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Quantitative Analysis with Desorption/Ionization on Silicon Mass Spectrometry Using Electrospray Deposition

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Desorption/ionization on silicon mass spectrometry (DIOS-MS) is demonstrated as a quantitative analytical tool when coupled to electrospray deposition (ESD). In this study, we illustrate the utility of DIOS-MS in the quantitative analysis of a peptide and two amino acids with deuterated and structural analogues used as internal standards. An important feature of this approach is the incorporation of ESD to improve sample homogeneity across the porous silicon surface. ESD allowed for a marked improvement in quantitative analysis due to its applicability to LC-DIOS, and because of the absence of matrix, sample can be deposited at very low flow rates (150 nL/min). Experiments comparing the traditional dried droplet and ESD methods show that ESD samples exhibit significantly improved quantitation and much higher sample-to-sample reproducibility.

Quantitative analysis with electrospray ionization mass spectrometry has become a powerful analytical tool in clinical chemistry,[1] drug discovery, and recently, proteomics.[2,3] Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is also a versatile ionization technique due to its amenability to an array of molecules with good sensitivity, high salt tolerance, and ability to analyze complex mixtures. There has been an increasing effort to apply MALDI-MS to quantitative analysis of proteins and peptides,[4−6] antibiotics,[7] cyclosporin,[8,9] oligosaccharides,[10] oligonucleotides,[9,11] low molecular weight compounds in biological fluids and extracts,[12] and enzyme-catalyzed reactions.[13,14] However, due to matrix ion interference, direct analyte quantitation by MALDI-MS has been problematic and time-consuming, especially for analytes with masses below 700 Da.

Recently, the introduction of a matrix-free desorption/ionization on electrochemically etched silicon surfaces has allowed the analysis of analytes with mass as low as 100 Da.[15,16] In desorption/ionization on silicon mass spectrometry (DIOS-MS), analytes are deposited on the porous silicon surface and desorbed/ionized by the irradiation of ultraviolet light. The relatively soft and efficient ionization process provided by these surfaces enables the analysis of analytes in the femtomole-to-attomole range with little to no fragmentation. In addition, DIOS-MS offers several advantages such as minimal sample preparation, the ability for on-chip separation and reaction monitoring, high sensitivity, and high-throughput capability, thus making it an attractive technique for analyte quantitation.

Traditionally, DIOS-MS sample preparation entails pipet deposition of a 0.5-μL droplet of the analyte solution on the DIOS chip and then air-drying. This dried droplet method of DIOS-MS sample preparation suffers from heterogeneous ion signal due to inhomogeneous analyte distribution. To overcome this problem, we have incorporated electrospray deposition (ESD) into our sample preparation. In the ESD method, the deposition of very fine positively charged droplets yields a uniform thin layer of the analyte on the porous silicon surface. By employing this method we achieve three notable results: (1) quantitative analysis is markedly improved, (2) ESD can be applied to LC-DIOS, and (3) because of the absence of matrix, sample can be deposited at very low flow rates starting from 150 nL/min.

