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Abstract. Carotenoids are one class of naturally-occurring pigments with antioxidant properties. They can absorb light energy for use in photosynthesis for plants, and act as antioxidants to reduce risk of cancer for human. Carotenoids are confirmed to exist in agricultural products as the main source for human. Raman spectroscopy is a new technique for determination of carotenoids in agricultural products as it is both noninvasive and rapid. Four types of Raman spectroscopy could be used for contact measurement of carotenoids in fruits and vegetables: (1) Fourier transform Raman spectroscopy; (2) Resonance Raman spectroscopy; (3) Raman microspectroscopy; (4) Spatially Offset Raman spectroscopy. The experimental setups, advantages and applications of the above-mentioned Raman spectroscopies are discussed.

Keywords: Carotenoids; Fourier transform Raman spectroscopy; Resonance Raman spectroscopy; Raman microspectroscopy; Spatially Offset Raman spectroscopy

1   Introduction

Carotenoids are one class of naturally-occurring pigments with antioxidant properties. They have positive effect on the immune system in animals and plants [1]. Most carotenoids cannot be synthesized by human beings directly. They widely exist in agricultural products such as tomatoes, watermelons, grapes and carrots. All carotenoids are produced from eight isoprene molecules and contain forty carbon atoms [2]. The best known carotenoids include lycopene and beta-carotene which is the vitamin A precursor. They all belong to the class of carotene which contains only carbon and hydrogen and no oxygen. There is another class called xanthophyll which contains oxygen such as lutein, zeaxanthin and astaxanthin. Carotenoids as natural pigments also contribute to the plants color when chlorophyll is not present [3]. That

means carotenoids abound in ripe fruits and vegetables with the color of yellows, reds, or oranges [4].

At present, chromatography and spectroscopy are two common methods applied to determinate carotenoids in agricultural products [5-9]. High-performance liquid chromatography (HPLC) is the representative of chromatography. It is widely used for quantitative analysis due to its low detection limit and high accuracy [10]. However, this method may cost considerable amount of time for sample preparation because this method need to destroy sample. It also requires elaborately extracting the chromophore before experiment. However, it need not take any trouble about preprocessing in spectrum detection. Raman spectroscopy is a well-suited noninvasive method in this context. It is a non-destructive, non-contact analytical technique result in little sample preparation [11-13]. Based on that, many variations of Raman spectroscopies could be capable of enhancing the sensitivity, acquiring very specific information or improving the spatial resolution [14-17]. Raman spectroscopy has been proved to be a useful and powerful tool for carotenoids analysis.

2   Raman spectroscopy for carotenoids analysis

A whole Raman spectroscopy instrument includes a laser excitation source, a sample chamber, a determination system and a data analysis system [18]. The lasers as the excitation sources provide directional monochromatic lights in vast majority of Raman measurements. Monochromaticity, directionality, and coherence are three unique properties of Raman excitation light source. The laser excitation sources are usually provided by lasers at 488, 514, 532, 633, 780/785 or 1064 nm. Compared with the requirement of lasers for fluorescence excitation, the linewidth of lasers for Raman excitation needs to be narrower. Another indispensable part of Raman system is sample chamber, which generally consists of lens, filters and sample holders. The role of lens is to obtain the most effective irradiation on the sample, and to maximize the collection of scattered lights. Filters, normally including notch filters and edge filters, are used to prevent Rayleigh scattering into the detected signals. The specifications of the filters are of great concern to the low wavenumber performance of Raman spectroscopy. A detection system always be divided into two parts: spectrometer and detector. Many technical parameters of spectrometer, such as resolution, spectral range, diffraction efficiency, aberrations, and stray lights, have crucial impact on the whole Raman system. However, the spectrometer would cause great loss of light intensity, thereby resulting in the decrease of sensitivity. It might be a way to improve sensitivity that obtains the required lights directly from narrowband filters without spectrometer. The detector could use a charge-coupled device (CCD) or a photomultiplier detector to collect Raman spectrum. The data analysis system is responsible for data pre-processing, data computing and data storage.

