Published in final edited form as:

Rapid Commun Mass Spectrom. 2011 December 15; 25(23): 3527–3536. doi:10.1002/rcm.5262.

Global Optimization of the IR Matrix-Assisted Laser Desorption Ionization (IR MALDESI) Source for Mass Spectrometry Using Statistical Design of Experiments

Jeremy A. Barry and David C. Muddiman

W.M. Keck FT-ICR Mass Spectrometry Laboratory, Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695

Abstract

Design of experiments (DOE) is a systematic and cost-effective approach to system optimization by which the effects of multiple parameters and parameter interactions on a given response can be measured in few experiments. Herein, we describe the use of statistical DOE to improve a few of the analytical figures of merit of the infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) source for mass spectrometry. In a typical experiment, bovine cytochrome c (~12 kDa) was ionized via electrospray, and equine cytochrome c (~12 kDa) was desorbed and ionized by IR-MALDESI such that the ratio of equine:bovine was used as a measure of the ionization efficiency of IR-MALDESI. This response was used to rank the importance of seven source parameters including flow rate, laser fluence, laser repetition rate, ESI emitter to mass spectrometer inlet distance, sample stage height, sample plate voltage, and the sample to mass spectrometer inlet distance. A screening fractional factorial DOE was conducted to designate which of the seven parameters induced the greatest amount of change in the response. These important parameters (flow rate, stage height, sample to mass spectrometer inlet distance, and laser fluence) were then studied at higher resolution using a full factorial DOE to obtain the globally optimized combination of parameter settings. The optimum combination of settings was then compared with our previously determined settings to quantify the degree of improvement in detection limit. The limit of detection for the optimized conditions was approximately 10 attomoles compared with 100 femtomoles for the previous settings, which corresponds to a four order of magnitude improvement in the detection limit of equine cytochrome c.

INTRODUCTION

With the advent of electrospray ionization (ESI)[1] and matrix-assisted laser desorption ionization (MALDI),[2,3] mass spectrometry (MS) has played an ever increasing role in the analysis of biological systems.[4] These two ionization techniques have emerged as somewhat complementary in biological analysis. ESI generates primarily multiply-charged ions which are more amenable to tandem MS and can increase the mass range of most mass analyzers. This method is also more easily coupled to liquid chromatography. MALDI, on the other hand, is typically limited to singly- or doubly-charged ions which can limit the mass range available to most mass analyzers when sampling large biomolecules. MALDI has a much greater tolerance for high salt samples; however, the vacuum requirement places restrictions on the type of samples that can be analyzed. While both of these techniques

***Author for Correspondence.** David C. Muddiman, Ph.D., W.M. Keck FT-ICR Mass Spectrometry Laboratory, Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, Phone: 919-513-0084, Fax: 919-513-7993, david_muddiman@ncsu.edu.

provide a wealth of information, they usually require extensive sample preparation in order to make the sample amenable to analysis.

As MS has evolved, the restrictions and limitations of traditional ionization sources such as ESI and MALDI have led to the development of methods for ambient ionization. These methods are becoming increasingly popular due to their wide applicability to a variety of samples and substrates while requiring little to no sample preparation. The introduction of desorption electrospray ionization (DESI)[5] marked the beginning of the trend towards native sampling in mass spectrometry. The subsequent years led to the release of well over thirty 'novel' ambient ionization sources which have been reviewed extensively.[6–10] A few of these sources have been commercialized and are becoming more universally integrated into industrial and academic laboratories including, but not limited to, DESI, direct analysis in real time (DART),[11] atmospheric pressure solids analysis probe (ASAP), [12] and perhaps most recently laser ablation electrospray ionization (LAESI).[13]

A subdivision of ambient ionization can be dedicated to laser sampling/desorption followed by post-ionization by electrospray. A handful of these ionization sources have been described and include electrospray-assisted laser desorption ionization (ELDI),[14] matrix-assisted laser desorption electrospray ionization (MALDESI),[15] infrared laser assisted desorption electrospray ionization (IR LADESI),[16] laser ablation electrospray ionization (LAESI),[13] laser ablation mass spectrometry (LAMs),[17] and laser electrospray mass spectrometry (LEMS).[18] Most recently a technique was published called laser desorption spray post-ionization (LDSPI)[19] in which the authors state that perhaps all of these techniques could be merged under this one acronym. These methods are relatively similar but some differ by a few of their parameter settings (source geometry, laser wavelength, repetition rate, and pulse width). In MALDESI, it is proposed that the laser (UV or IR) is used to excite the sample matrix (endogenous or exogenous) thereby facilitating the desorption of the analyte (liquid or solid). The desorbed neutral molecules or particulate matter in the ablated plume are then entrained or are extracted into the electrospray plume which is parallel to the sample plate and on axis with the inlet to the mass spectrometer. Ionization is then presumed to proceed through an ESI-like mechanism where the analyte containing charged droplets undergo multiple events of desorption and fission ultimately resulting in the ionization of the analyte. A major advantage of this technique is that it combines certain benefits of MALDI and ESI. The high spatial resolution, salt tolerance, and extended interrogation capabilities of MALDI along with the multiple charging effects and ambient nature of ESI are also realized in MALDESI. ESI post-ionization provides MALDESI with the ability to include reagents or standards in the electrospray solvent in order to manipulate the analyte or allow for quantitation. This niche of the post-ionization techniques has been used to facilitate protein unfolding, reduce disulfide bonds, super-charge proteins,[20] define neutral capture efficiency,[15,21] determine the source of the charging protons using deuterated solvents,[22] increase lipid dissociation by generating lithiated ions,[23] and as a means of introducing internal calibrants without disrupting the analyte solution.[15,24,25]