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(1) Chace, D. H. *Chem. Rev.* **2001**, *101*, 445−77.
(2) Griffin, T. J.; Goodlett, D. R.; Aebersold, R. *Curr. Opin. Biotechnol.* **2001**, *12*, 607−12.
(3) Aebersold, R.; Goodlett, D. R. *Chem. Rev.* **2001**, *101*, 269−95.
(4) Mirgorodskaia, O. A.; Koz'min, IuP.; Titov, M. I.; Savel'eva, N. V.; Korner, R.; Sonksen, C.; Miroshnikov, A. I.; Roestorff, P. *Bioorg. Khim.* **2000**, *26*, 662−71.
(5) Mirgorodskaya, O. A.; Kozmin, Y. P.; Titov, M. I.; Korner, R.; Sonksen, C. P.; Roepstorff, P. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1226−32.
(6) Nelson, R. W.; Mclean, M. A.; Hutchens, T. W. *Anal. Chem.* **1994**, *66*, 1408−15.
(7) Ling, Y. C.; Lin, L.; Chen, Y. T. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 317−27.
(8) Muddiman, D. C.; Gusev, A. I.; Proctor, A.; Hercules, D. M.; Venkataramanan, R.; Diven, W. *Anal. Chem.* **1994**, *66*, 2362−68.
(9) Wu, J.; Chatman, K.; Harris, K.; Siuzdak, G. *Anal. Chem.* **1997**, *69*, 3767−71.
(10) Harvey, D. J. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 614−19.
(11) Bruenner, B. A.; Yip, T. T.; Hutchens, T. W. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1797−801.
(12) Duncan, M. W.; Matanovic, G.; Cerpa-Poljak, A. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 1090−94.
(13) Kang, M. J.; Tholey, A.; Heinzle, E. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1972−78.
(14) Kang, M. J.; Tholey, A.; Heinzle, E. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1327−33.
(15) Shen, Z.; Thomas, J. J.; Averbuj, C.; Broo, K. M.; Engelhard, M.; Crowell, J. E.; Finn, M. G.; Siuzdak, G. *Anal. Chem.* **2001**, *73*, 612−19.
(16) Wei, J.; Buriak, J. M.; Siuzdak, G. *Nature* **1999**, *399*, 243−46.

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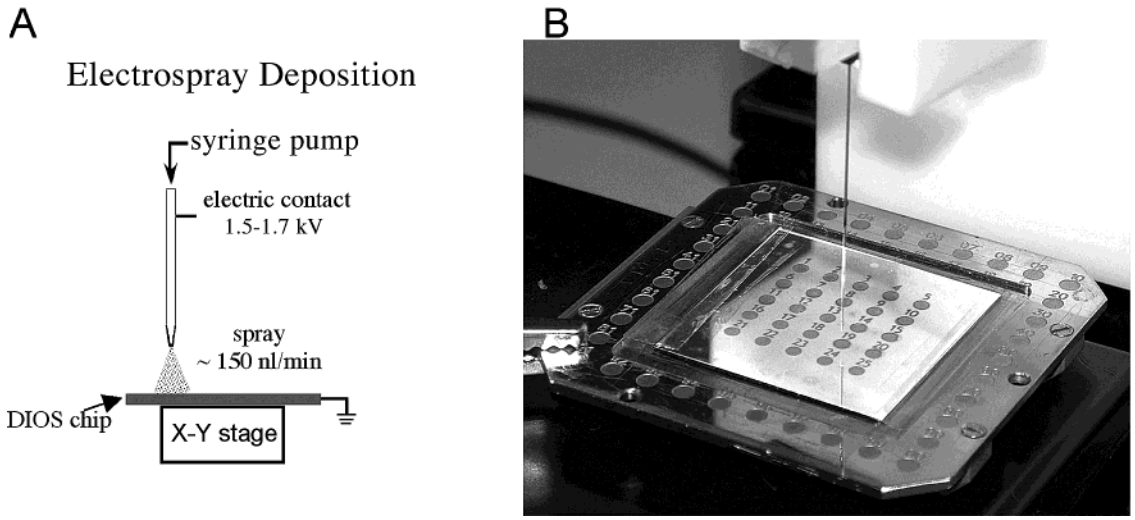

Figure 1. (A) Schematic diagram of the electrospray sample deposition setup. (B) Photograph of the electrospray setup showing the photopatterned DIOS chip and the capillary tip.

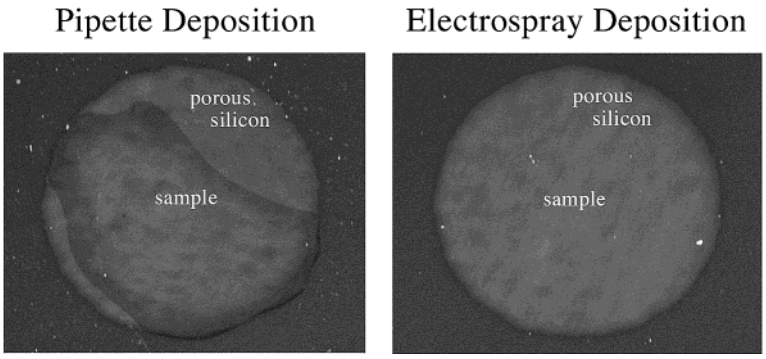

Figure 2. Micrographs of pipet and ESD samples depicting sample distribution on the DIOS surface. The dried droplet sample shows a localized analyte region while the ESD sample exhibits a homogeneous distribution of sample over the entire DIOS spot.

