as it may contain totally dissimilar types of compounds. However, they can react concurrently when perturbation is applied. The degree of sensitivity, either from the user or the system is very much relied on the experience and practice, e.g. two possible interpretations based on the similar spectrum. Therefore, the analysis on the mixture with FTIR and 2DIR must be accurate and detailed initially to prevent subsequent misinterpretation.

6.3. Strengthening of data

It is not advisable to use the asynchronous spectrum in 2DIR for the mixture due to the tremendous overlapping of assignment which is confusing. Other types of data generated from HPTLC, HPLC, GCMS, LCMS and NMR could be additional evidences for mixture complex analysis.

7. Limitations of FTIR and 2DIR

FTIR and 2DIR technique used in analysis has their limitations that could reduce the degree of efficiency. Fractions in solution form are not eligible for 2DIR analysis except using liquid sample cell, since special sample cell is needed to create high temperature changes and the rate of evaporation of the natural product solution is unknown. However, liquid sample cell is expensive. Besides, there is no appropriate FTIR equipment to capture the changes of spectrum within a few seconds, such as oxidation from the natural product. The thermal perturbation is commonly utilised for analysis of different substances and difficult to interpret compound with more than

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one isomers structure. However, no standardise method exist to determine the degree of peak differences. Some researchers agree that the range 2–3 cm^{-1} is for detecting the pure compound and the fraction, while the range 5–8 cm^{-1} is for the raw material and other materials. However, the acceptance of these ranges is still debatable. Lastly, there will be confusion on spectra for comparison when the quality of extraction is not the same especially the fractions.

8. Conclusion

In conclusion, much research on FTIR and 2DIR is still ongoing in order to overcome the various limitations confronting their widespread use. With accumulated experience and sophisticated innovation such as imaging microscope connect to FTIR, the techniques will be improved and enhanced in the near future.

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