Water has a relatively high absorption cross section in the mid-IR due to the asymmetric O-H stretching modes, thus the use of a mid-IR laser allows for endogenous water in the sample to act as a matrix and facilitate analyte desorption.[26] It has been well studied that IR laser ablation at atmospheric pressure leads to particularly low ionization yields[27] where a larger portion of the ablated plume consists of neutral particles.[28] MALDESI is believed to have higher ionization efficiency than direct laser ionization due to the post-ionization of the more abundant neutral molecules. This hypothesis is supported by the observations of Nemes and coworkers where LAESI was found to produce greater intensity ion signal compared with AP IR-MALDI.[29]

One aspect that is common among all of these ionization sources is the vast experimental space which needs to be explored. There is a multitude of parameters and settings which can have a dramatic impact on the quality and intensity of the signal. Traditionally, the optimization of a multi-parameter system would be performed by what has been called the 'One Factor at a Time' (OFAT) approach by which one factor is varied at a time while the others are held constant in order to achieve the desired response. The OFAT approach makes the assumption that all of the factors are independent from each other which is almost never valid in experimental data.[30] This assumption could then lead to highly confounded and suboptimal results which may result in optimization of signal to a false maxima. The other extreme would be to test all possible combinations of each level for each factor in an approach known as a full factorial design of experiments (DOE). A more feasible approach to this problem would be a fractional factorial DOE in which a large portion of the experimental space is explored but with a significantly smaller number of experiments. Usually this subdivision of DOE arrives at the same conclusions of a full factorial design but in a fraction of the time and cost. DOE is a statistical tool which can be used to efficiently optimize multiple factors in order to obtain the desired response(s) of a given system while recognizing higher order interactions. In DOE, experiments (different combinations of factor levels) are statistically constructed so the greatest amount of information about the importance of each factor can be extracted from the results of each experiment. It is through this process that DOE can efficiently evaluate a large experimental space. There are many great resources which more thoroughly describe the mathematics behind this principle.[31–33] A tutorial by Riter and coworkers provides a detailed description of DOE and its conceivable role as an important contrivance in mass spectrometry.[34] Our lab has applied DOE to optimization to hydrophobic tagging reactions for glycans,[35] LTQ-Orbitrap instrumental parameters in order to increase proteome coverage,[36] and in the design and development of an Air Amplifier.[37] Other groups have demonstrated this approach's utility in optimizing the operation of ESI and APCI ion sources.[38,39] Statistical experimental design has also been used for optimizing separation conditions in liquid chromatography (LC) and to develop a general LC-MS method.[40] Here, we used fractional factorial DOE in order to efficiently explore and optimize the combination of parameter settings for our IR-MALDESI source for mass spectrometry in order to decrease our detection limits for small to medium sized proteins.

EXPERIMENTAL

Materials

Bovine cytochrome c, equine cytochrome c, and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and water were purchased from Burdick & Jackson (Muskegon, MI, USA). All materials were used as received without further purification.

Methods

The electrospray solution was prepared by mixing 15:85 acetonitrile and water (v/v) with 0.1 % formic acid. A stock solution of bovine cytochrome c was prepared by dissolving the protein in the electrospray solution to give a 100 nM solution which was used as the electrospray solvent in the DOE and spot down experiments. Solutions of equine cytochrome c were prepared by dissolving the protein in HPLC grade water to give a stock concentration of 100 μ M which was serially diluted down to 1 pM for the spot down experiments.

IR-MALDESI Source and LTQ-FTICR Mass Spectrometer

The UV-MALDESI source has been described in detail previously[41] and has been recently modified to accommodate a Mid-IR laser.[42] Briefly, the electrospray solution containing 100 nM bovine cytochrome c was supplied at a flow rate of 400 nL/min – 3 μ L/min using a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA) through a tapered silica emitter tip (75 μ m ID, 30 μ m tip, New Objective, Woburn, MA, USA). High voltage, applied to a stainless steel union (MU1XCS6, VICI Valco, Houston, TX, USA) just prior to the emitter tip, was varied between 2.0 and 3.5 kV such that the electrospray was stabilized in cone-jet mode (visual affirmation). This mode of electrospray has been shown to yield higher ionization efficiency.[43,44] The ESI emitter tip was positioned within 5–10 mm of and on axis with the inlet to the mass spectrometer. A 1 μ L droplet of 100 μ M equine cytochrome c was deposited onto the stainless steel target which was biased (0–1.0 kV) and held 3–20 mm below the electrospray axis. The sample was ablated using an Nd:YAG pumped, wavelength tunable (2.7 – 3.1 μ m) optical parametric oscillator (IR Opolette, Opotek, Carlsbad, CA, USA) with a pulse width of 7 ns. The wavelength was tuned to 2.94 μ m for all experiments and the repetition rate was varied between 5 and 20 Hz. The laser power was attenuated using an external attenuator to values of 210 μ J/pulse – 1.6 mJ/pulse. The laser beam was directed using two gold coated Pyrex mirrors (Newport Corporation, Irvine, CA, USA) and was focused using a calcium fluoride plano convex lens (Edmund Optics, Barrington, NJ, USA) to a spot size of 200–300 μ m in diameter, corresponding to fluence values of 0.5 – 1.5 J/cm². The sample droplet was ablated at varying distances from the inlet to the mass spectrometer (1–10 mm). After the interaction of the ablated plume with the ESI plume, the resulting ions were then sampled by an extended inlet capillary, biased at 42 V and heated to 175 °C, for a Thermo LTQ-FT-ICR Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The tube lens was set to 125 V. The resolving power of the FT-ICR was set to a value of 50,000_{FWHM} at m/z 400 and a maximum injection time of 500 ms was used which corresponds to ~1 s/scan. The automatic gain control (AGC) limit was reached for every scan implying that the maximum injection time was never reached. These resolving power and ionization time settings reflected a trade off between resolution and duty cycle of the instrument. The instrument was mass calibrated just prior to conducting the fractional factorial and full factorial DOEs. Mass spectra were collected in positive-ion mode over 20 scans for a mass range of 500–2000 m/z. Intensity values for both forms of cytochrome c were obtained from single acquisition spectra.