EXPERIMENTAL SECTION

Materials. Optima grade methanol, tribasic ammonium citrate, thymopentin (MW 679.2), splentin (MW 693.2), L-phenylalanine (MW 165.19), and L-tyrosine (MW 181.19) were all obtained from Sigma. Stable isotopes of [$^2H_5$]-L-phenylalanine (MW 170.23) and [$^2H_4$]-L-tyrosine (MW 185.23), both with isotopic purity of >99%, were obtained from Cambridge Isotopes. Butanolic HCl (3 N) was purchased from Regis.

Sample Preparation. Primary stock solutions of the two peptides, thymopentin and splentin, were freshly prepared at a concentration of 1 mg/mL in deionized water. Standard solutions of each peptide were prepared at 100 μM by diluting the primary stock solutions with deionized water, followed by serial dilution to obtain five working solutions. The working solutions were prepared in 50% methanol and 2 mM ammonium citrate (pH 7.5) with peptide concentration ratio of 10−0.1 corresponding to a concentration range of 10−0.1 μM. The buffered solutions were prepared by adding an increasing concentration of thymopentin (peptide 1) and a constant concentration (1 μM) of splentin (peptide 2, internal standard). Stock solutions of phenylalanine, tyrosine, and their deuterated analogues as internal standards were freshly prepared at a concentration of 1 mg/mL in 50% methanol/water. A series of working solution of the amino acids and their deuterated analogues were prepared at a concentration ratio of 10−0.1 and slowly evaporated to dryness. The amino acids were derivatized to butyl esters by dissolving the dried samples in 250 μL of 3 N butanolic HCl and incubating at 65 °C for 30 min. Excess butanol was removed by evaporating the reaction mixture to dryness in a Speedvac. The derivatized samples were reconstituted with 50% methanol/2 mM ammonium citrate buffer (pH 7.5).

Electrospray Sample Deposition. Electrospray sample deposition was performed using fused-silica columns that were constructed from 100-μm-i.d. capillary (Agilent) pulled to a diameter of ~5 μm. A continuous supply of the analyte solution is introduced to the capillary tip by a syringe pump with an infusion rate of 10 μL/h. Deposition was accomplished by applying a potential difference between the capillary and the DIOS chip, which was mounted on a grounded modified MALDI target plate. Typical voltage used for the deposition was in the range of 1.5−1.7 kV. A stable electrospray was achieved by optimizing the voltage and the distance between the capillary tip and the DIOS chip. Typically, 0.5 μL of sample was deposited on a DIOS spot, which generated a thin circular layer on the DIOS spot.

Time-of-Flight Reflectron Mass Spectrometer. Experiments were conducted on a Micromass M@LDI-R (Manchester, U.K.) mass spectrometer operated in the positive ion mode. This instrument is equipped with automated and multisampling capabilities for rapid sample analysis. The DIOS chips were attached to a modified MALDI target plate with a conductive tape, and

samples were irradiated with a 337-nm N_2 laser operated at 5 Hz. Data acquisition was performed in the autosampler mode. Mass spectra were generated by averaging 65 individual laser shots into a single spectrum. Total acquisition time for each sample was ~1 min. During acquisition, the laser energy percentage was incrementally increased until acceptable signal-to-noise levels were obtained. Mass spectral data were considered acceptable if the signal was 5−10 times of the background noise and < 80% of saturation.

The details of DIOS chip preparation have been described elsewhere.[15,16] Briefly, photopatterned DIOS chips were prepared by etching low-resistivity (0.005−0.02 Ω·cm) n-type Si(100) wafers in 25% v/v HF/ethanol (Acros and Sigma, respectively) under white light illumination for 2 min at a current density of 5 mA/cm^2. Photopatterning of the DIOS chips allow for the analysis of 25 sample spots on each chip. Following the first etching, the surface was oxidized with ozone and re-etched with 5% v/v HF/H_2O solution.

