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The influence of laser fluence on ion yield in matrix-assisted laser desorption ionization mass spectrometry

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Abstract

Ion yields in matrix-assisted laser desorption ionization have been measured as a function of laser fluence using pulse-counting and analog detection methods in the same TOF instrument configured in the usual axial geometry. In addition, measurements have been made using pulse-counting methods in an orthogonal-injection TOF instrument, which uses collisional cooling to spread the plume along the injection axis. This allows pulse-counting methods to be used without ambiguity as to the number of ions striking the detector, and extends the dynamic range over which pulse-counting can be used reliably without changing the instrument transmission. All measurements indicate a steep power-law dependence of the ion yield on fluence with an exponent in the range of 7–9. The same power-law dependence extends below the fluence usually associated with a threshold in analog measurements by at least a factor of two, and the data show no indication of physical fluence threshold. (Int J Mass Spectrom 221 (2002) 67–81)

Keywords: MALDI; Threshold; Laser fluence; Ion yield

1. Introduction

In the last 10 years ultraviolet matrix-assisted laser desorption/ionization mass spectrometry (UV MALDI-MS) [1] has become an important method for analysis of biomolecules, but the mechanisms are still not completely understood. To help develop a quantitative model, it is important to determine the dependence of the ion yield (defined here as the number of ions produced per laser pulse) on laser fluence. It is particularly interesting to examine the low fluence regime to understand the nature and statistics of the ion generation process.

The term “threshold” is commonly used in MALDI to refer to the fluence or irradiance above which analyte signals can be obtained above noise from a single laser shot using analog detection methods with a transient recorder. This definition does not correspond to a well-defined, absolute fluence because it depends on many parameters such as analyte–matrix combination and their molar ratio in the preparation, laser spot size, spectrometer transmission, detector gain, noise level, and a subjective interpretation of the statistics of the event. Nevertheless, the term has been sufficiently

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unambiguous to have found a useful role in the field. The existence of an operational threshold, particularly at a reasonably consistent fluence (given comparable experimental conditions), reflects the very steep dependence of the ion yield on laser fluence, which has been reported by several groups [2–9]. Whether a true threshold in the physical process exists, or whether this observed threshold is simply a result of limitations in the measurement methods had remained uncertain.

If the observed threshold results from the limited signal-to-noise (S/N) ratio obtained from single laser shots using analog detection methods, then it is difficult to determine whether a true physical threshold for desorption exists at the same or some lower fluence using the same detection method. For this reason, earlier investigations of MALDI at the University of Manitoba were performed using pulse-counting methods with a time-to-digital converter [3]. Those results were interpreted as evidence for a true fluence threshold near 200 $\mathrm{J/m^2}$ for insulin molecular ions desorbed from a sinapinic acid matrix. Above this threshold, the ion signals were observed to follow a power-law with an exponent between 4 and 6.

The dependence of the ion signal on laser fluence in MALDI was subsequently measured in Münster [9]. The Münster experiment introduced several improvements in the optics compared to the Manitoba experiment: (i) To separate the effects of beam spot-size and laser fluence, a fibre-optic was used to produce a flat-top laser profile, rather than the Gaussian or near-Gaussian profile obtained with standard optics. Increasing the fluence when the profile is Gaussian, as was the case in the Manitoba experiment, will not only result in an increased local irradiance, it will also increase the total area of substantial ion emission. Indeed, a steep dependence of the signal on fluence would be observed from this increase in area alone, if the signal per unit area were a simple step-function at some threshold [10]. (ii) Secondly, the laser energy was monitored for each laser shot, and the measurement was carefully calibrated to get an accurate absolute fluence measurement. (iii) Finally, an attenuator with a much finer step-size was used to control the fluence.

The Münster results also showed a steep power-law dependence of the ion yield with fluence, with exponents larger than 7, but showed no evidence for a true fluence threshold. Moreover, using laser post-ionization, the experiment also showed that many neutrals are generated per laser shot, even at fluence values well below the analog threshold. The yield of such neutrals showed a similar steep dependence on fluence, with exponents only slightly smaller than those found for ions. However, the experiment evaluated single shot spectra using analog detection. Thus, although an accurate measure of the absolute fluence was not available for the earlier Manitoba experiment, it seemed likely that it had probed a lower fluence range by using pulse-counting detection, and the possibility remained that this difference accounted for the observed threshold in the Manitoba results.

Here we report collaborative experiments at Münster [11] and similar measurements at Manitoba [12], in which we combined the improved optical configuration with pulse-counting detection and compare the results directly with measurements made using analog detection in the same instrument with directly comparable fluence. Both methods of data acquisition have limitations in the usual axial TOF geometry. The analog measurements are limited at low fluence by the S/N ratio, at least for single-shot spectra, and at high fluence by detector saturation, if a microchannel plate detector is used. The pulse-counting measurements are difficult to interpret, especially at high fluence, because they require that only individual ions are detected. Therefore, we have also made measurements in an orthogonal-injection MALDI/TOF instrument with collisional cooling [13]. This new instrument enables pulse-counting over a much wider fluence range, without ambiguity as to the number of ions striking the detector.

2. Experimental

Measurements were performed on two different types of mass spectrometer: linear time-of-flight (axial TOF), and orthogonal-injection TOF. Axial TOF measurements were made in Münster and at Manitoba in instruments that differ in detail only. The measurements on the orthogonal-injection instrument were made in Manitoba. Both pulse-counting and analog detection were used in the axial TOF experiments; all the measurements in the orthogonal TOF system were made using pulse-counting. All the measurements depend on the acceleration of desorbed ions to keV energies, so desorbed neutrals do not contribute to the measurements. Instrument transmission and detection efficiency depend on the details of the experimental configuration and the ion energies, but for a given experiment, these are kept constant and are not expected to affect the measurement of the fluence dependence.

2.1. *Optics*

The optical arrangement was essentially the same for all of the experiments. The target was irradiated with a nitrogen laser (VSL-337ND: Laser Science, Inc., Franklin, MA, USA) with a wavelength of 337 nm and a pulse duration of about 3 ns. To achieve a flat-top distribution of energy on the target, a fiber-optic arrangement, similar to the one described and characterized previously [9,12], was used. The laser beam was coupled into a high-power optical fiber (Münster: FG-200-LAT, 3M, West Haven, CT, USA; Manitoba: F-MCC-T, Newport, Mississauga, Canada) with a short focal length lens. The fibers were both 200 μm in diameter, had numerical apertures of 0.18 (Münster) and 0.22 (Manitoba), and were 1.5 m (Münster) or 4 m (Manitoba) in length. A homogeneous intensity profile at the fiber exit face is formed because of multiple reflections of the laser beam inside the fiber. The surface of the fiber tip was then imaged 1:1 onto the target by two identical planoconvex lenses arranged in infinite conjugation ($f = 120$ mm Münster; 150 mm Manitoba).

