The Matrix Suppression Effect in Matrix-assisted Laser Desorption/Ionization: Application to Negative Ions and Further Characteristics

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Abstract

As recently shown for positive ions (*Rapid Commun. Mass Spectrom.* 10, 871 (1996)), small to moderate-sized analyte ions (<20 000 Da) can fully suppress all matrix signals in matrix-assisted laser desorption/ionization (MALDI) mass spectra at appropriate matrix:analyte mixing ratios. The technique is useful for removing matrix interferences and led to a model for primary ion formation mechanisms. We here extend the method to negative ions, and investigate the effects of analyte type and size on the matrix suppression concentration range. For a given matrix/analyte pair, suppression was observed in either positive- or negative-ion mode, but not both. Some matrices do not show the effect at all, which is attributed to unfavourable thermodynamics. The analyte concentration where suppression appears is correlated with the analyte molecular weight and supports the proximity requirement of the model. The dependence of matrix signals on analyte concentration is very similar in both modes, and may have implications for the role of plume reactions at low analyte concentrations. Delayed extraction was found to extend matrix suppression to lower analyte concentrations. Lack of homogenous matrix/analyte co-crystallization at low analyte concentrations is shown to prevent the appearance of matrix suppression or to modify the shape of the concentration curves in some cases. Evidence is presented for the activity of electronic excitations in IR as well as in UV MALDI.

Introduction

As discussed in Ref. 1 and references therein, when analytes of moderate size (<20 000 Da) are mixed with common matrices in relatively high molar ratio (10:1 to about 2000:1 matrix to analyte ratio), all positive matrix ions in the mass spectrum can be completely suppressed. Along with the fact that analyte fragmentation is typically weak in MALDI, this leads to nearly ideal mass spectra: strong analyte parent ions but no other signals of any kind. The effect has been observed in positive ion mode with a number of matrices[2,3] including nicotinic acid, a-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid. (DHB), and seems to be a widespread phenomenon in matrix-assisted laser desorption/ionization (MALDI).

In Ref. 1 it was noted that matrix suppression involves the disappearance of *all* matrix ions, including radical cations, protonated and alkali cationized species, regardless of the analyte ion form (protonated or cationized). This not only rules out simple competition models of the effect,[2] but led to the hypothesis that, of the many possibilities previously proposed,[4,5] multiply excited matrix aggregates (most likely pairs) are the primary ion generators in UV MALDI. A simplified scheme leading to protonated analyte ions is (m = matrix, m* = electronically excited matrix, A = analyte):

$$\underset{\textit{protonation}}{2m^* + A \rightarrow [m\text{-}H]^- + AH^+ + m} \qquad (1)$$

If processes like this are necessary for the creation of MALDI analyte ions, there should also be a similar pathway to matrix ions such as mH, when analyte is not in the vicinity:

$$2m^* \rightarrow m^+ + m^- \text{ and/or } [m\text{-}H]^- + [m+H]^+, \text{ m + H, etc} \qquad (2)$$

This provides an efficient mechanism for production of primary matrix ions and radicals without direct multiphoton ionization. Although m^- may be weak and $[m\text{-}2H]^-$ is also observed, all products listed in reaction (2) are observed for DHB, including neutral H atoms.[6] Corroborating evidence for matrix–matrix reactions has also recently been obtained from optical spectra of MALDI plumes.[7] Primary ions not immediately in contact with analyte may continue to react in the gas phase, leading to further secondary analyte

signal, as is suggested by observations of an optimum delay for post-desorption extraction[8] and by angle and velocity resolved mass spectra.[9] Recent results using two different methods also show clear evidence for both prompt and delayed ion generation.[10,11]

It is important to note that the detailed mechanism of these reactions is still not known. It is possible that reaction (1) proceeds in two or more steps, with matrix–matrix reactions, such as reaction (2), as intermediates. Fragments of the primary matrix ions may also be active in protonating the analyte.[1,2] The hypotheses represented by the reactions (1) and (2) were tested in Ref. 1 by various means and found to be consistent with the data. However, in that work the possibility of matrix suppression in negative-ion mode was not addressed. Indeed, it was observed that strong negative matrix signals were present under positive ion suppression conditions, and this was taken as support for the model.

