

# Development of a multi-frequency laser for use in MALDI-TOFMS

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Received 11 November 2004; received in revised form 28 April 2005; accepted 11 May 2005

Available online 13 June 2005

## Abstract

The application of a multi-frequency laser source for the use in matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) is described. An elliptically polarized beam of a Nd:YAG laser emitting at 355 nm (200 mJ) is focused into a Raman shifter, filled with high pressure hydrogen. As a result, numerous Raman lasers (including vibrational and rotational Raman emissions for hydrogen; 4155 and 587  $\text{cm}^{-1}$  shifts, respectively) were generated with a total power of  $\sim 100$  mJ. Using this multi-frequency laser as an ionization source, methionine enkephalin (MW 573.7), angiotensin I (MW 1296.5) and oxidized insulin chain B (MW 3495.9) were examined, as model compounds using  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA) and activated charcoal as the matrix, respectively. As a result, the S/N ratios were improved when the multi-frequency laser was used, compared to the single light source of the Nd:YAG laser (355 nm), irrespective of the type of matrix used. This is because the multi-frequency laser provides multi-line for absorption, where the traditional  $\text{N}_2$  laser only provides single wavelength (at 337 nm) for absorption.

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**Keywords:** Multi-frequency laser; MALDI-TOFMS

## 1. Introduction

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) has become a very popular and powerful tool for the mass analysis of both biological [1–9] and small molecules [10–17]. In this technique, the use of a matrix in laser desorption could circumvent some of the limitations, associated with the ionization of high molecular weight bio-molecules. A requirement of the matrix is that, at a minimum, it should have a strong absorbance at the wavelength of an ionization laser. In this technique, the analyte is uniformly dispersed throughout a solid or liquid matrix, deposited on the surface of a substrate, and a pulsed laser beam is then directly focused on the surface. Although the mechanism of MALDI is not clearly understood, the ionization process is thought to involve several

steps. Initially, a solid solution is formed where the matrix absorbs energy from the excitation laser. Following this, the energy can be rapidly transferred from the matrix to the analyte, leading to its ionization. These ionization reactions occur in the first tens of nanoseconds after irradiation, and the matrix/analyte is desorbed. Thus, the selection of a suitable matrix for the transfer of energy to specific analytes is very important. A number of matrices have been examined at various wavelengths of different type of lasers, including nicotinic acid for 266 nm [2,18],  $\alpha$ -cyano-4-hydroxycinnamic acid/2,5-dihydroxybenzoic acid for 337 nm [19–21] or for 355 nm [17,22–23], graphite plate or rhodamine 6G/glycerol for 532 nm [24–26] and 3-hydroxypicolinic acid for 1064 nm [27–28]. Each of the above matrices has unique advantages and disadvantages with respect to energy transfer efficiency, sensitivity for mass detection, precision and simplicity of use. For an actual analysis case, in fact, a “trial and error” step for acquiring an adequate matrix is sometimes necessary, if the wavelength of the laser, such as a nitrogen laser (337 nm) is fixed, which is the most commonly used laser.

The generation of a multi-frequency laser source is not a new technique. Imasaka and co-workers demonstrated this

*Abbreviations:* CHCA,  $\alpha$ -cyano-4-hydroxycinnamic acid; MALDI-TOFMS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; SA, sinapinic acid

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technique for several applications [29–35]. In general, hydrogen is frequently used as the Raman medium because of its excellent Raman efficiency, but other Raman media can also be used, such as O<sub>2</sub> and N<sub>2</sub> but the conversion efficiency is poor. The exciting source (i.e. fundamental beam) for a Raman scattering experiment can be any type of high power laser, such as a Nd:YAG laser, Ti:sapphire laser, optical parametric oscillators (OPO) laser or two-color dye lasers. Even though, to the best of our knowledge, no reports have appeared on the use of a multi-frequency laser in MALDI-TOFMS. Therefore, we propose herein, for the first time, the use of a multi-frequency laser source in MALDI-TOFMS. The wide spectral region of this multi-frequency laser emission may be used in conjunction with almost any type of matrix, such as  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA; two common matrices used in commercial instruments, equipped with a N<sub>2</sub> laser emitting at 337 nm; absorbance range: UV) and activated charcoal (absorbance range: UV–vis–IR) used in this study. This possibility was examined and some results obtained are reported herein.