As we known, Raman spectroscopy is characterized by strong bands produced from stretching vibration of specific nonpolar groups. Based on conventional Raman determination, the C-C and C=C bonds in carotenoids just belong to nonpolar groups, and it is easy to observe strong bands within 1150-1170 cm^{-1} and 1500-1550 cm^{-1}, respectively. Additionally, a polyene chain with CH_3 groups attached could generate

Raman shift in the 1000-1020 cm^{-1} region. However, there are some deficiencies in the process of in vivo conventional Raman determination: (1) fluorescence of samples is strong during the determination; (2) Raman spectrum is not abundant to distinguish various carotenoids; (3) it is unable to observe the structure of carotenoids in this resolution level; (4) it cannot detect internal Raman spectrum of solid samples. To solve above problems, Fourier transform Raman spectroscopy (FT-Raman), Resonance Raman spectroscopy (RRS), Raman microspectroscopy, and spatially offset Raman spectroscopy (SORS) are introduced into in vivo determination of carotenoids. These techniques will be discussed below. Besides, surface-enhanced Raman spectroscopy (SERS) is another rapid detection method. It depends on enhancement module contacting with samples to enhance the Raman scattering. This paper is mainly concentrated on the introduction of non-invasive and in vivo Raman methods, so SERS method is not discussed in this paper.

3   Fourier Transform Raman Spectroscopy

Raman spectroscopy relies on Raman scattering which is very weak excited by intense laser light [19]. Normally, the wavelength range of laser is in the visible, near infrared or near ultraviolet range [20]. When an intense laser light illuminating on carotenoids, the electrons in molecules of carotenoids would emit light with low energy, leading to both fluorescence emission and Raman scattering in a wide wavelength range. Under this background, FT-Raman is developed to reduce the interference of fluorescence. FT-Raman uses an Nd:YAG laser (1064 nm) to obtain the maximum information available, moreover, the problem of fluorescence background could be circumvented. In addition, a longer integration time and higher power lasers are required at the same time [21].Figure 1 shows one type of FT-Raman experimental setup. The monochromatic light (1064 nm) emitting by the laser is collimated by the lens, reflected by the mirror, and irradiated on the sample. The Raman signal is collected by a Michelson interferometer after filtering by a dichroic filter. The Michelson interferometer could produce a relative improvement in signal-to-noise ratio. Then, the scattering light passed through a dielectric filter and finally focused into a liquid N$_2$ cooled detector.
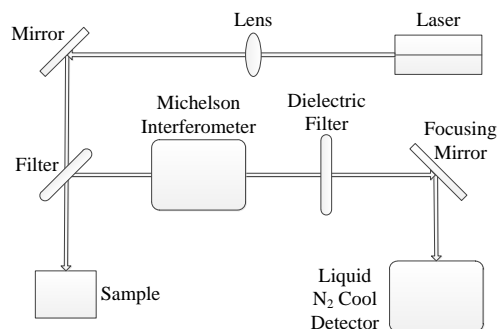

Fig.1. Schematic diagram of FT-Raman spectrometer

The application of FT-Raman is determining special samples with strong fluorescence. FT-Raman eliminates the fluorescence background effectively by using excitation laser in near-infrared wavelength (1024 nm). It is an easy way to distinguish between similar isomers. Compare to Fourier transform infrared spectrum (FT-IR), FT-Raman shows good applicability on aqueous solution. For this reason, FT-Raman could determine carotenoids directly in vivo, such as plant epidermis, fruits, and human skin. H. Schulz et al. demonstrated the feasibility of FT-Raman spectroscopy applied on carotenoids determination in floristic kingdom.It presented an especial way to detect the distribution of several carotenoids and distinguish various quantity of double bonds conjugated carotenoids in the intact plant tissue [22]. Similarly, Vanessa E. de Oliveira studied on natural carotenoids in more than 50 samples of plant tissue by FT-Raman. They recorded the characteristic bands of C-C and C=C stretching and $C-CH_3$ bending in each sample. They also found that the characteristic key bands were arose from their own molecular interactions. [23]. Compared to ATR-IR and NIR reflection spectroscopy, FT-Raman spectrum of tomato products showed significant wavenumber shifts of targeted carotenoids, and it showed a good reliability on lycopene (R^2=0.91 and $SECV$=74.34) and beta-carotene (R^2=0.89 and $SECV$=0.34) [24]. Based on Raman images, the spatial distribution of carotenoids in intact carrot roots of different colors was evaluated by Malgorzata Baranska et al. [25]. They also defined tissue specific accumulation of several variants of carotenoids by Raman images in the carrot root cross section [26]. However, the laser device at 1064nm wavelength is always large and expensive, and this is a limiting factor of FT-Raman to be portable.