The laser beam fluence, defined as energy per unit area per pulse, was controlled by a circular gradient neutral density disk (Manitoba) or by a rotatable dielectric attenuator (Münster). The fluence of individual laser pulses was monitored in the axial TOF measurements by placing a quartz beam splitter between the two imaging lenses. In the orthogonal TOF measurements, a reflection from the quartz window (which admits the laser beam into the spectrometer) was used to monitor the fluence. A small fraction of the laser beam was thus reflected onto a pyroelectric detector (420: Eltec Instruments Inc., Daytona Beach, FL, USA) connected to a custom preamplifier (built by the Münster group). The signal from the pyroelectric detector preamplifier was fed into a linear amplifier (1416: Canberra Industries, Meriden, CT, USA) and recorded by the data acquisition system for each laser shot.

Calibration of the pyroelectric detector was done with commercial energy meters (Münster: Laser Precision Corp., Yorkville, USA; Manitoba: J3-02, Molectron, USA). The absolute fluence used in the Manitoba measurements remains somewhat uncertain (but probably within ±50%) because accuracy of the energy meter was not well-known, the size of the beam spot was not directly measured (only inferred from the optics) and for the orthogonal TOF, losses resulting from entry into the spectrometer could not be exactly reproduced for the calibration. Therefore, the absolute fluence in the different instrumental geometries, for which different optics were used, should not be directly compared. However, the optics were identical for the pulse-counting and the analog measurements for a given TOF geometry, so the comparison between these measurements is valid. The calibration in Münster was done more precisely, so those fluence measurements are expected to be accurate to within about ±20%. It should be emphasized that the measurements of the fluence dependence are not affected by the uncertainty in the absolute fluence, since the measurements of the relative fluence in any given experiment are accurate to within about ±3%; the pulse-counting experiments used a 5% fluence window (see below).

The incident laser beam struck the sample target at an angle of incidence of 30° (Münster) and 65° (Manitoba) so the spot size in the Manitoba measurements was about two times larger than in the Münster measurements. This is likely to affect the absolute signal intensities but should not have an appreciable effect on the fluence dependence.

2.2. Sample preparation

Sample preparation was similar for all the measurements. The pulse-counting experiments require a comparison of ion signals from many different sample spots (see below), so it was essential to obtain uniform sample coverage over a fairly large area. For this reason a fast-drying technique similar to the one introduced by Vorm et al. [14] was used. In our case, in order to give a signal which persists for more laser shots, a much thicker layer was produced, and the analyte was mixed in solution with the matrix prior to depositing on the target. Matrix was dissolved to a concentration of about 10 g/L in acetone. The analyte was first dissolved to a concentration of about 10^{-3} M in 0.1% aqueous TFA, and then this solution was mixed approximately 1:20 with the matrix solution. Between 5 and 20 μL of this mixed solution was then applied to the metal targets of the different instruments. The acetone evaporated within seconds leaving behind a quasi-homogeneous crystallized coating of the matrix/analyte mixture with an analyte-to matrix molar ratio of about 5×10^{-4} and a sample size between 0.5 and 1.0 cm^2. All samples and matrices were used as purchased from commercial suppliers.

2.3. Mass spectrometry

2.3.1. Axial geometry

Simple linear time-of-flight instruments with the usual ion-injection geometry were used for most of the measurements. The positive ions produced at the target are accelerated by a constant electric field established between the target at a dc potential of 6 kV (Münster) or 10 kV (Manitoba) and a grounded grid 10 mm (Manitoba) away and a three element einzel lens of 3 mm aperture, 6 mm away (Münster). The flight path was approximately 1 m in the Manitoba instrument, and either 0.9 or 2 m in the Münster instrument.

In the pulse-counting measurements, the objective was to count single ions to improve the sensitivity and linearity of the measurements. In the higher fluence range, it was therefore necessary to reduce the transmission of the instrument with an aperture placed near the detector. In the Münster spectrometer an array of selectable apertures with diameters 1, 2, 4, 10 and 20 mm was used. At Manitoba, a continuously variable aperture was used with a maximum opening of about 1 cm^2. It consisted of a tapered slit cut into a rotatable half-cylinder, placed in front of a stationary vertical slit with a width of 5 mm.

Each measurement cycle was initiated by a pulser, which triggered the laser and supplied a start signal for the timing measurement. The ions were detected in the Manitoba instrument by secondary-electron emission from a conversion plate 25 mm in diameter, coated with CsI. The secondary electrons were accelerated between the conversion plate at −600 V and a grounded 95%-transmission grid, and then directed through 135° of a circular arc by a uniform magnetic field onto a dual microchannel-plate (MCP) detector (Fig. 1). The conversion plate potential was pulsed to avoid MCP saturation from the abundant low-mass matrix ions. In the Münster instrument the ions were detected directly by a dual microchannel plate detector after post-acceleration by about 6 kV to give a final energy of about 12 keV [9]. Some analog measurements in the Münster instrument were also made with a conventional secondary electron multiplier for comparison. No matrix suppression was used in the Münster measurements.