## 2. Materials and methods

### 2.1. Reagents

Methionine enkephalin (MW 573.7), angiotensin I (MW 1296.5), oxidized insulin chain B (MW 3495.9), cytochrome C, sinapinic acid and  $\alpha$ -cyano-4-hydroxycinnamic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Glycerol and activated charcoal were obtained from Yakuri Pure Chemicals (Osaka, Japan) and Saimakyu's Pure Chemicals (Osaka Japan), respectively. Acetonitrile, methanol and trifluoroacetic acid were purchased from Acros (NJ, USA). Hydrogen gas was supplied by Echo Chemical Co. Ltd. (Taipei, Taiwan).

### 2.2. Multi-frequency laser system

Fig. 1 shows the experimental apparatus used in this study. A Raman shifter (50-cm in length/1-in. in diameter/two arms equipped with 1-cm thick quartz window) was used for the

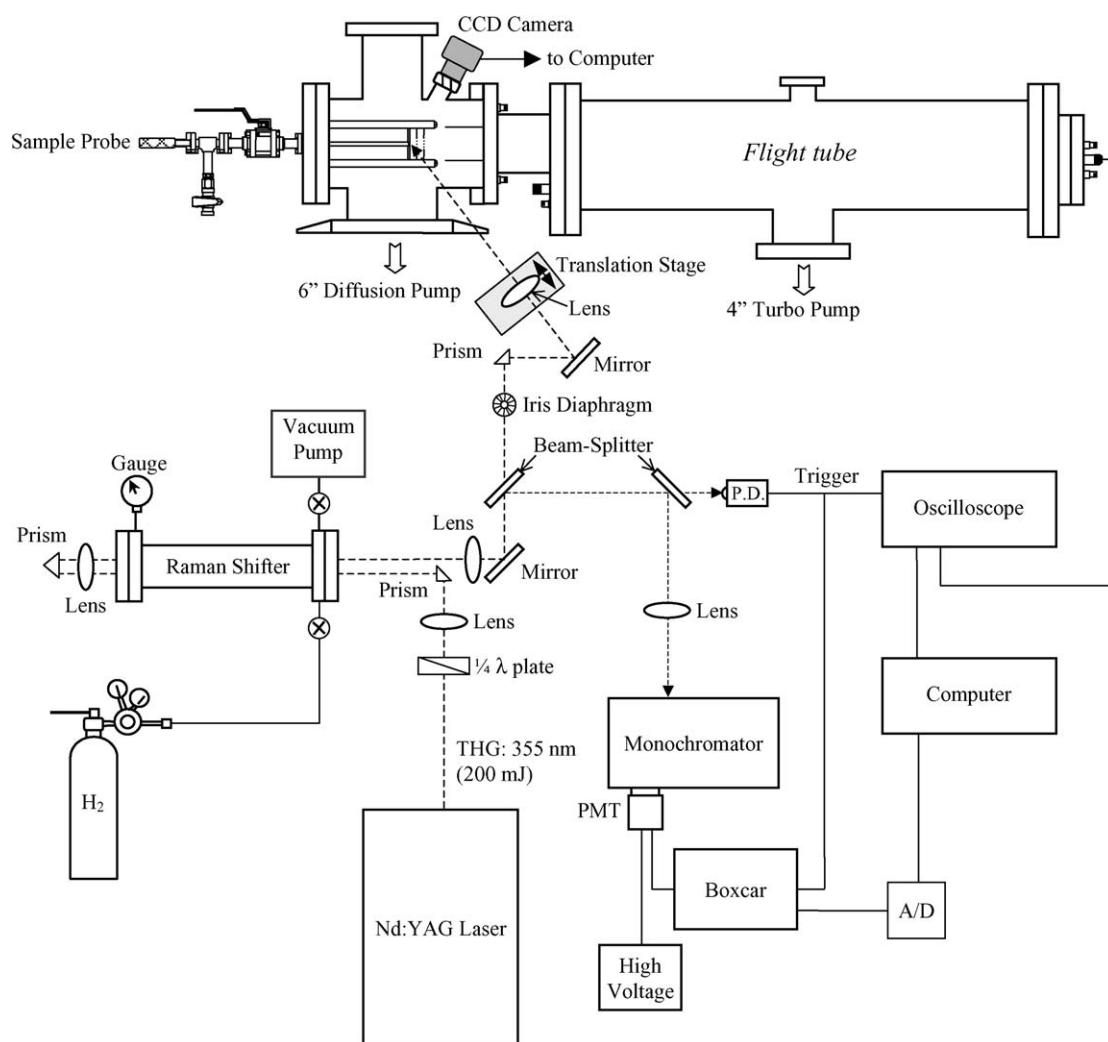


Fig. 1. The experimental apparatus used for the generation and detection of multi-frequency laser emission.

generation of the multi-frequency laser when high pressure hydrogen was used as the Raman medium. The 355 nm (200 mJ, 10 Hz) radiation, generated from a Nd:YAG laser (Spectraphysics GCR-170, Mountain View, CA, USA) was used as the fundamental beam. In this technique, the beam is passed through a quarter-wave plate (CASIX, WPL1212-1/4 $\lambda$ -355) to rotate the beam polarization for the generation of the first-Stokes beam by the effect of “stimulated Raman scattering”. The elliptically polarized beam is focused on the Raman shifter by a quartz lens (focus length, 50 cm). The angle of the quarter-wave plate can be rotated, so as to change the beam polarization from circular to elliptical to acquire the generation of higher-order Stokes and anti-Stokes beams; the rotational Raman shift frequency of which is 587  $\text{cm}^{-1}$ . In order to improve the conversion efficiency, the beam is passed through the Raman shifter twice. The spectrum of the output beam was collected by a monochromator (ARC, Acton Research Corporation; Model SP-500, 1200 grooves/mm grating) equipped with a photomultiplier (Hamamatsu, R928). The output signal was measured by a boxcar integrator (SRS 250, Stanford Research Systems, Inc., CA, USA). The analog signal was converted to a digital signal by an A/D converter (ADAM-4012 module, Advantech Co., Ltd., Taiwan), and then recorded with a data acquisition system connected to a personal computer.