Data Analysis

A seven factor, three level D-optimal screening DOE was designed using JMP 9.0 (SAS Institute, Inc., Cary, NC, USA). The parameters that were studied including their ranges and the motivation for testing them can be found in Table 1. A pictorial description of the factors is shown in Figure 1. In a given experiment a solution of bovine cytochrome c is electrosprayed while a 1 μ L droplet of equine cytochrome c is desorbed and ionized by IR-MALDESI. The presence of bovine cytochrome c in the electrospray solution provided a measure of electrospray stability throughout the experiments. Bovine and equine cytochrome c share about 97% of their primary sequence. An equimolar mixture of these two species analyzed by ESI shows similar charge states and charge state distributions which are close to a 1:1 ratio (bov:equ).[45] Due to this sequence homology, the ratio of ions produced by IR-MALDESI to those produced by ESI (i.e. equine:bovine) was used as the experimental response which was set to be maximized in the DOE. Responses for average charge state and ion injection time were included in the design as non-influential responses so that fluctuations in their values could be monitored under the various parameter settings. All single factor, two-factor, and second order interactions were included in the design to be resolved from confounding with each other. Experiments were performed in duplicate and their order was randomized to decrease the influence of experimental and

systematic bias respectively. The design table for this analysis is included in the supplemental information (Figure S1). Upon completion of the allotted experiments, signal intensities for the various charge states ($[M+8H]^{8+}$ – $[M+19H]^{19+}$) of both bovine and equine cytochrome c were extracted from a single mass spectrum. Intensities were then normalized for ion injection time and charge state (due to the increased current induced by higher charge states in ICR). The normalized intensities for each charge state were then summed for both forms of cytochrome c. The ratio of the summed normalized intensities of equine cytochrome c to bovine cytochrome c (EQU:BOV) was then calculated and input into JMP in order to determine the impact of each factor setting. The factors which were deemed to be significant in the screening DOE were then used as the main factors in a full factorial DOE which was designed in JMP.

RESULTS and DISCUSSION

Screening DOE

A subgroup of DOE known as fractional factorial DOE allows for systematic examination of multiple factors and interactions simultaneously in order to determine their degree of significance requiring only a small number of experiments. This is in contrast to a full factorial DOE in which the number of experiments required can be represented as L^f , where f is the number of factors (parameters) being studied and L signifies the number of levels (settings) at each factor. For example, a system with 7 factors being tested at 3 levels would require 3^7 or 2,187 experiments in order to test all possible factor combinations. Replicates of these experiments would drive the number of required experiments even higher (duplicate = 4,374 or triplicate = 6,561). This is an astoundingly large number which, due to economic and temporal restraints, is not feasible for most systems. Fractional factorial DOE only requires a fraction of the experiments and will generally still arrive at the same conclusion; however, there could be some degree of confounding between the main factors and higher order interactions depending on the design resolution. In a typical fractional factorial DOE, two levels are chosen for each factor, a maximum (+) and minimum (–), which reflect that factor's practical range. Here we have also included center points (0) for each factor to account for the possibility that the response may not be linear over the entire range. After incorporating all of this information (factors, levels, responses), the desired resolution of the design is selected. Design resolution represents what degree of aliasing will be allowed. The lowest meaningful resolution is a resolution III design where estimated main effects are confounded with two factor and higher order interactions. In a resolution IV design the main effects may be confounded with three factor or higher interactions but are unconfounded from two factor interactions and two factor interactions may be confounded with other two factor interactions. A resolution V design has main effects and two factor interactions unconfounded from themselves and each other but there may be confounding with three factor or higher order interactions. The highest resolution is a resolution VI design where there is no confounding between main effects, two factor interactions, and three factor interactions but some confounding is possible with higher order interactions. Since our objective was to estimate main effects and two factor interactions, a resolution V design would provide the required information. After selecting the desired resolution the program (JMP) generates a list of experiments to be conducted from which each factor's effect on the system response can be extrapolated. Within the program, the levels for each factor is assigned as a dimensionless variable with a value of –1 (minimum factor level) to 1 (maximum factor level) with the center point as zero. This approach accounts for differences in the magnitude of the factor range so that it will not influence its significance. The design that was used required 128 total experiments including duplicates and the order of the experiments was randomized.