2.3.2. Orthogonal geometry

A schematic diagram of the orthogonal-injection MALDI/TOF instrument is shown in Fig. 2. In this instrument, the ejected MALDI plume enters an rf-quadrupole ion guide which is filled with nitrogen at a pressure of about 10 Pa. Collisional cooling in the ion guide produces an ion beam with a low energy distribution and small cross-section, well-suited for orthogonal injection into a reflecting TOF instrument. Details of this spectrometer and its performance have been reported previously [13]. For this experiment, the relevant characteristic is that the collisional-cooling spreads the MALDI plume out along the quadrupole axis, which allows many injection pulses to be used for each laser shot. Therefore, pulse counting can be used reliably even if multiple ions generated from

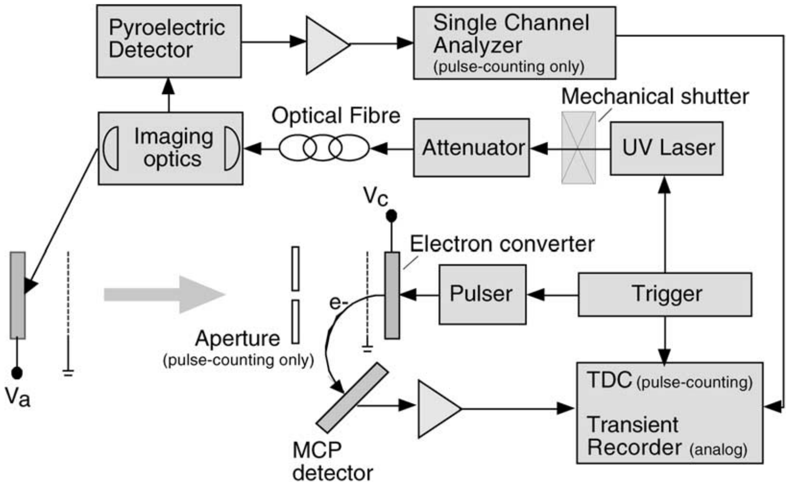

Fig. 1. Schematic diagram of the axial TOF spectrometer showing both analog and pulse-counting data system. The detector used at the University of Manitoba is illustrated. In the Münster instrument, an MCP detector was used in the position of electron converter, without a pulser. An array of apertures, 1, 2, 4, 10, and 20 mm, was used in Münster. In Manitoba a variable opening with an area between 0 and 1 cm^2 was used.

a single laser pulse are detected. After many laser shots, the total number of ions counted is simply proportional to the total number of ions produced, and normalized to the number of laser shots, represents the average number of ions per laser shot, or the average ion yield [9].

In these experiments, the laser was pulsed at 20 Hz, and ions were injected into the spectrometer at a repetition rate of 3400 Hz. In most cases, even for the highest fluence, on average fewer than one ion of a given species was injected into the spectrometer per injection pulse, so the instrument is well-suited for pulse-counting. Since there are about 170 injection pulses for each laser shot, this arrangement allows pulse-counting to be used with a 170-fold increase in data-rate over the pulse-counting measurement done in an axial MALDI/TOF instrument. This allows almost the entire fluence range to be examined without artificially reducing the ion transmission, and without ambiguity as to the number of ions striking the detector. Although the ion transmission was not deliberately reduced, the overall transmission of the orthogonal injection geometry is small, probably around 1%. This low transmission limits the accessible fluence range at the low end.

The ions enter the acceleration column perpendicular to the TOF axis, with about 6 eV energy, defined by the potential applied to the aperture plate, which admits ions into the column. They are injected into the TOF spectrometer by pulses applied to the back electrode of the acceleration column. The ions are accelerated to an energy of about 4 keV, and then travel toward an electrostatic ion mirror which reflects them onto the microchannel plate detector. At the detector the ions undergo post-acceleration through ∼1 kV so their total energy when they strike the detector is about 5 keV (for the singly-charged ions considered here).

2.4. Data acquisition

2.4.1. Analog measurements

Analog measurements were made only in the axial geometry (Fig. 1). For these measurements the aperture is removed or left open, and the procedure is similar to the usual analytical application of MALDI; a complete mass spectrum is recorded for each laser shot with a digital oscilloscope or transient recorder (LeCroy 9450AM (Manitoba), 8400 (Münster): LeCroy Corp., NY, USA). The signal from the microchannel-plate detector is either amplified first

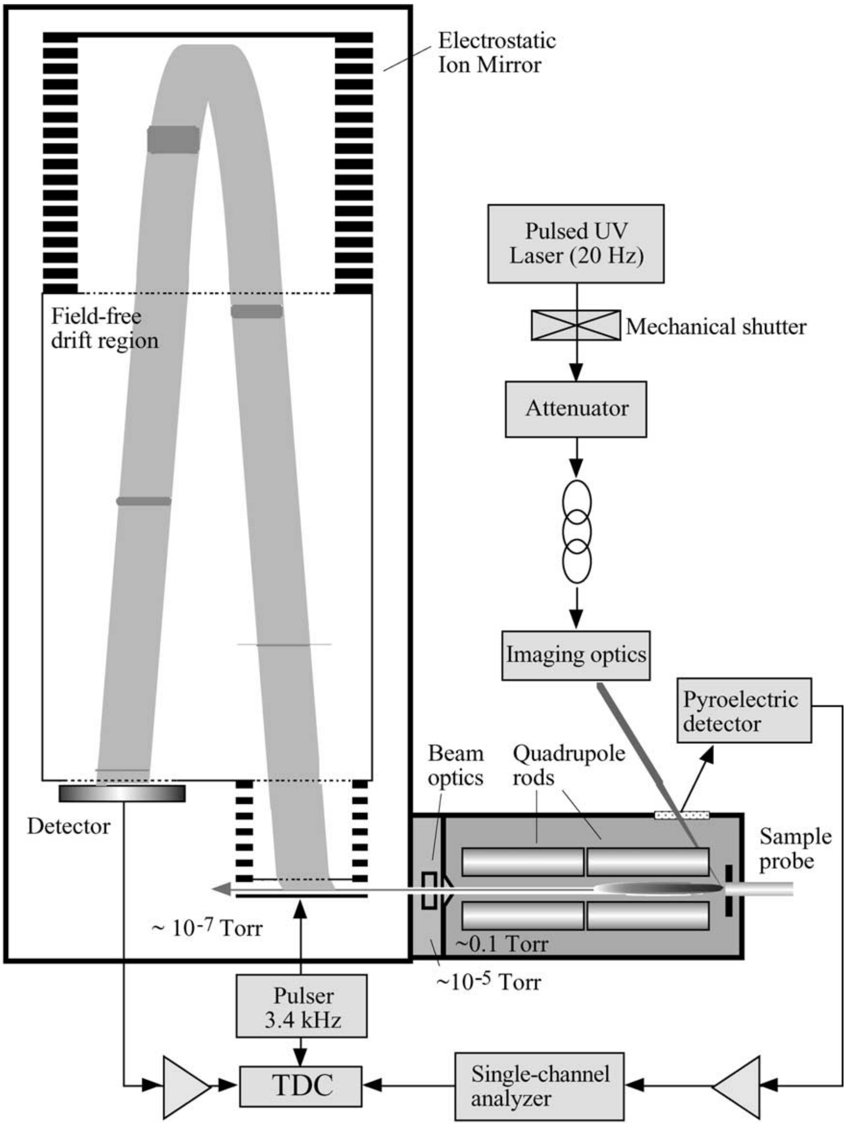

Fig. 2. Schematic diagram of the orthogonal MALDI/TOF instrument and pulse-counting data system.

with a gain of 10 (Münster) or fed directly (Manitoba) to the input of one channel of the oscilloscope. The energy signal from the pyroelectric detector is fed into the second channel of the oscilloscope directly (bypassing the single-channel analyzer in Fig. 1). Both transients are transferred to a computer for further processing. Thus, for each laser shot, the ion signal and a measure of the laser fluence are recorded.