4   Resonance Raman Spectroscopy

The feature of RRS is to define characteristic vibration bands of corresponding model compound. RRS requires a special wavelength of the laser which is used to obtain the selective spectra. The excitation frequency must be close in energy to the electronic transition of the sample. For carotenoids, the optional excitation wavelength range depends on the numbers of C=C in molecules from 410 to 520 nm. Carotenoids like lycopene, alpha-carotene, beta-carotene and their isomers, which own 11 conjugated carbon double-bonds, have approximate Raman shift at 488 nm. However, only lycopene owns special peak position at 514 nm with the maximal difference [27]. In other carotenoids, the maximum of absorption values are located at 515 and 490 nm for diadinoxanthin and diatoxanthin, respectively [28]. By comparing C=C band at different excitation wavelength for further study, the peak positions are very similar to those of lutein at 514, 496 or 476 nm. The peak position of violaxanthin is at 502 nm and another variant neoxanthin is at 488 or 458 nm [29]. But in practical applications, agricultural products contain more than one type of carotenoids in most instances [30]. Based on that, the optimal excitation wavelength for most carotenoids is determined at 488 or 514 nm. Figure 2 shows a typical RRS experimental setup applied to carotenoids. The constitutive requirement of RRS is similar to FT-Raman. The main distinction is the exciting beam filtered by a BPF (band pass filter) and focused onto the sample, then using a dichroic filter and a LPF

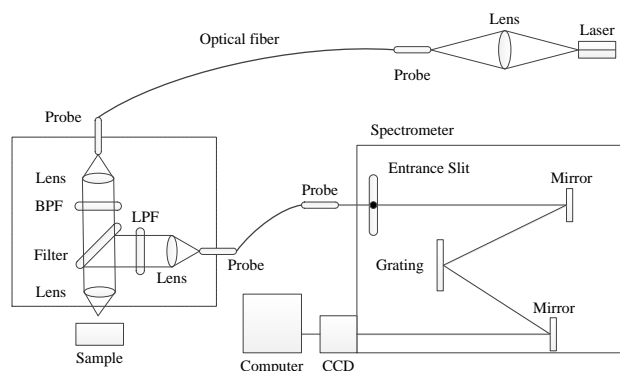(low pass filter) to eliminating the laser. The band of BPF and LPF are according to the sample under test.

Fig.2. Schematic diagram of RRS experimental setup

The main advantage of RRS method is its high sensitivity. It makes the determination of a small quantity of carotenoid molecules in different agricultural products realized. Dane Bicanic et al. used RRS method combined with tristimulus colorimetry to rapidly detect beta-carotene in mango homogenates. At a low concentration of carotenoids, RRS featured the characteristic bands of beta-carotene at 1008, 1158 and 1523 cm^{-1}. The correlation between beta-carotene content and relative intensity of Raman scattering was linear with R^2=0.962 [31]. Bhosale P et al. also measured the levels of carotenoids contents in farm products especially fruit juice by RRS and compared to HPLC [32]. It shows complementation between the two methods: HPLC had a good performance in evaluating juices, while RRS was suitable for determining content of carotenoids in the same maturity of intact tomatoes. Based on resonance Raman effects, Ouyang S L, et al. obtained the content of lycopene and beta-carotene by measuring the second harmonic of stretching vibration of C=C directly in lycopene and beta-carotene, respectively [33].Yan J, et al. also confirm that *9-cis*-beta-carotene exists in cytochrome *b6f* complex of spinach by HPLC and RRS [34]. Compared to chromatography, the measurement of RRS is quick, usually not exceeding 5 seconds [35]. However, in practical applications, the measurement may maintain for some time to decrease the influences of fluorescence background. In addition, the high universalizable of RRS methods could be another advantage [36].