### 2.3. MALDI-TOFMS apparatus

The linear type of time-of-flight mass spectrometer (TOFMS) used in these experiments was a modified Wiley–McLaren design by the R.M. Jordan Corporation (Grass Valley, CA, USA). The flight distance was 1.1 m; a 4-in. turbo (flight tube) and a 6-in. diffusion (ionization region) pump was used to maintain a vacuum below  $\sim 5 \times 10^{-7}$  Torr during the experiments. A stainless steel target was used as the MALDI substrate and the samples were deposited directly on it. The instrument was equipped with a CCD camera, displaying the sample image on a monitor, thus permitting the laser to be focused on a specific spot within the area of the target. The power of the multi-frequency laser was measured by a power meter and can be adjusted by an Iris diaphragm or attenuation filters. The lasers were then focused by a quartz lens on the target. The focus lens was located on a translation stage; the focus length of each laser is different, so that the focusing zone can be optimized by moving the stage. The ions formed by MALDI were produced in a field-free region and then directly migrated toward the detector, where a 25 mm triple microchannel plate (MCP) was used for ion detection. Data were recorded using a LeCroy 9350A digital oscilloscope (500 MHz) and processed by a personal computer. All spectra were obtained as 200-shot averages.

### 2.4. Sample preparation

Angiotensin I, methionine enkephalin and oxidized insulin chain B (1.0 mg of each) were dissolved in 1.28,

3.33 and 0.47 mL water, respectively, to give stock solutions (600  $\mu\text{M}$  each). After this, a 0.1 mL aliquot of each stock solution was mixed with 0.9 mL water to give three standard solutions (each 1.0 mL, 60  $\mu\text{M}$ ). Cytochrome C (5.0 mg) was dissolved in 0.6 mL of water and the following preparation was the same as described above. The remainder of the stock solutions were stored in the freezer for future use.