Quantitative Analysis. All of the data analysis was performed by averaging 65 successive signals into a single spectrum. Each spectrum was accumulated from 5 shots at 13 different locations within the 2-mm DIOS spot. Data processing involved linear background subtraction resulting in a flat baseline with intensity at zero. Calibration curves were constructed by plotting the logarithm of the peak intensity of the analyte (A) to the internal standard (IS) versus the logarithm of the concentration ratio of A to IS. All calibration curves were fitted to a nonweighted linear regression. The precision of the method was measured as relative standard deviation (RSD), which was obtained by calculating the percentage ratio of the standard deviation to the mean of eight replicate measurements.

RESULTS AND DISCUSSION

Sample preparation plays a critical role in determining the sensitivity and quality of the mass spectral data. ESD coupled with MALDI-MS has been reported to markedly improve sample homogeneity, resulting in enhanced sensitivity and signal reproducibility.[17,18] However, with MALDI-MS the incorporation of matrix not only adds another step in sample preparation but also compromises detection sensitivity. In this study, ESD coupled with DIOS-MS was achieved using the configuration shown in Figure 1. The electrospray is generated by applying an electric field to the analyte solution passing through the capillary tubing. Electrospraying results in the formation of very fine positively charged droplets that dry quickly and are dispersed into a homogeneous circular pattern on the DIOS surface. Spray stability is dependent on the flow rate, the shape and size of the capillary tip, the potential difference, the solvent system, and the distance between the capillary tip and the DIOS chip. Best results are achieved with a flow rate of 150 nL/min, small capillary tip (<5 μm), potential difference of 1.6 kV, analyte solutions in 50% methanol, and separation distance of ~1 mm between the capillary tip and the DIOS chip. With these conditions, ESD yields a circular spot with a diameter of 2.5 mm on the DIOS surface (Figure 2). Comparison of the images in Figure 2 clearly shows that there are no discernible features in the ESD sample, indicating a homogeneous distribution of the sample across the DIOS surface.

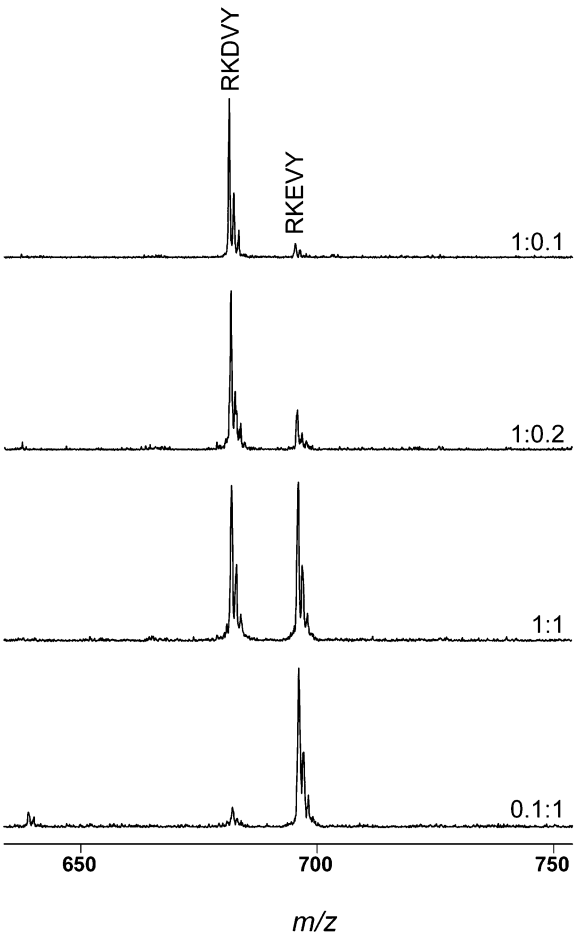

Figure 3. DIOS mass spectra of the peptide mixture as a function of increasing thymopentin (RKDVY) concentration and a constant splentin (RKEVY) concentration.