5   Raman Microspectroscopy

Traditionally Raman microspectroscopy is used to measure Raman spectrum of a micro-scale region on a sample. The main application is to observe the molecular concentration profiles. The system is usually equipped with an optical microscope combined with an excitation laser, a sensitive detector and some other optical devices. This method could focus on the distribution of carotenoids in tiny samples with a high spatial resolution of about 250 nm [37]. Since the size of laser beam is only several

micrometers in diameter, it could reduce the fluorescence during the experiment. For this reason, Raman microspectroscopy is suitable for determining carotenoids in emulsions or homogenates [38]. To further increase the optical resolution, Confocal Raman microscopy is designed to investigate carotenoids, the principle of which is to eliminate out-of-focus lights by means of adding a spatial pinhole placed at the confocal plane of the lens. At the expense of long exposures, the optical resolution could confine in hundreds of nanometers [39]. A characteristic block diagram of Raman microspectroscopy experimental setup is shown in Fig 3. A microscope objective is added to a common Raman spectrum measuring system. In the process of detection, the laser (785 or 514 nm) need to pass through the objective and focus on the sample. The scattered signal caused by laser is also collected by the objective. Therefore, a laser rejection filter need to attenuate the laser in a very low level. After that, the scattered light can be collected into the spectrometer. The spectrum is obtained from a CCD camera, analyzed and displayed on the computer similarly.
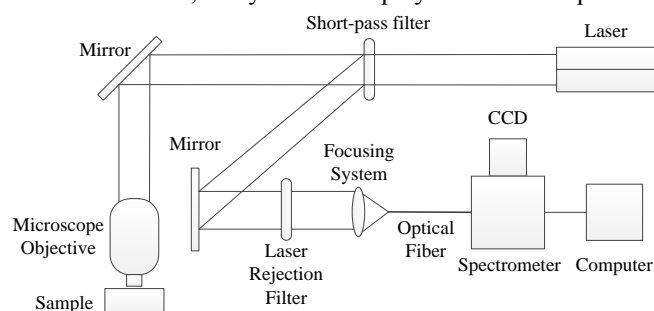

Fig.3. Schematic diagram of Raman microspectroscopy experimental setup

The advantage of Raman microspectroscopy is the nano-scale resolution and high accuracy. Based on that, Pudney P D A, et al. showed the spatial locations of three main carotenoids within tomato cells, and also demonstrated the follow changes of carotenoids [40]. It is confirmed that the confocal Raman microscope is a powerful tool to measure the green tomatoes. It can offer a full-scale Raman spectrum of the organic composition in unripe tomatoes. This method was also applied to detect carotenoids in ripe tomatoes in which the major carotenoid is lycopene [41]. In addition, the influence factors of external environment were discussed. Cecilia A. Svelander et al. evaluated the microstructure of carotenes in tomato and carrot emulsions under varying degrees of high pressure homogenization. [42]. Paolo Camorani et al. proposed confocal micro-Raman spectroscopy as a spatially resolved method to evaluate the changes of carotenoid pattern after thermal treatment of carrots. They have used steaming, boiling and microwaving way to cook carrot. The difference before and after are measured by Raman spectroscopy and HPLC [43]. Combined with density functional theory, the molecular structure are also analyzed by comparing the calculated and determination results [44].