Following the procedure of Dreisewerd et al. [9], all ion signals were measured from spectra generated from single laser shots to ensure precise fluence correlations and to facilitate comparison to the pulse-counting data. In the Manitoba experiments, to minimize the usual spot-to-spot fluctuations in ion signals, complete signal-versus-fluence datasets were collected from single sample spots. There is some depletion of the signal during irradiation, especially after the first few shots. However, by ignoring the first 10 or 20 shots on a spot, and by careful monitoring of the signal at a given fluence, this effect could be made small enough to be neglected. Relative ion yields were obtained from the height of the peaks of interest in the digitized transient. Absolute yields are expected to be proportional to these peak-heights, but the proportionality constant, determined by instrument transmission and the detector gain, was not evaluated, so the units are arbitrary. In the Münster measurements, many different spots were used to generate the datasets, taking only the first few shots from each spot, and peak areas were used to calculate relative signal intensities.

2.4.2. Pulse-counting measurements

The arrangements for the pulse-counting measurements in the two geometries are shown in Figs. 1 and 2. In both cases, a 255-stop time-to-digital converter (TDC) (CTN-M2: Institut de Physique Nucléaire, Orsay, France) was used instead of a transient recorder to measure the time between the start signal and the detector pulses, produced, in the ideal case, by individual ions. In the axial geometry, the start signal for the TDC is coincident with the laser trigger, and both are supplied by the trigger pulser. The small jitter between the trigger pulse and the laser shot is not significant for these experiments. In the orthogonal geometry, the start signal corresponds to the injection pulse which is supplied at 3400 Hz by a digital delay generator; the laser is triggered internally at 20 Hz.

To detect single ions, the microchannel-plate voltage is set so that the signal from individual ions is substantially larger than the electronic noise which is typically only a few mV. The detector signal is amplified with a preamplifier (9301: EG&G ORTEC, Wellesley, MA, USA), and then fed into a constant-fraction discriminator (axial TOF: Philips Scientific 715, Ramsey, NJ, USA; orthogonal TOF: ENERTEC Schlumberger 7174, Quimper, France) with the threshold set high enough to eliminate noise (about −60 mV). Timing signals from the discriminator are fed into the TDC, which is connected to a computer which accumulates the time-of-flight histogram. For the axial TOF measurements, an Atari TT030 computer was used with a custom interface, and for the orthogonal TOF measurements, a PowerMac 7300/180 was used connected by a PCI digital I/O card (FPCI-DIO: Fishcamp Engineering, Orcutt, CA, USA).

Measurements made using pulse counting (in both geometries) clearly require accumulation of data from many laser shots. Therefore, a protocol was developed to ensure that the laser fluence for a series of laser shots on different sample sites was within a selected range. The amplified signal from the pyroelectric detector, which is proportional in amplitude to the laser fluence for each shot, was fed into a single-channel analyser (SCA) (Ortec 455: EG&G ORTEC, Wellesley, MA, USA). The SCA generates an output pulse if the input pulse-amplitude is within a selected range. For these measurements, the SCA was always set to accept laser shots within a 5% fluence window. The output of the SCA is connected to a second channel of the TDC and the data acquisition software is configured to record data from the ion detector only if a pulse from the SCA is detected; that is, only if the corresponding laser shot is within the 5% energy window. Operating at a constant repetition-rate, the laser used for these measurements has approximately 10% fluctuations in the energy from shot-to-shot. Thus, the SCA improves the precision in the fluence value for a given measurement by a factor of about 2, and perhaps more importantly, ensures that no anomalous laser shots, with significantly higher fluence, contribute to the measured signal.

Each spectrum is the accumulation of data from several different target spots, each spot exposed to the laser for 1 or 2 s (20–40 laser shots). Since the stability of the laser fluence is better if the laser runs continuously at a constant repetition rate, the 2-s exposure was achieved with a custom mechanical shutter, connected to a standard darkroom timer. After about 100 ms from the time the shutter closes, there are no ions remaining in the quadrupole, so there is no cross-talk between spectra taken at different fluence. To ensure that the laser spot-size remains constant, the optics are left fixed for all measurements, and the target is moved to expose different sample sites. Thus, the identical laser focus is used for all the target sites. In the Manitoba instruments, the targets were rotatable probes, so only a ring could be exposed without moving the laser spot. In the axial TOF instrument, this limited the number of spots to about 50 spots per target, corresponding to about 2000 laser shots. In the orthogonal TOF instrument, the probe was smaller, and at most about 30 spots per target were used. Fewer target spots were needed for higher fluence since the count rate is higher. After one full rotation of the target, a fresh target-sample was prepared. In the Münster instrument, the target could be rastered so that more of the target could be used, and up to 5000 laser shots per target could be used. The spot-to-spot fluctuations mentioned above tend to average out when data from many spots, and many laser shots, are accumulated. The relative signals are determined by integrating the peak of interest and dividing by the number of laser shots, and are therefore reported as counts per laser shot.

3. Results and interpretation

3.1. Measurements in the axial TOF geometry: analyte signals

Experiments were first performed at Münster using the Münster axial MALDI/TOF instrument with a TDC and data system from the Manitoba group. Most of these measurements were made with the analyte angiotensin I, and the matrices α-cyano-4-hydroxycinnamic acid (αCHCA) and 2,5-dihydroxybenzoicacid (DHB). One measurement was also made with insulin and αCHCA. The experiment in the axial TOF mode was later repeated at Manitoba to extend it to include insulin in sinapinic acid, the sample used in the 1990 measurements [3], and to check for a possible instrument dependence. These measurements at Manitoba also provided benchmark for the optical system, which was then used in the orthogonal TOF measurements described below.