A screening fractional factorial DOE was used to explore the vast experimental space presented within the many possible parameter settings of the IR-MALDESI source. The seven parameters that were tested are listed in Table 1 including the ranges over which they were studied. The range of values for each parameter was established to reflect the settings that have been used by other labs as well as our own. These seven parameters as well as all two-factor and second order interactions were included in the design. As mentioned previously, two-factor interactions are often ignored in the OFAT approach but can play a significant role in global optimization. A pictorial representation of a typical experiment is depicted in Figure 1. A 100 nM solution of bovine cytochrome c was electrosprayed through the direct infusion line of the IR-MALDESI source while a 100 μ M equine cytochrome c solution (1 μ L) was ablated from the sample target then was captured, and ionized in the ESI plume. Both bovine and equine cytochrome c were therefore present in the same mass spectra; one ionized by ESI (BOV) and the other ionized by IR-MALDESI (EQU). This allowed for some degree of internal calibration and provided a means of monitoring the stability of the electrospray under each set of conditions. The ratio (EQU:BOV) accounts for differences in electrospray ionization efficiency which may arise under the different factor settings and was therefore used as the response metric for the DOE.

The results for the screening DOE are shown in Figure 2. The half normal probability plot (Figure 2a) orders the estimated effects of the variables and compares them with a normal quantile distribution. Those variables which diverge from the normal quantile curve represent those factors (circled in red) which contribute the most to the variance observed in the response. A full list containing all of the main factors and interactions is shown in Supplemental Figure S2. The significant variables (highlighted in blue) along with their corresponding contrasts, *t*-ratios, and *p* values can also be found in S2. A parameter is considered to be significant if its *p* value is smaller than 0.1 (90 % confidence). Those which have *p*-values which are marked with an asterisk define the variance in the data within 95 % confidence (*p*-value smaller than 0.05). The contrasts which are listed give an indication as to which factor setting was preferred as well as the degree to which it influences the response. Those with large, in magnitude, contrast values induced the greatest effect on the EQU:BOV ratio. The sign for contrast (+/−) provides some indication as to whether the maximum (+) or minimum (−) setting provided the best response.

Four of the seven main factors were found to be significant. Among these four were sample to mass spectrometer inlet distance, stage height, flow rate, and laser fluence. The sample to inlet distance defines where the sample is ablated in relation to the mass spectrometer inlet and larger distances were favored. One could reason that this increased distance provides more time and area for the ablated plume to interact with the electrospray plume. Stage height was also found to be statistically significant and has previously been shown to have a rather drastic impact on ion intensity.[29] Nemes and coworkers have shown that signal can be obtained up to 30 mm away from the electrospray axis but the maximum signal intensity was observed around 15 mm for bradykinin. They hypothesized that perhaps such a large distance was required due to the large momentum of the ejected particles from the ablated plume. Our results, however, suggest that a shorter stage height led to slightly better ionization efficiency, but this could be attributed to differences in instrumentation. Laser fluence was also concluded to be significant which is not very surprising considering that higher fluence rates, which were favored, impart more energy into the target and result in the ablation of more material. Plume shielding at higher fluence rates could result in decreased energy deposition in subsequent pulses and would allow explanation for the favoring of short stage heights and the observation that laser repetition rate was found to be insignificant.[46] The final main factor which was found to be significant was the electrospray flow rate. In particular, low flow rates were most influential. Electrospray conducted at low flow rates (nL/min) produces smaller droplets which can lead to more

efficient desolvation and ionization in ESI.[47] Small droplets have a larger surface area to volume ratio compared to their larger counterparts which could provide the laser desorbed particles with a greater interaction surface thereby yielding more efficient analyte encapsulation/extraction. A few two factor interactions were found to be vital as well as several second order interactions all of which would have been overlooked if the OFAT approach was conducted. For example, the interactions of sample to inlet distance with both stage height and ESI to inlet distance would have otherwise been ignored. The prediction profiler plot, shown in Figure 2b, uses standard least squares analysis to model the behavior of the system based on the results of the measurements which were obtained. Using this model and an iterative approach, the global response can be maximized and the coefficients for each factor that give rise to the maximum response can be obtained. This combination of values, shown in red above each factor, represents the predicted globally optimal settings. It should also be noted that these values are estimates based on a model of the data and therefore may not be accurate out to the number of decimal places shown. A surprising observation is that the lowest repetition rate tested, 5 Hz, was predicted to yield the best response. This prediction could be due to the prolonged observation of signal resulting from fewer laser pulses per scan or the somewhat reduced effects of plume shielding at lower repetition rates. Optimization at 5 Hz also implies that as few as five laser pulses per scan were required to observe appreciable signal. Despite this prediction, it should be mentioned that that laser repetition rate was not considered a significant factor. In fact the estimated response for 5 Hz repetition rate is statistically the same over the entire range tested as shown in Figure 2b where the maximum response (horizontal dashed red line) is within the 95% confidence interval (blue dashed lines) over the entire range.