The utility of DIOS-MS for quantitative analysis using ESD as the method for uniform sample deposition was demonstrated with a peptide and two amino acids as model systems. To monitor reproducibility and measure quantitation, internal standards were employed. Specifically, a structural analogue was utilized for peptide quantitation and deuterium-labeled isotopes for analysis of the amino acids. The sample-to-sample reproducibility of the analyte ion signal was monitored, and the results for both dried droplet method and ESD were compared.

In the first set of experiments, the dynamic range of the peptide mixture was monitored. The limit of quantitation for both peptides used in this study was 50 fmol, and the linear dynamic range is ~$2^1/_2$ orders of magnitude. Using 1 μM of the internal standard (peptide 2), the relative signal intensities of peptide 1 were measured within 2 orders of magnitude. Figure 3 shows analyte ion signal intensities of the two peptides as a function of concentration measured by DIOS-MS. The spectra show the monoisotopic peaks at m/z 680.2 for peptide 1 and at m/z 694.2 for peptide 2 (internal standard) with high signal-to-noise ratio.

Signal reproducibility was monitored by comparing the signal intensity from the dried droplet and the ESD deposited samples. ESD samples exhibited more uniform analyte ion signal intensities

(17) Axelsson, J.; Hoberg, A. M.; Waterson, C.; Myatt, P.; Shield, G. L.; Varney, J.; Haddleton, D. M.; Derrick, P. J. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 209−13.

(18) Hensel, R. R.; King, R. C.; Owens, K. G. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1785−93.

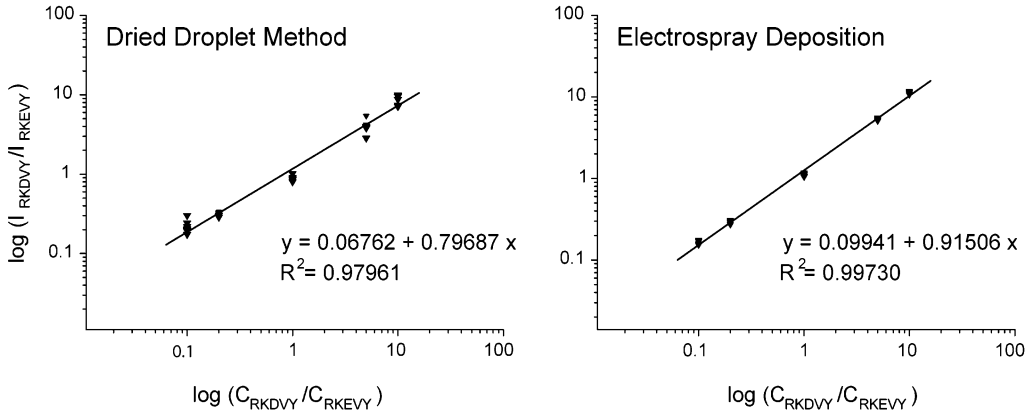

Figure 4. Peak intensity vs concentration ratios in the logarithmic scale of the peptide mixture for both the ESD and dried droplet methods.

Table 1. Comparison of the Sample-to-Sample Reproducibility between the Dried Droplet and ESD Method of Sample Deposition

		RSD (%)	
sample mixture	ratio	dried droplet method	electrospray deposition
peptide	10	12.6	3.5
	5	20.7	2.9
	1	8.2	4.3
	0.2	6.1	4.0
	0.1	19.2	5.7
Tyr/Tyr-d_4	10	18.9	6.7
	5	10.0	3.8
	1	12.2	1.8
	0.2	18.6	5.6
	0.1	17.9	5.2
Phe/Phe-d_5	10	16.5	3.9
	5	14.4	6.2
	1	5.6	1.8
	0.2	16.2	7.1
	0.1	17.8	4.8