## 3. Results and discussion

Fig. 2 shows the spectrum of multi-frequency laser emissions generated by a Nd:YAG laser when 355 nm radiation was used as the fundamental beam. Numerous high-order vibrational (4155  $\text{cm}^{-1}$  shifts) as well as rotational (587  $\text{cm}^{-1}$  shifts) lines are observed; the pressure of hydrogen was 5 atm. Among these vibrational lines (from AV<sub>3</sub> to V<sub>3</sub>), spreads of numerous rotational lines are observed. The detectable lines can cover from 242.6 to 865.9 nm with different intensities. The spectral intensity decreased in the long-wavelength region since the grating used was a UV-blaze; the spectral response of the photomultiplier used in this study decreased at wavelengths beyond 900 nm. Nevertheless, the

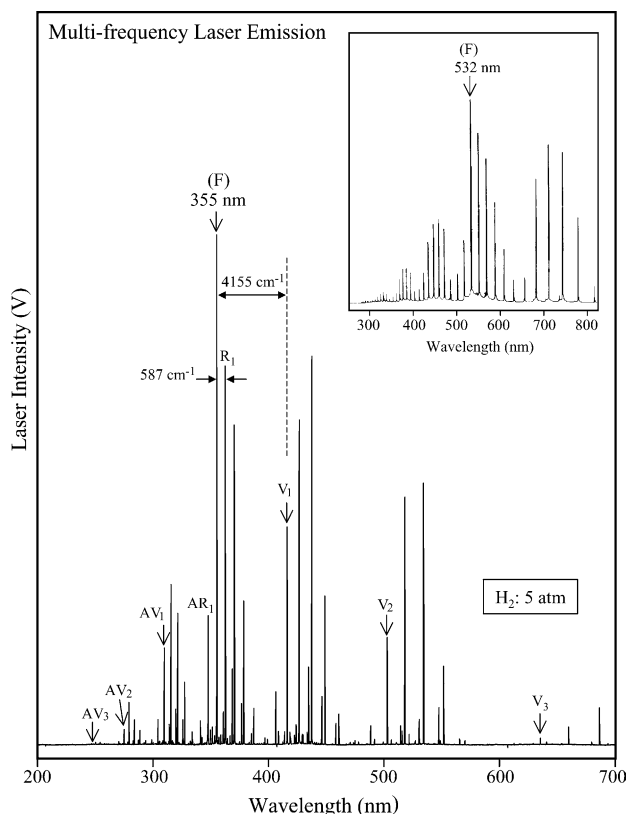


Fig. 2. Spectrum of multi-frequency laser emission ( $\text{H}_2$ : 5 atm). Inset, spectrum obtained when a second harmonic generation (532 nm) of the Nd:YAG laser was used. R, rotational lines; V, vibrational lines; AV, anti-Stokes vibrational lines; AR, anti-Stokes rotational lines. The subscript indicates the order of the vibrational or rotational line.

total energy of these emissions is  $\sim 100$  mJ. Since a high power laser (greater than 1 mJ) is not necessary for MALDI, all of the multi-frequency lines can be used after attenuation, or they can be separated to a single beam by a prism, such as a Pellin Broca prism. In the former case, the focal lengths of these lines are different, so that the focus points are multiple. Since these lines are very close (separated from each other by only  $587\text{ cm}^{-1}$ ), the focus points should be overlapped because of the beam waists and the confocal lengths of these lines can be as much as several micrometers. A focus lens (focus length: 20 cm at 500 nm), equipped on a translation stage, was used to select the most favorable focusing zone. Meanwhile, the intensity of the detected ions was monitored by moving the translation stage from front to back. In

the other case, a multi-frequency laser also can be used as a single beam after prism separation, or as multiple lines by band-pass filters, depending on the wavelengths needed. Furthermore, when a second harmonic generation (532 nm) of the Nd:YAG laser was used, the wavelength covering range can be up to the infrared region (data not shown). This would be useful if an IR-excited matrix were to be necessary for a specific analyte. In either of the cases mentioned above, using this approach for finding a suitable exciting wavelength to a particular matrix, searching for an appropriate matrix to a fixed wavelength of laser is no longer necessary. In order to investigate the effects when different hydrogen pressures are used, under exactly the same experimental conditions, various hydrogen pressures (1–10 atm) were examined. When