Full Factorial DOE

The variables which were found to be significant from the screening DOE were then analyzed at higher resolution using a full factorial DOE with shorter ranges for each factor. Those factors which were insignificant over the range of values tested were held constant at the setting which was predicted to yield the optimum response (Figure 2b). Shorter ranges for the significant variables were established by limiting the range to those values which were within the 95 % confidence interval (where the horizontal red dashed line crosses the blue dashed line in Figure 2b). These factors including their new ranges are shown in Table 2. The maximum value for sample to inlet distance was limited to 8 mm to avoid ablating a spot which is behind the optimal ESI emitter distance (9 mm). A summary of the results of the higher resolution full factorial DOE can be found in Supplemental Figures S3 and S4. A half normal quantile plot (Supplemental Figure S3) shows the factors which were found to be significant (red dashed circle) under higher resolution. Their contrasts, *t-ratios*, and *p* values are shown in Supplemental Figure S4. As stated previously, those which account for the variance within 90 % confidence are highlighted in S4 and circled in S3. Those factors that account for the variance within 95 % confidence are marked with an asterisk on the *p*-values in S4. The factor which was found to be most influential was the sample to inlet distance. The maximum value for this setting was favored which suggests that ablating a spot closer to the ESI emitter generated the greatest ion signal. A large sample distance may provide a greater area for interaction of the two plumes. The position along the electrospray axis where the sample is ablated can also reflect the degree of desolvation which the charged electrospray droplets have undergone. This would, at least partially, control the size of the charged droplets which interact with the ablated neutrals. Laser fluence was found to be important which, again, is not very surprising for the reasons discussed earlier (more ablated material). The prediction profiler was used to model the combination of tested settings which would yield the best response (Figure 3). The optimal combination is shown in red above each factor. A list of the optimal combination of settings for the all of the parameters that were tested in both DOEs can be found in Table 3. The lowest flow rate (400 nL/min)

was preferred which is likely to be due to higher efficiency of ESI at low flow rates. It would be interesting to see if perhaps even lower flow rates or possibly static nanospray could provide an even better response. Stage height was optimized at 5.5 mm; however, it can be deduced that larger stage heights, at least up to 15 mm, do not produce a significantly different response. This conclusion is supported in Figure 3 where the slope of the modeled line for stage height (black line) is sufficiently close to zero such that the predicted maximum response (horizontal dashed red line) falls within 95 % confidence interval (dashed blue lines) over the entire tested range (5 – 5 mm). It is also important to note that the optimal conditions predicted by the model from the screening DOE (Figure 2b) nearly matches the results which were obtained from the higher resolution full factorial DOE (Figure 3). This implies that enough information was collected from the screening fractional factorial DOE (128 experiments for 7 factors at 3 levels and performed in duplicate) to accurately predict the overall optimal conditions without strictly requiring the extra experimental results obtained from the higher resolution full factorial DOE (162 experiments for 4 factors at 3 levels and performed in duplicate). We, however, felt it necessary to test the validity of the fractional factorial DOE prediction by conducting the full factorial DOE with the significant variables.

Spot Down Experiment

In order to quantify the validity of our global optimization, a spot down experiment was performed using the best, worst, and previous parameter settings in our lab to determine the limit of detection for equine cytochrome c. The chart in Table 3 lists the parameter settings for the best and worst conditions that were investigated as well as our previous settings which were determined using the OFAT approach. As in the DOE experiments, the EQU:BOV ratio was used as the response. Triplicate measurements were obtained for IR-MALDESI of equine cytochrome c at concentrations ranging from 100 μ M to 1 pM under each set of conditions. The results shown in Figure 4 demonstrate that the lowest detection limit (10 amol) was realized using the DOE optimized parameter combination (**4d**) and provided an improvement of at least a four orders of magnitude over our previous settings (100 fmol) (**4b**). Detection limits were calculated by determining the total amount of material spotted for the lowest detected signal. For example, 10pM equine cytochrome c was detected from a 1 μ L droplet for the best set of conditions yielding a 10 amol detection limit ($1.0\text{E-}11 \text{ M} \times 1.0\text{E-}6 \text{ L} = 10.0\text{E-}18 \text{ mol}$). These detection limits are conservative estimates since they represent the total amount of material spotted on the surface. The worst parameter combination failed to yield any signal over the range tested. This observation was somewhat surprising given that relatively similar settings, with the exception of laser fluence, were used for LAESI imaging.[48] However, due to inherent differences in the various types of mass spectrometers from one lab to another, it is unlikely that there would be only one set of conditions which works best for all labs. Prior to this work, detection limits for the similarly sized protein ubiquitin (~8.5 kDa) were demonstrated to be around 100 fmol using the IR-ELDI source to ablate the analyte from a water containing droplet.[49] While the differences in detection limits can be attributed to the type of the mass analyzer used, this study clearly demonstrates that ionization source parameters can profoundly influence the detection limits.