6   Spatially Offset Raman Spectroscopy

SORS is an unconventional method specialized in obtaining special information which beneath the surface of objects, such as drugs [45], explosives [46] and plastic [47]. Raman spectroscopies above-mentioned have pimping near-surface detection limits. In contrast, SORS has an effective retrieval of Raman spectra at deep subsurface layers. The basic principle of SORS is to obtain the Raman scattering information using two spatially offset points: one is the location of illumination and the other is the location of determination [48]. There are several millimeters separation distance between two places. The purpose is to avoid the Raman scattering at domination excitation region [49]. The experimental setup of SORS is unlike conventional Raman instruments. The main distinction is the mode of collecting Raman scattering. Figure 4 shows the distinctions of conventional Raman and SORS approach. As shown in Fig 4, the laser excitation point and the sample collection point are at the same location in conventional Raman. In contrast, Raman spectra in standard SORS approach are collected from the spatial regions away from the place of illumination, and the direction of laser irradiation is opposite to the probe which collects the Raman scattering. When detecting transparent or liquid samples, an adapted SORS approach is applied for analyzing variety of samples [50]. This method enhances the scattering signal of the liquid or transparent sample directly under the Raman detection area. It realizes detecting bottled liquid directly cause of Raman scattering of liquid could not be easily lost within the noise. As the offset distances increasing, however, the Raman signal would be barely discernible. In this case, this method may require adding multivariate analysis.
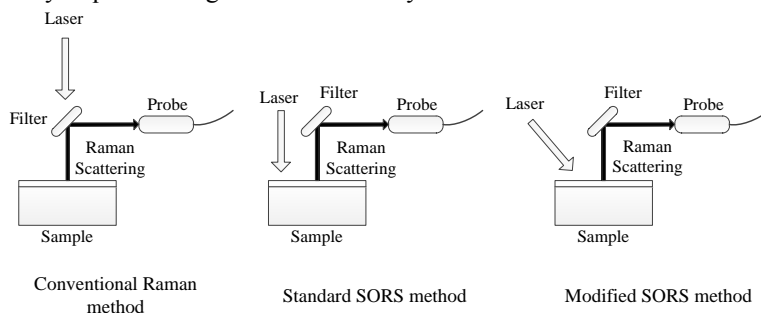

Fig.4. Schematic diagrams of conventional Raman and SORS

The advantage of SORS method is non-invasive determination of subsurface. Three Raman spectroscopies above can measure near-surface only with a few hundred micrometers depth. By contrast, the determination depth of SORS could reach several millimeters. Due to the complexity of SORS instrument, the application of SORS for carotenoids detection is not widespread. Qin et al. developed a benchtop point-scan Raman chemical imaging system to show the detail distribution of lycopene during postharvest ripening of tomatoes utilizing SORS technique. The tomato samples were selected and cut open from green stage to red stage. The results showed that the SORS technique was capable of obtain internal Raman chemical information in tomato [51]. Afseth N K., et al. explored the possibility of determination carotenoids

of salmon through the skin [52]. At an offsets around 5 mm with an 830 nm laser, the results clearly showed that information regarding carotenoids content could be extracting from both sides of the skin. These applications demonstrated the potential of SORS technology as a new method for subsurface determination of carotenoids.

7   Conclusion

Raman spectroscopy is a powerful tool to research on carotenoids due to its unique capability of fingerprint for identifying and characterizing the structure of carotenoids molecules. Raman spectroscopy also shows great potential in qualitative and quantitative analysis on carotenoids. In recent years, with advances in optical instruments, several variations of Raman spectroscopies is increasingly widespread such as FT-Raman, RRS, Micro-Raman and SORS. The contrast of these four methods is shown in Table 1. However, new technologies and ideas should be provided to improve insufficient. Many features such as background fluorescence, category distinction of carotenoids, interference and absorbance of reaction mixture still affect the detection results. For different agricultural products, the most crucial is to explore proper detection mode of non-invasive, non-destructive or in vivo. In addition, new perspectives such as femtosecond stimulated Raman spectroscopy and transmission resonance Raman spectroscopy are promising for improvement of the precision and applicability of carotenoids study. The combination of Raman spectroscopy and chromatography will be promising for overcoming many defects. It is expected that Raman spectroscopy will applied in on-line fast determination of carotenoids in the future.

Table.1. The contrast of four variations of Raman spectroscopy

Techniques	Principles	Advantages
Fourier Transform Raman Spectroscopy	Operation with FTIR spectrometer at 1064 nm	Fluorescence suppression
Resonance Raman Spectroscopy	Special excitation wavelength at 488 or 514 nm	Raman signal enhancement
Raman Microspectroscopy	Combination with an optical microscope	Higher spectral resolution
Spatially Offset Raman Spectroscopy	Uses two spatially offset points to obtain subsurface spectra	More subsurface information

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