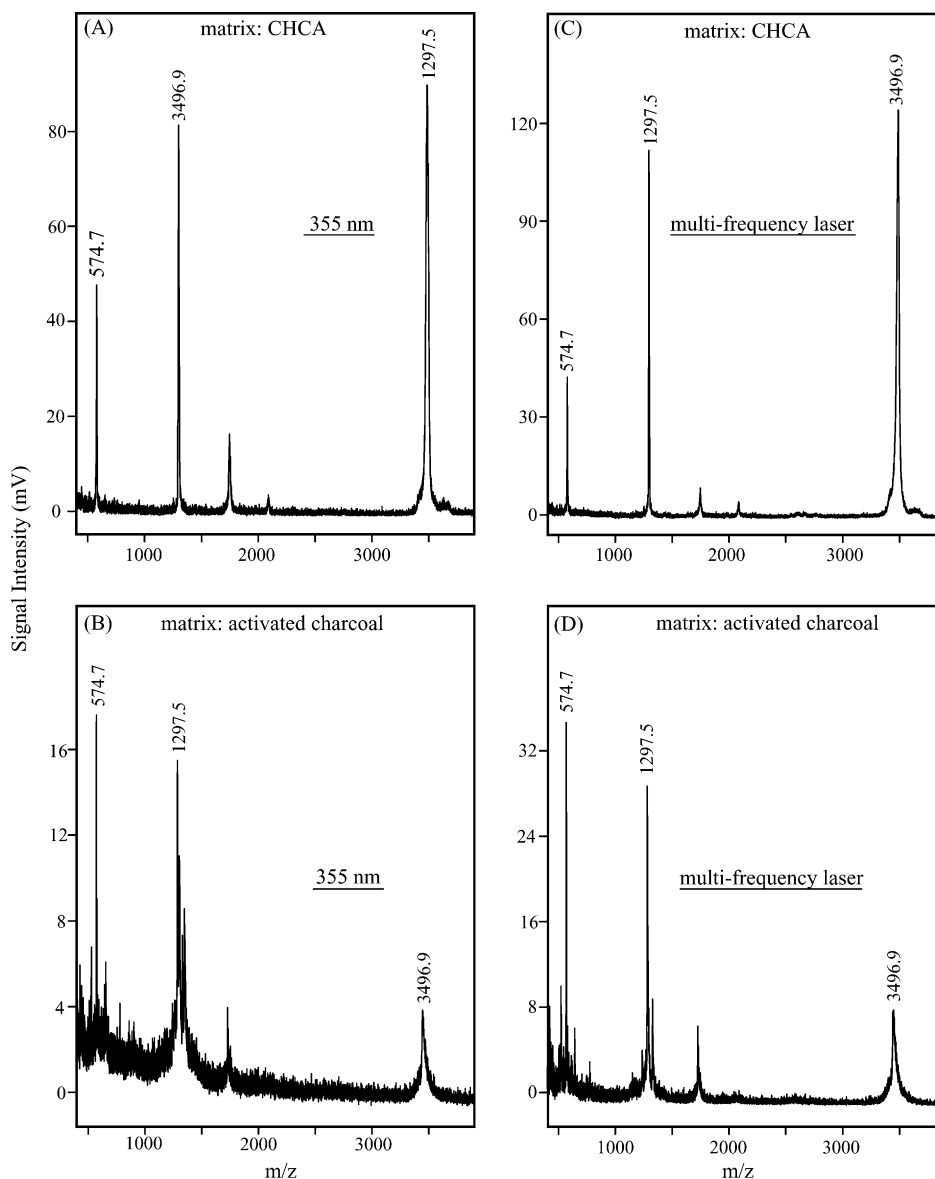


Fig. 3. Frames A–D show MALDI-TOFMS results. Ionization source: frames A and B, the third harmonic generation of Nd:YAG laser (355 nm); frames C and D, multi-frequency laser. Matrix (frames A and C), CHCA 10 mg/mL in a mixed solution (water/acetonitrile = 50/50, v/v, with 1% trifluoroacetic acid); analytes: a mixture of methionine enkephalin (15 pmol), angiotensin I (0.01 pmol) and oxidized insulin chain B (8 pmol). Matrix (frames C and D), activated charcoal/glycerol; analytes: a mixture of methionine enkephalin (40 pmol), angiotensin I (0.05 pmol) and oxidized insulin chain B (30 pmol), respectively.