Table 2. Comparison of the Linear Correlation Coefficient (R^2) between Dried Droplet and ESD Method of Sample Deposition

sample mixture	dried droplet method	electrospray deposition
Peptide	0.97961	0.99730
Tyr:/Tyr-d_4	0.98686	0.99764
Phe/Phe-d_5	0.97763	0.99864

across the DIOS spot. In addition, reproducibility between samples was monitored by measuring the variation in the peak height ratios of two peptides at a given concentration ratio on eight different DIOS spots. The optimal precision, which is also related to sample reproducibility, was determined by calculating the RSD. In order for a technique to be suitable for quantitative analysis, RSD values for any measured response should be low and consistent. Figure 4 shows the peptide mixture calibration curves for both ESD and the dried droplet method. The curves were constructed by plotting the peak intensity versus concentration ratios using a logarithmic scale. The calibration curves for both methods show a linear response in the working concentration ratio range. It is apparent, however, that ESD samples ($R^2 = 0.997$) exhibit higher linearity than the dried droplet samples ($R^2 = 0.980$). The data also show high precision at a given concentration ratio for both methods. However, the measured RSD values ($n = 8$) for the ESD samples are much lower than the dried droplet samples at a given concentration (Table 1). The RSD of the ESD method is 5% or better compared to 6−20% obtained from the dried droplet method. All of the results indicate higher sample-to-sample reproducibility for the ESD method.

To illustrate the capability of DIOS-MS for the quantitative analysis of low molecular weight molecules (<500 Da), the amino acids phenylalanine ($MH^+ = 165.2$) and tyrosine ($MH^+ = 170.2$) were analyzed using their deuterated analogues as internal standards. The spectra obtained for the amino acids showed isotopically resolved peaks with high specificity. To facilitate the analysis, the amino acids and their deuterated analogues were derivatized to butyl esters.[19,20] The processed peak area ratios of the derivatized ions were determined as a function of the concentration ratio corresponding to phenylalanine/phenylalanine-d_5 (222:227) and tyrosine/tyrosine-d_4 (238:242). The calibration curves were generated for both ESD and dried droplet samples, and the least-squares data are summarized in Table 2. The data show that both methods exhibit a linear response. However, the ESD samples showed higher linearity than the dried droplet samples over the entire working concentration range. In addition, the spot-to-spot reproducibility is better for the ESD samples as indicated by lower RSD (Table 1) than the pipet spotted samples, demonstrating better reproducibility between DIOS spot for electrosprayed samples.

The improvement in deposition homogeneity obtained by ESD results in better signal reproducibility. This inherent characteristic of ESD illustrates the suitability of this sample deposition method for quantitative analysis with DIOS-MS. However, analysis of complex samples is often compromised due to signal interference and ion suppression effects. The combination of off-line LC separation with ESD can be implemented to address this problem. Recently, the combination of ESD LC-DIOS-MS has been demonstrated to improve the sequence coverage of two model protein systems compared to traditional LC ESI-MS/MS analysis.[21] Presently, we are extending the utility of this technique in the analysis

(19) Chace, D. H.; Millington, D. S.; Terada, N.; Kahler, S. G.; Roe, C. R.; Hofman, L. F. *Clin. Chem.* **1993**, *39*, 66−71.
(20) Tholey, A.; Wittmann, C.; Kang, M. J.; Bungert, D.; Hollemeyer, K.; Heinzle, E. *J. Mass Spectrom.* **2002**, *37*, 963−73.
(21) Prenni, J. E.; Shen, Z.; Trauger, S.; Chen, W.; Siuzdak, G. *Spectroscopy*, in press.

of small molecules in human plasma and urine samples. Experiments are being performed, and the results will be compared to LC−MS to further explore its utility for the analysis of a variety of complex biological mixtures.

CONCLUSIONS

DIOS-MS is an attractive analytical tool in the quantitative analysis of biomolecules with masses in the range 100−3000 Da. The technique offers advantages such as high sensitivity, high-throughput capability, rapid sample analysis, minimal sample preparation, and on-chip separation and reaction monitoring. Unlike MALDI-MS, this matrix-free technique does not require the additional step of matrix addition, which contributes to analyte signal variability. Since sample preparation plays an important role in achieving reliable analyte quantitation, two sample preparation methods were compared, ESD and the dried droplet method. Overall, linear calibrations with $R^2 > 0.99$ were obtained in the analysis; typical RSD values of <7% were routinely obtained for ESD samples, compared to the RSD values of <18% for dried droplet samples. Because of better sample homogeneity, ESD samples result in higher linearity and signal intensity within a sample and high sample-to-sample reproducibility.

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