The measured relative ion signals for insulin with the matrices αCHCA and sinapinic acid, for leutinizing hormone releasing hormone (LHRH) with the matrix αCHCA, and for angiotensin I with αCHCA and DHB, obtained with the axial MALDI/TOF configuration are shown in Fig. 3. Each of the plots shows the data obtained both with the analog system and with pulse-counting. The analog measurements were accepted for laser shots that produced a S/N ratio ≥ 2:1, and for fluences where the probability of obtaining this ratio was at least 50%. The pulse-counting data are shown for large and small aperture sizes as indicated, to probe different fluence ranges.

The datasets are fit to a simple power-law. The open data points at higher fluence, which deviate from the power-law dependence, are excluded from the fit. The slope for each curve is indicated in Fig. 3.

3.1.1. Interpretation of the analog measurements

The vertical scale for the analog measurements (shown on the right axis) represents the molecular ion peak intensities determined from the recorded transient. In the Manitoba measurements, these were derived simply from the peak heights because this gave more reproducible results compared to when peak areas were used. The intensities of the peaks in the Münster measurements were derived from the peak areas. The assumption is that the intensities derived are proportional to the number of ions produced in “successful” laser shots, as defined above. The validity of this assumption depends on the linearity of the detector and the subsequent electronics. In the Manitoba measurements (Fig. 3d–f), the slopes are somewhat higher than observed in the Münster measurements or in the pulse-counting measurements. This may reflect the difference in transmission optics between the two instruments, if the radial velocity has a fluence dependence, which is not unreasonable at higher fluence where the plume dynamics are more significant. When peak areas were used instead of peak heights, even steeper slopes were observed. No significant effort was spent in trying to further resolve the discrepancy because the main purpose of the analog measurements in these supplemental measurements was to establish the approximate “threshold” fluence for comparison to the pulse-counting results.

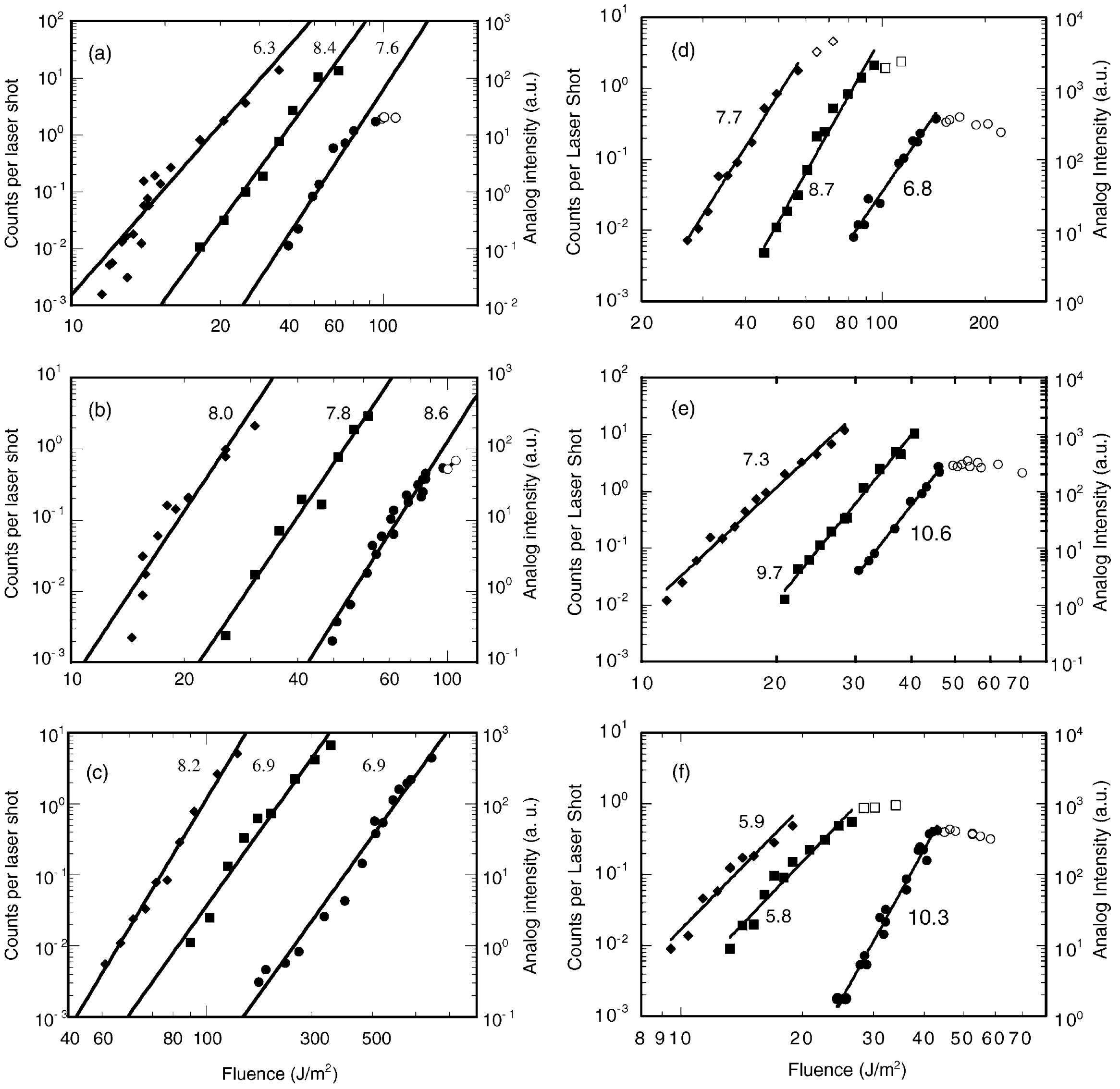

Fig. 3. Measured signal intensity as a function of laser fluence in the axial TOF geometry, for molecular ions of (a) bovine insulin using the matrix αCHCA, (b) angiotensin I using αCHCA, (c) angiotensin I using DHB, (d) insulin using sinapinic acid, (e) insulin using αCHCA, (f) LHRH using αCHCA. Parts (a–c) were measured at Münster, and (d–f) at Manitoba. In each case, circles represent analog measurements, squares represent pulse-counting measurements with a small iris (about 1 mm diameter), and diamonds represent pulse-counting measurements with an open iris (20 mm diameter in the Münster instrument; about 1 cm^2 in the Manitoba instrument). Each set of data was fit to a simple power-law, and the exponents are indicated. The open symbols represent data points thought to be influenced by saturation; they were not used in the fit.