the hydrogen pressure was adjusted to 1 atm, only a few lines are observed around the fundamental beam because the efficiency of Raman scattering is dependent on the density of the medium. When the density of medium is increased (up to 10 atm), numerous lines appear. However, at higher hydrogen pressures, the vibrational emissions are more effective than rotational emissions. In addition, when the quarter-wave plate was rotated at various angles, the beam pattern would be altered but the total intensity remained unchanged (data not shown). We selected a pressure of 5 atm as optimal in subsequent experiments. The inset shows the result obtained when the fundamental beam was changed to 532 nm, multi-frequency lasers was observed, and the range can cover from 250 to 800 nm.

Fig. 3 shows the MALDI-TOFMS spectra when the three smaller peptides was selected as the test sample (frames A and B: laser, 355 nm/matrixes, CHCA and activated charcoal, respectively; frames C and D: laser, multi-frequency laser/matrixes, CHCA and activated charcoal, respectively). Herein, the concentration of CHCA was 10 mg/mL in a mixed solution (water/acetonitrile = 50/50, v/v; with 1% trifluoroacetic acid). To maintain the ion intensity at the same scale, the concentrations of analytes used are different since various ion intensities are produced when CHCA and the multi-frequency lasers were used. Compared to a single beam (355 nm), the S/N ratios are improved. This is because the multi-frequency laser can provides a greater opportunity for absorption to occur. Fig. 3B and D shows a similar experiment