Conclusions

We have demonstrated the utility of fractional factorial design of experiments as an efficient approach to globally optimizing complex systems which include a wide variety of parameters and settings. Such a vast experimental space would be insufficiently explored using the OFAT approach and would be uneconomically examined by conducting an exhaustive full factorial DOE. The globally optimized combination of parameter settings was validated by comparing it to the worst set of conditions and perhaps more importantly to

our previous settings. A limit of detection of 10 attomoles for equine cytochrome c was obtained using our optimized settings. This detection limit corresponds to an improvement of four orders of magnitude over our previous parameter settings[41] which were determined using the OFAT method. The improvement in signal provides evidence of the influences of higher order interactions which are usually not taken into consideration and can have a profound effect on the response of a system. It is possible that these optimal parameter settings may specifically favor ionization of small to medium sized proteins from liquid droplets. However, we have demonstrated that fractional factorial DOE offers a systematic approach to global optimization which can be applied to efficiently and economically enhance conditions for any molecular class or sample state (solid, liquid, tissue) of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Guillaume Robichaud, Genna Andrews, and Hunter Walker for their helpful recommendations and discussions. The authors would also like to gratefully acknowledge the financial support received from the National Institutes of Health (R01GM087964), the W. M. Keck Foundation, and North Carolina State University.

References

1. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray Ionization for Mass Spectrometry of Large Biomolecules. *Science*. 1989; 246(4926):64–71. [PubMed: 2675315]
2. Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T, Matsuo T. Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 1988; 2(8):151–153.
3. Karas M, Hillenkamp F. Laser desorption/ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal. Chem.* 1988; 60(20):2299–2301. [PubMed: 3239801]
4. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003; 422(6928):198. [PubMed: 12634793]
5. Takats Z, Wiseman JM, Gologan B, Cooks RG. Mass Spectrometry Sampling Under Ambient Conditions with Desorption Electrospray Ionization. *Science*. 2004; 306(5695):471–473. [PubMed: 15486296]
6. Cooks RG, Ouyang Z, Takats Z, Wiseman JM. Ambient Mass Spectrometry. *Science*. 2006; 311(5767):1566–1570. [PubMed: 16543450]
7. Van Berkel GJ, Pasilis SP, Ovchinnikova O. Established and emerging atmospheric pressure surface sampling/ionization techniques for mass spectrometry. *J. Mass Spectrom.* 2008; 43(9):1161–1180. [PubMed: 18671242]
8. Chen HW, Gamez G, Zenobi R. What Can We Learn from Ambient Ionization Techniques? *J. Am. Soc. Mass Spectrom.* 2009; 20(11):1947–1963. [PubMed: 19748284]
9. Covey TR, Thomson BA, Schneider BB. Atmospheric Pressure Ion Sources. *Mass Spec. Rev.* 2009; 28(6):870–897.
10. Weston DJ. Ambient ionization mass spectrometry: current understanding of mechanistic theory; analytical performance and application areas. *Analyst*. 2010; 135(4):661–668. [PubMed: 20309440]
11. Cody RB, Laramée JA, Durst HD. Versatile new ion source for the analysis of materials in open air under ambient conditions. *Anal. Chem.* 2005; 77(8):2297–2302. [PubMed: 15828760]
12. McEwen CN, McKay RG, Larsen BS. Analysis of Solids, Liquids, and Biological Tissues Using Solids Probe Introduction at Atmospheric Pressure on Commercial LC/MS Instruments. *Anal. Chem.* 2005; 77(23):7826–7831. [PubMed: 16316194]