3.1.2. Interpretation of the pulse-counting measurements

The vertical scale for the pulse-counting experiment (shown on the left axis) represents the number of pulses counted in the time-window corresponding to the molecular ion, divided by the number of laser shots. The time window was defined to include the salt adducts to improve the counting statistics and was several hundred nanoseconds in width. No significant dependence of the relative intensity of these adducts on fluence was observed. The number of counts in a window with equivalent width in a background region near the peak was less than 10% of the molecular ion signal, and independent of fluence, so no background subtraction was performed.

If more than one ion is detected within the dead time of the counting system (about 20–50 ns), only one pulse is counted. Although several ions can still be counted from a single laser pulse within a 500 ns window, the probability for a coincidence only becomes significant above about one count per laser shot, assuming the number of counts per pulse follows a simple Poisson distribution. Such coincidences lead to saturation of the observed intensity, as is evident in some of the experiments in this range from the deviation from a power-law dependence.

If the probability for the detection of two ions within the instrumental dead time can be neglected, the measured intensity curve is simply proportional to the average number of ions desorbed per laser pulse, and is therefore directly comparable with the analog measurements, apart from a normalization constant, and to the extent that the detector has a linear response in the analog measurements. It is therefore not surprising that the analog and pulse-counting measurements show very similar fluence dependence in the same fluence range. The measurements also indicate that the power-law dependence persists with essentially the same slope for a fluence range well below the analog threshold. The measured slopes for the pulse-counting measurements range between about 6 and 10, somewhat higher than reported by Ens et al. [3], but consistent with those reported by Dreisewerd et al. [9].

The normalization constant is determined by the transmission of the instrument (including the aperture) and the detector efficiency. These values are not accurately known, but nevertheless, a rough estimate can be made of the average number of ions produced per laser in the analog measurements. Extrapolating the count rate curve with the open aperture indicates count rates above 10 per laser shot are consistently found at the analog threshold fluence. Assuming an overall efficiency of 10% to at most 50%, this clearly shows that throughout the full range of fluence probed by analog detection, on average at least 20–100 ions are produced per laser shot. At the lowest fluence probed by pulse counting only one ion was counted in every 10^3 laser shot. Even assuming a difficult-to-rationalize TOF transmission of only 1% this still means that on average a single ion is *produced* in only every 10th laser shot.

It should be mentioned that these data alone do not rigorously justify the assumption of a simple Poisson distribution of the counts per laser pulse, and that the interpretation of the measured intensity as being proportional to the average number of ions per laser shot depends on this assumption. A different interpretation would apply if, for example, desorption events resulted from an avalanche phenomenon, where either no ions or a large number of ions were produced. In this case, with the large aperture, each event that produces many ions would produce a coincidence of ions and be counted. The decrease in the measured intensity at low fluence would then result from laser shots that produced no ions at all, and measured intensity would represent the probability of a desorption event, and not the average number of ions detected per laser shot. However, the similarity in the slope with both aperture sizes makes this interpretation considerably less plausible.

Regardless of the interpretation, the results in the axial geometry show clearly that the pulse-counting experiment probes a fluence range substantially lower than that accessed with analog measurements. In the data for insulin measured with the larger aperture in Münster, there is an indication of a steeper slope (up to about 10) at low fluence, but this may be a result of poor statistics, or an artifact; there is no indication of a change in slope in the Manitoba data. In any case, there is no evidence in any of the curves for a physical fluence threshold. The *measured* graph for a true threshold would not have infinite slope because of the finite laser energy window, but would have a slope between 20 and 40, clearly higher than observed in any of the measurements.

3.2. Measurements in the axial geometry: matrix ions

The dependence of the molecular ion signals for the matrix was also measured in Münster for DHB and αCHCA using pulse-counting. The results are shown in Fig. 4. The rather similar signal intensities for analyte and matrix is characteristic of the particular matrix-to-analyte ratio used, and made the comparison of fluence dependence straightforward. For αCHCA, the matrix ion signals have a slightly steeper dependence on fluence than do the analyte ion signals, throughout the fluence range. For DHB, the fluence dependence is nearly identical for analyte and matrix ions at fluence values above the analog threshold, but for low fluence, the matrix signal drops off much more slowly than for the analyte. The different behavior for the two matrices is not yet understood, but it appears that another mechanism such as molecular desorption may play a role in the desorption of