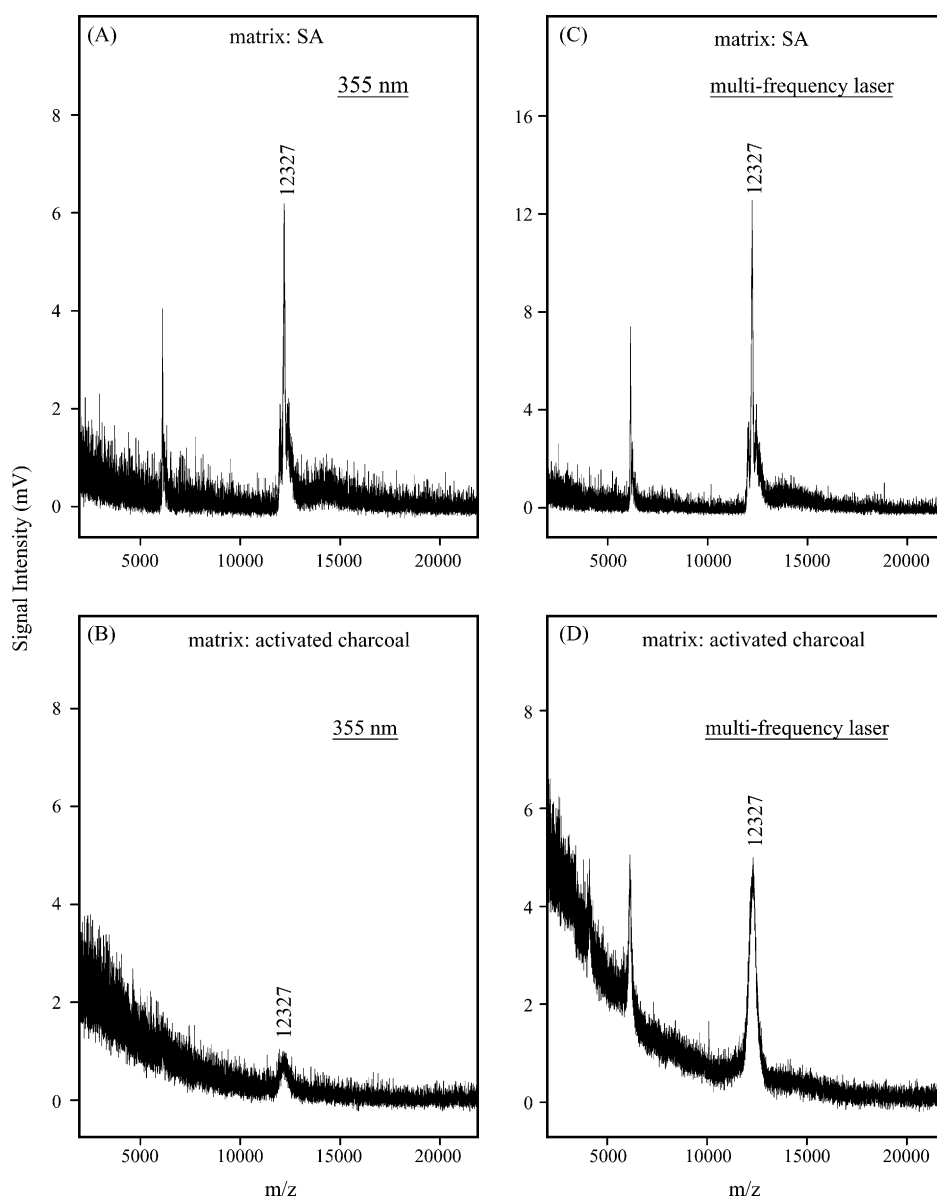


Fig. 4. Frames A–D show MALDI-TOFMS results. Ionization source: frames A and B, the third harmonic generation of Nd:YAG laser (355 nm); frames C and D, multi-frequency laser. Matrix (frames A and C), SA 10 mg/mL in a mixed solution (water/acetonitrile = 50/50, v/v, with 1% trifluoroacetic acid); analyte: cytochrome C (5 pmol). Matrix (frames C and D), activated charcoal/glycerol; analyte: cytochrome C (5 pmol).

in which a mixture of activated charcoal/glycerol was used to examine the analytes (methionine enkephalin, 40 pmol; angiotensin I, 0.05 pmol; oxidized insulin chain B, 30 pmol). Activated charcoal absorbs both in the UV and NIR regions. Because of this, the use of a multi-frequency laser as the ionization source is preferable to that of a single laser. As a result, this method also permits a better S/N ratio for the mass spectra. These data demonstrate that the use of a multi-frequency laser can be very useful when for CHCA or activated charcoal is used as the matrix. The detection limit (S/N = 3) of methionine enkephalin, angiotensin I and oxidized insulin chain B were 200, 5 and 15 pM, respectively. In order to compare the ionization efficiency on larger protein, cytochrome C (5 pmol) was selected as a model compound. The MALDI-TOFMS spectra (as shown in Fig. 4) and similar experiments were carried out (frames A and B: laser, 355 nm/matrixes, SA and activated charcoal, respectively; frames C and D: laser, multi-frequency laser/matrixes, SA and activated charcoal, respectively). As it can be seen again, the S/N ratios were improved when the multi-frequency laser was used, irrespective of the type of matrix. The detection limit (S/N = 3) of cytochrome C was 0.5  $\mu$ M.

#### 4. Conclusions

This work describes the successful application of a multi-frequency laser for use in MALDI-TOFMS. Compare to a single beam (355 nm) or a nitrogen laser (337 nm), the multi-frequency laser provides a greater opportunity for absorption to occur. Although the utility of the multi-frequency laser was investigated using CHCA and activated charcoal as matrices, the performance could be extended to any type of matrix for a specific analyte. The method is a rapid, simple and economic technique, and also suggests that such a type of laser has great potential for use as a new light source in the field of MALDI-TOFMS, not only for ionization needs but for other purposes, such as mechanism studies.

#### Acknowledgment

This work was supported by a grant from the National Science Council of Taiwan under Contract No. NSC-92-2113-M-003-023.

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