13. Nemes P, Vertes A. Laser ablation electrospray ionization for atmospheric pressure, in vivo, and imaging mass spectrometry. *Anal. Chem.* 2007; 79(21):8098–8106. [PubMed: 17900146]
14. Shiea J, Huang M-Z, Hsu H-J, Lee C-Y, Yuan C-H, Beech I, Sunner J. Electrospray-assisted laser desorption/ionization mass spectrometry for direct ambient analysis of solids. *Rapid Commun. Mass Spectrom.* 2005; 19(24):3701–3704. [PubMed: 16299699]
15. Sampson JS, Hawkrigge AM, Muddiman DC. Generation and detection of multiply-charged peptides and proteins by matrix-assisted laser desorption electrospray ionization (MALDESI) Fourier transform ion cyclotron resonance mass spectrometry. *J. Am. Soc. Mass Spectrom.* 2006; 17(12):1712–1716. [PubMed: 16952462]
16. Rezenom YH, Dong J, Murray KK. Infrared laser-assisted desorption electrospray ionization mass spectrometry. *Analyst.* 2008; 133(2):226–232. [PubMed: 18227946]
17. Jorabchi K, Smith LM. Single Droplet Separations and Surface Partition Coefficient Measurements Using Laser Ablation Mass Spectrometry. *Anal. Chem.* 2009; 81(23):9682–9688. [PubMed: 19886638]
18. Brady JJ, Judge EJ, Levis RJ. Mass spectrometry of intact neutral macromolecules using intense non-resonant femtosecond laser vaporization with electrospray post-ionization. *Rapid Commun. Mass Spectrom.* 2009; 23(19):3151–3157. [PubMed: 19714710]
19. Liu J, Qiu B, Luo H. Fingerprinting of yogurt products by laser desorption spray post-ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 2010; 24(9):1365–1370. [PubMed: 20391610]
20. Peng IX, Loo RRO, Shiea J, Loo JA. Reactive-electrospray-assisted laser desorption/ionization for characterization of peptides and proteins. *Anal. Chem.* 2008; 80(18):6995–7003. [PubMed: 18683952]
21. Judge EJ, Brady JJ, Dalton D, Levis RJ. Analysis of Pharmaceutical Compounds from Glass, Fabric, Steel, and Wood Surfaces at Atmospheric Pressure Using Spatially Resolved, Nonresonant Femtosecond Laser Vaporization Electrospray Mass Spectrometry. *Anal. Chem.* 2010; 82(8):3231–3238. [PubMed: 20334359]
22. Dixon RB, Muddiman DC. Study of the ionization mechanism in hybrid laser based desorption techniques. *Analyst.* 2010; 135(5):880–882. [PubMed: 20419234]
23. Shrestha B, Nemes P, Nazarian J, Hathout Y, Hoffman EP, Vertes A. Direct analysis of lipids and small metabolites in mouse brain tissue by AP IR-MALDI and reactive LAESI mass spectrometry. *Analyst.* 2010; 135(4):751–758. [PubMed: 20349540]
24. Sampson JS, Hawkrigge AM, Muddiman DC. Direct characterization of intact polypeptides by matrix-assisted laser desorption electrospray ionization quadrupole Fourier transform ion cyclotron resonance mass spectrometry. *Rapid Commun. Mass Spectrom.* 2007; 21(7):1150–1154. [PubMed: 17318926]
25. Sripadi P, Nazarian J, Hathout Y, Hoffman EP, Vertes A. In vitro analysis of metabolites from the untreated tissue of *Torpedo californica* electric organ by mid-infrared laser ablation electrospray ionization mass spectrometry. *Metabolomics.* 2009; 5(2):263–276.
26. Berkenkamp S, Karas M, Hillenkamp F. Ice as a matrix for IR-matrix-assisted laser desorption/ionization: mass spectra from a protein single crystal. *Proc. Natl. Acad. Sci. U.S.A.* 1996; 93(14):7003–7007. [PubMed: 8692933]
27. Laiko VV, Taranenko NI, Berkout VD, Yakshin MA, Prasad CR, Lee HS, Doroshenko VM. Desorption/ionization of biomolecules from aqueous solutions at atmospheric pressure using an infrared laser at $3\frac{1}{4}\mu\text{m}$. *J. Am. Soc. Mass Spectrom.* 2002; 13(4):354–361. [PubMed: 11951973]
28. Jackson SN, Kim J-K, Laboy JL, Murray KK. Particle formation by infrared laser ablation of glycerol: implications for ion formation. *Rapid Commun. Mass Spectrom.* 2006; 20(8):1299–1304. [PubMed: 16548069]
29. Vertes A, Nemes P, Shrestha B, Barton AA, Chen ZY, Li Y. Molecular imaging by Mid-IR laser ablation mass spectrometry. *Appl. Phys. A: Mater. Sci. Process.* 2008; 93(4):885–891.
30. Fisher, RA. *Statistical Methods for Research Workers.* New York: Hafner Pub. Co.; 1973.
31. Tamhane, AC. *Statistical Analysis of Designed Experiments: Theory and Application.* New Jersey: John Wiley & Sons, Inc.; 2009.

32. Launsby, RG.; Weese, DL. Straight Talk on Designing Experiments: An Introductory Text for Scientists and Engineers. 2nd Ed.. Colorado: Launsby Consulting; 1995.
33. <http://www.itl.nist.gov/div898/handbook/index.htm>.
34. Riter LS, Vitek O, Gooding KM, Hodge BD, Julian RK Jr. Statistical design of experiments as a tool in mass spectrometry. *J. Mass Spectrom.* 2005; 40(5):565–579. [PubMed: 15880604]
35. Walker SH, Papas BN, Comins DL, Muddiman DC. Interplay of Permanent Charge and Hydrophobicity in the Electrospray Ionization of Glycans. *Anal. Chem.* 2011; 82(15):6636–6642. [PubMed: 20590124]
36. Andrews GL, Dean RA, Hawkrige AM, Muddiman DC. Improving proteome coverage on a LTQ-Orbitrap using design of experiments. *J. Am Soc. Mass Spectrom.* 2011; 22(4):773–783. [PubMed: 21472614]
37. Robichaud G, Dixon RB, Potturi AS, Cassidy D, Edwards JR, Sohn A, Dow TA, Muddiman DC. Design, modeling, fabrication, and evaluation of the air amplifier for improved detection of biomolecules by electrospray ionization mass spectrometry. *Int. J. Mass Spectrom.* 2011; 300(2–3):99–107. [PubMed: 21499524]
38. Huang SK, Glancy SB, Stansbury WF. Optimization of a commercial liquid chromatography/mass spectrometry interface by statistical experimental design. *Rapid Commun. Mass Spectrom.* 1993; 7(8):722–724.
39. de Sousa ND, Ellett CJ, Gilbert M, Wright AD. Use of Chemometrics To Optimize the Operation of an Ion Source. *Anal. Chem. (Washington, DC, U. S.).* 2008; 80(3):873–877.
40. Seto C, Bateman KP, Gunter B. Development of generic liquid chromatography-mass spectrometry methods using experimental design. *J. Am. Soc. Mass Spectrom.* 2002; 13(1):2–9. [PubMed: 11777196]
41. Sampson JS, Hawkrige AM, Muddiman DC. Construction of a Versatile High Precision Ambient Ionization Source for Direct Analysis and Imaging. *J. Am. Soc. Mass Spectrom.* 2008; 19(10):1527–1534. [PubMed: 18657438]
42. Sampson JS, Murray KK, Muddiman DC. Intact and Top-Down Characterization of Biomolecules and Direct Analysis Using Infrared Matrix-Assisted Laser Desorption Electrospray Ionization Coupled to FT-ICR, Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* 2009; 20(4):667–673. [PubMed: 19185512]
43. Valaskovic GA, Murphy JP, Lee MS. Automated orthogonal control system for electrospray ionization. *J. Am. Soc. Mass Spectrom.* 2004; 15(8):1201–1215. [PubMed: 15276167]
44. Nemes P, Marginean I, Vertes A. Spraying Mode Effect on Droplet Formation and Ion Chemistry in Electrosprays. *Anal. Chem. (Washington, DC, U.S.).* 2007; 79(8):3105–3116.
45. Gordon EF, Mansoori BA, Carroll CF, Muddiman DC. Hydropathic influences on the quantification of equine heart cytochrome c using relative ion abundance measurements by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. *J. Mass Spectrom.* 1999; 34(10):1055–1062. [PubMed: 10510428]
46. Nahen K, Vogel A. Plume dynamics and shielding by the ablation plume during Er:YAG laser ablation. *J Biomed Opt.* 2002; 7(2):165–178. [PubMed: 11966301]
47. Valaskovic GA, Kelleher NL, Little DP, Aaserud DJ, McLafferty FW. Attomole-Sensitivity Electrospray Source for Large-Molecule Mass Spectrometry. *Anal. Chem.* 1995; 67(20):3802–3805. [PubMed: 8644926]
48. Nemes P, Barton AA, Li Y, Vertes A. Ambient molecular imaging and depth profiling of live tissue by infrared laser ablation electrospray ionization mass spectrometry. *Anal. Chem.* 2008; 80(12):4575–4582. [PubMed: 18473485]
49. Peng IX, Ogorzalek Loo RR, Margalith E, Little MW, Loo JA. Electrospray-assisted laser desorption ionization mass spectrometry (ELDI-MS) with an infrared laser for characterizing peptides and proteins. *Analyst.* 2010; 135:767–772. [PubMed: 20349541]