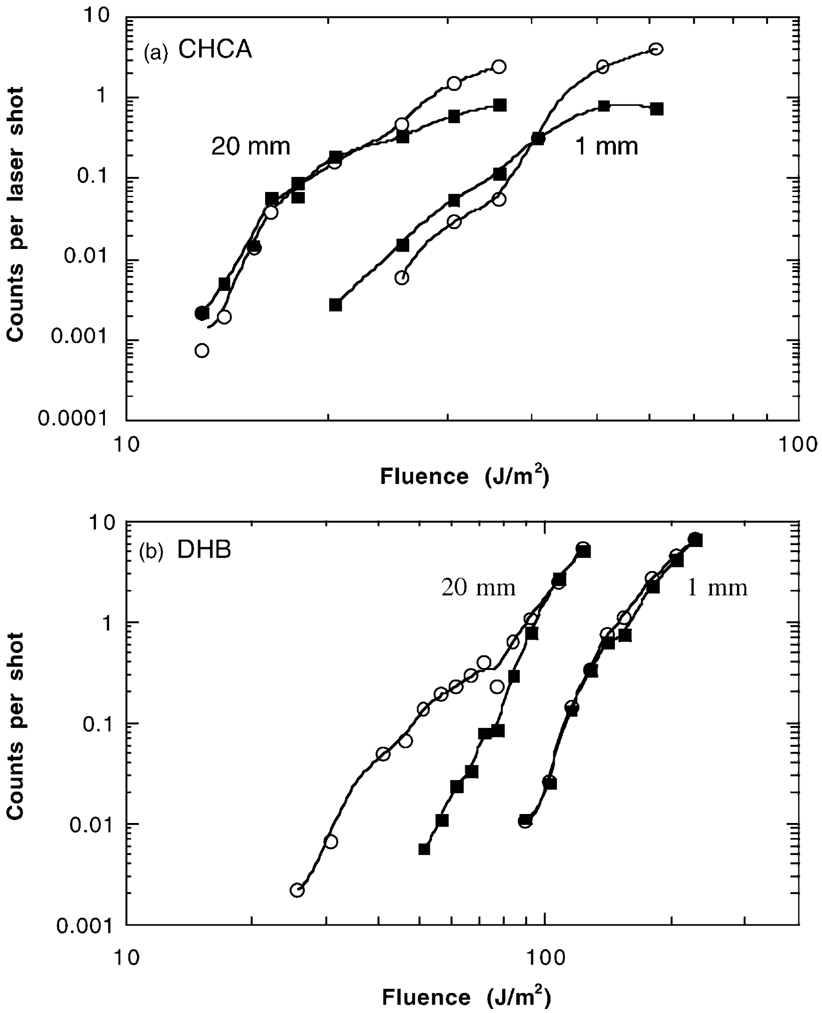

Fig. 4. Signal intensities of matrix molecular ions (circles) and analyte molecular ions (squares) for angiotensin I in (a) αCHCA and (b) DHB. Measurements were made in the axial reflecting TOF instrument in Münster, using pulse-counting with 1 and 20 mm diameter apertures.

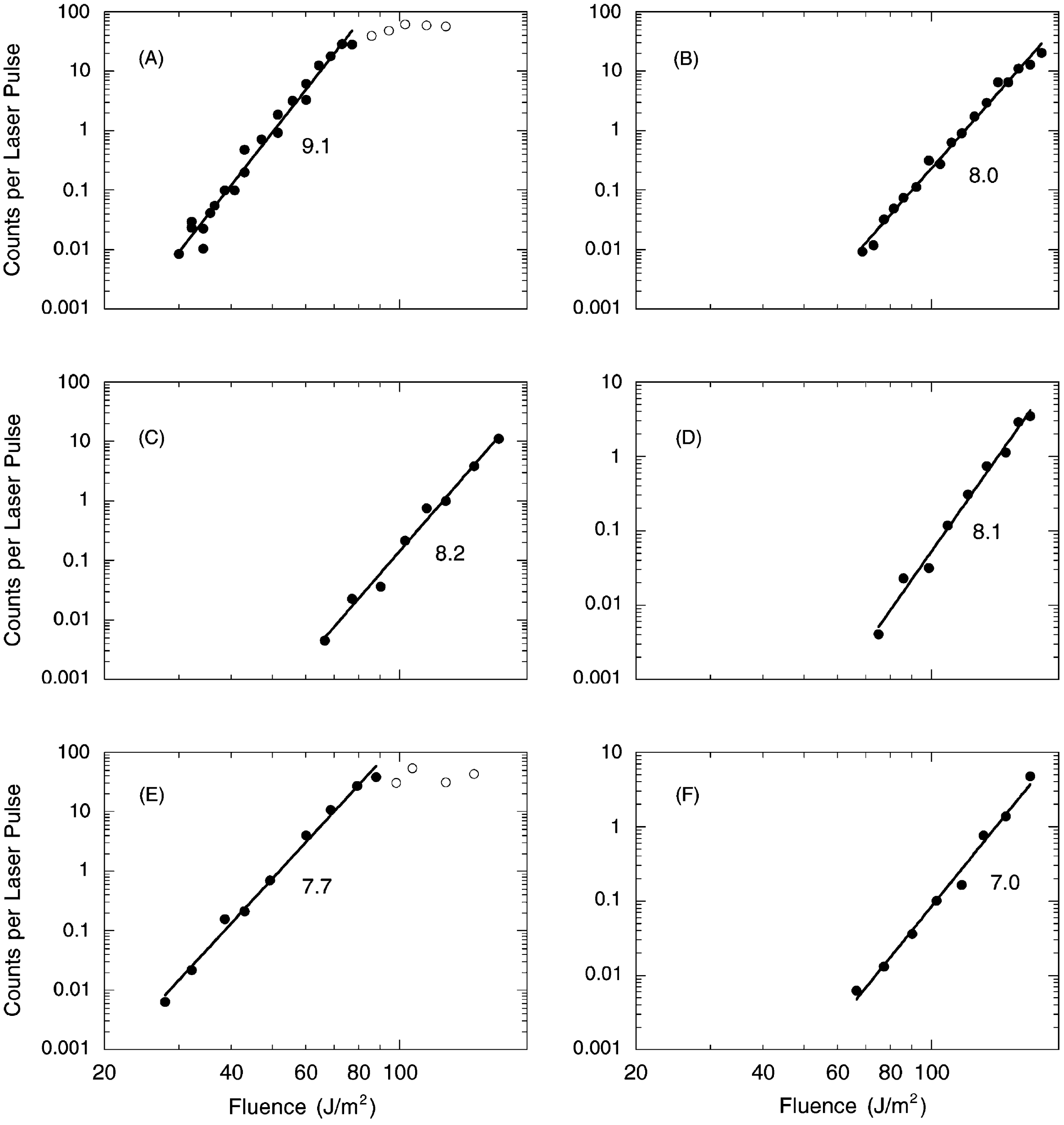

Fig. 5. Ion intensities as a function of laser fluence obtained using pulse-counting methods in an orthogonal-injection MALDI/TOF instrument. (A) LHRH in αCHCA, (B) LHRH in sinapinic acid, (C) LHRH in 2′,6′-dihydroxyacetophenone, (D) LHRH in DHB, (E) γ-endorphin in αCHCA, (F) insulin in sinapinic acid. Each dataset is fit to a simple power-law and the exponents are indicated. The open symbols in (A) and (E) represent data influenced by saturation of the counting system and are excluded from the fits.

DHB matrix ions at low fluence. The less significant difference between the matrices (relative to analytes) at higher fluence may reflect subtle differences in the ionization processes. The different dependence of analyte and matrix ions on fluence, particularly for DHB, may be a useful probe to understanding the nature of chemical noise in MALDI spectra, since it is widely believed to be associated with the matrix.

3.3. Measurements in the orthogonal TOF geometry

The measured relative ion signals for LHRH with four different matrices, for γ-endorphin with the matrix αCHCA, and for insulin with sinapinic acid, obtained with the orthogonal-injection MALDI/TOF instrument are shown in Fig. 5. As above, the datasets are fit to a simple power-law, and the exponents are indicated in the figure. The open data points at higher fluence in A and E, which deviate from the power-law dependence are excluded from the fit. These are influenced by saturation effects and possibly by increased ion fragmentation. The measured exponents are between 7 and 9. Only the slopes can be compared directly with the measurements in the axial TOF instrument. The transmission is unknown in both cases so the signals are only relative; the absolute fluence is estimated here as described in Section 2, but the uncertainty is rather high.