Figure 1.

a) A pictorial description of the experiments which were performed. The inclusion of bovine cytochrome c (BOV) in the electrospray solution during the ionization of equine cytochrome c (EQU) by IR-MALDESI provided a means of tracking electrospray stability and semi-quantitatively determining the ionization efficiency of IR-MALDESI under each set of conditions. **b)** A mass spectrum showing the charge state distributions of both BOV (blue) and EQU (red) captured from a typical experiment. **c)** A zoomed-in portion of the spectrum in showing the isotopic distributions for both analyte and internal standard as well as the observation of salt adduction for the electrosprayed internal standard but not the analyte ionized by IR-MALDESI.

Figure 2.

Summary of the results of the screening fractional factorial DOE. a) A half-normal quantile plot that describes which factors and interactions were found to be significant (hashed red circle). b) Prediction profiler which models the system and predicts the optimal combination of settings (shown in red above each factor).

Figure 3. Prediction profiler which models the system and predicts the optimal combination of settings (shown in red above each factor).

Figure 4.

Single acquisition mass spectra from the spot down experiment zoomed in on the $[M + 16H]^{16+}$ charge states for both equine and bovine cytochrome c. The top spectra (a, c) represent the 100 pmol equine cytochrome c spotted on the target and analyzed. The bottom spectra (b, d) represent the smallest amount spotted which resulted in detectable signal.

Table 1

List of the factors and the meaningful ranges over which they were tested. Also noted are some of the motivations for their inclusion in the screening fractional factorial DOE.

Factor	Min.	Mid.	Max	Motivation
Stage Height (mm)	3	11.5	20	Stage height can determine the degree of overlap between the ablated plume and the electrospray plume.
ESI-Inlet Distance (mm)	5	7.5	10	This factor can control the stability of the electrospray as well as the area available for interaction with the ablated plume.
Sample-Inlet Distance (mm)	1	5.5	10	This factor can also affect the degree of overlap between the two plumes as well as the size of the ESI droplets which interact with the ablated plume.
Flow Rate (nL/min.)	400	1700	3000	Flow rate can control the size of the electrospray droplets as well as the efficiency of ionization.
Plate Voltage (V)	0	500	1000	This factor could be used to account for the presence of a grounded stainless steel plate in the electric field created by electrospray.
Laser Fluence (J/cm ²)	0.5	1	1.5	Will probably determine the amount of material which is ablated from the surface as well as the allowable sampling time.
Laser Repetition Rate (Hz)	5	10	20	Repetition rate would affect the number of laser pulses which would be required in order to observe appreciable signal as well as to some degree determining the amount of sample ablated in a given time frame.

Table 2

List of the factors and the range of settings which were tested at higher resolution in the full factorial DOE.

Factor	Min.	Mid.	Max
Stage Height (mm)	5	10	15
Sample-Inlet Distance (mm)	4	6	8
Flow Rate (nL/min.)	400	1000	1500
Laser Fluence (J/cm ²)	0.8	1.1	1.5

Table 3

The best, worst, and previously used combination of factor settings

Factor	Best	Previous	Worst
Stage Height (mm)	5.5	5	20
ESI-Inlet Distance (mm)	9	5	10
Sample-Inlet Distance (mm)	8	5	9
Flow Rate (nL/min.)	400	800	1800
Plate Voltage (V)	550	300	0
Laser Fluence (J/cm ²)	1.5	2.2	0.5
Laser Repetition Rate (Hz)	5	10	10