As described above, the spectra are accumulated with pulse-counting methods in this instrument, but the results are much simpler to interpret than the pulse-counting measurements in the axial TOF instrument, and a wider fluence range can be accessed than in the analog measurements without changing instrumental conditions. Because each MALDI plume is spread out along the quadrupole axis, only a few ions are injected into the TOF instrument in a given measurement cycle, and the probability that more than one ion of a given species is detected in one cycle is negligible, except at the highest fluence in some cases. When the count rate for a given species approaches the repetition rate of the injection pulser, then saturation does occur, as in Fig. 5A and E. Such saturation of the counting system is readily observed in this instrument because the isotopic pattern is resolved. The dead-time of the TDC is sufficient to spoil the expected pattern for a given species if multiple ions of that species are detected. In the other cases, where the ion signal was lower, the full fluence range could be used with a reliably linear detector response, and the measured intensity. The fluence for these measurements was varied by close to an order of magnitude, corresponding to a range of ion signal intensity nearly four orders of magnitude in most cases, exceeding all previous measurements.

4. Discussion

As expected, pulse-counting measurements can give ion signals for considerably lower fluence values than are accessible with analog measurements, at least if only single shots are analyzed. However, contrary to the previous observations made with pulse-counting [3], these measurements indicate no evidence for a true fluence threshold.

Even though the mechanistic details of the desorption and ion formation are not fully understood, it is reasonable to assume that the overall result is statistical in nature, governed to first approximation by Poisson statistics. This in particular given the evidence that a large number of neutral molecules are desorbed per laser shot throughout the whole fluence range examined [3]. Thus, in the fluence range above threshold for analog detection, where on average >10 ions are detected and at least 20 ions are generated per laser shot, the actual number of ions and thereby the amplitude of the analog analyte signal are expected to fluctuate from shot to shot with a standard deviation equal to the square root of the mean, in qualitative agreement with experimental observation, at least close to the threshold fluence. At lower fluence, where on average one ion is generated per pulse, Poisson statistics predicts a single ion generated in about every second shot. The probability for generating two or more ions per shot is already $<30\%$ in this case. For even lower fluence, where the corresponding average is substantially below unity, the statistics simply describe the probability for generation of a single or any other number of ions for a certain fraction of laser shots, with the probability for more than one ion generated quickly dropping to very low values. For an average of 10^{-2}, the probability of generating zero ions is 0.99, for one ion it is about 10^{-2}, and for more than one ion it is below 5×10^{-5}. The curves in Figs. 3–5 then simply describe the decrease of the average number of ions generated per laser pulse as a function of fluence. No absolute numbers can be derived, of course, because of the unknown transmission of the TOF spectrometer and efficiency of ion detection. As already mentioned, the signal vs. fluence curves do not exclude more complicated statistical processes, but the consistent slope throughout the range of fluence, and for different methods of measurement, makes this seem unlikely. Further investigation is needed to determine the detailed statistical nature of the process at low fluence.

It is of course possible that a physical threshold exists at lower fluence, but the data do not suggest this, and it is clear that the practical threshold normally associated with analog measurements in MALDI is not a physical threshold. This is particularly evident in the comparison of analog measurements and pulse-counting measurements with the small aperture in the same instrument. Here the interpretation of both sets of measurements is reasonably straightforward, the fluence is known to be the same, and the pulse-counting measurements clearly extend to lower fluence with essentially the same slope.

It is not surprising that an apparent threshold is associated with MALDI in view of the steep fluence dependence. If at some fluence value, the probability of observing a peak with analog measurements is proportional to the average number of ions per laser shot, then a 30% increase in fluence increases the probability of observation from 10% (near zero) to about 90% (near unity). In practice, such an increase looks like a threshold. However, from these results it is clear that the fluence at which this apparent threshold is observed depends on the detection efficiency and instrument transmission, so that the “threshold fluence” will not in general represent a completely unambiguous value.

The dependence of the ion signals on fluence are qualitatively consistent with the earlier experiments [3,9] which show a steep power-law dependence. In a given experiment, where only the fluence is changed, there is very little scatter of the data points, but the variation in the slope from different experiments, especially with different instruments gives a more realistic indication of the reproducibility of the measurement. The data in Fig. 3 are representative of the fluctuation observed in all the measurements. The most reliable measurements indicate an exponent of about 7–9, but as mentioned, it should be kept in mind that the exponent can depend on a variety of parameters such as the particular matrix and possibly even the analyte, particularly if ones goes to higher masses. Another parameter, expected to influence the slope, which has not been investigated yet, is the molar analyte-to-matrix ratio in the sample. The exponent also depends on other details of the measurement. Dreisewerd et al. showed previously that the exponent decreases for smaller spot sizes [9]. We have found that if the laser is simply focused directly onto the target with a single lens, giving a Gaussian distribution, the dependence is somewhat weaker, and this may account partially for the lower exponent observed previously by Ens et al. [3]. Moreover, the exponent was found to depend slightly on the number of shots per spot in the pulse-counting measurements. By changing the shutter time by a factor of 10, there is a ∼15% increase in the exponent. This reflects the fact that the ion signal from a single spot persists longer at higher fluence, so the higher fluence data points benefit more from the longer exposure.

In recent years, after completion of the experiments reported here several papers have appeared in the literature on molecular modeling of the desorption process in MALDI [15]. The results of these calculations predict a change in the process at some fluence, defined by a transition from a predominantly molecular desorption to that of an ablation of clusters. The authors interpret this as some sort of a MALDI desorption “threshold”. No indication of such a threshold was found however for any of the samples in the fluence range tested in the experiments reported here. Although the average number of ions detected per laser shot is small at the lowest fluence used in this experiment, this does not necessarily mean the desorption is non-ablative, since as already mentioned, there are many neutrals desorbed below the usual analog threshold for ion emission [9]. Of course it is possible that a threshold effect is present on a much smaller scale, such as those used in the numerical simulations, but is obscured by statistical averaging over a much larger inhomogenous surface. Nevertheless, it is important to emphasize that experimental evidence for a threshold phenomenon appears at present to be absent.

5. Conclusions

The average number of molecular ions of peptides desorbed per laser pulse in MALDI has a steep power-law dependence on fluence, with an exponent between 7 and 9. No evidence was observed for a fluence threshold in the desorption ion yield.

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