Such observations nevertheless do not rule out a suppression effect for negative ions using other matrices than those studied in Ref. 1. In general, the matrix can react amphoterically with analytes. Experimentally, spectra are often obtainable in both positive- and negative-ion modes. A parallel scheme to (1) can be proposed for the analyte deprotonation (m = matrix, m* = electronically excited matrix, A = analyte):

deprotonation

$$2m^* + A \rightarrow mH^+ + [A\text{-}H]^- + m \qquad (3)$$

Once again, if all the excited matrix is consumed (directly or via intermediate steps) in reaction with analyte, suppression will be observed in the negative-ion mass spectrum, while copious matrix signal will appear in the positive spectrum.

The polarity of matrix suppression (positive- or negative-ion mode) will be determined by the thermodynamics and kinetics of the respective reaction pathways. If one route is sufficiently favourable, every encounter with analyte will result in analyte ionization, and suppression will tend to appear in only one polarity. It must be remembered, however, that suppression is a property of the analyte/ matrix pair and not of the matrix alone. If neither pathway is strongly favoured, no suppression may be observed at all.

The present study is concerned with several aspects of the matrix suppression effect. Matrix suppression is demon- strated in negative-ion mode, as is the case of non-suppression in both polarities. Parameters influencing the effect are also studied, including the matrix-to-analyte concentration ratio, sample crystallization and the use of delayed extraction.

Experimental

The MALDI experiments were performed in a conventional 2 m linear time-of-flight instrument which was built in our laboratory. The total 2-stage acceleration voltage was 25 kV. Desorption was peformed with a pulsed nitrogen laser (Laser Science Inc., Newton, MA, USA, model VSL- 337ND-T). The laser was attenuated by glass plates and an adjustable iris. Typical power densities were on the order of 5×10^6 W/cm^2 with fluences of 15 mJ/cm^2 for strong MALDI signal. Delayed extraction was performed by pulsing the repeller plate using a home-built circuit with a high voltage switch. The repeller voltage was 25 kV, the extractor was held at 21 kV, and the delay time was 650 ns.

Valinomycin (98%), DHB, sinapinic acid, adenosine-5'-monophosphate monohydrate (AMP), polyethyleneglycols 1000, 2000, 3000 and cyclodextrin were from Fluka. Bovine ubiquitin, cyctochrome C and human insulin were from Sigma. Reagents were used as received, except DHB which was purified by sublimation. The solvent for the biomole-cules was generally 1:1 v/v 2-propanol/water, with 0.1% trifluoroacetic acid (TFA) added. AMP was prepared in water. Valinomycin was dissolved in methanol, and substance P was prepared in 3:7 v/v acetonitrile/water with 0.1 % TFA. The matrices were dissolved in methanol at a concentration of about 0.1 M; except for sinapinic acid, which was dissolved in 2:1 acetonitrile/water.

Solutions were mixed immediately prior to use, 1–2 mL was deposited on the holder, and dried under a warm air flow and then in vacuum. The operating pressure during MALDI measurements was typically 1×10^{-6} mbar or less. Between 50 and 100 laser shots were summed to improve the signal-to-noise ratio for each measurement. The sample was also rotated during summation to yield a more representative spectrum.

Matrix-to-analyte intensity ratios were evaluated by integration of the relevant peaks in the mass spectrum. All known matrix signals were summed, including, for example m^+, mH^+ and MNa^+ species in positive-ion mode.

Results and Discussion

Negative mode matrix suppression

As first examples for matrix suppression in negative mode we consider the analytes MOPS (3-morpholino-propane-sulfonic acid, MW = 209), and HEPES (4-(2-hydro-xyethyl)-piperazine-1-ethansulfonic acid). These compounds have been used as buffering agents in MALDI sample preparation[13] and are readily observed in negative-ion mode. In aqueous solution, MOPS buffers between pH = 6.2 and 8.2 (pK_a = 7.2), and HEPES between pH = 6.5 and 8.5 (pK_a = 7.55).

Using DHB as matrix, at a matrix/analyte molar ratio (M/A) of 10:1 matrix suppression by HEPES or MOPS is observed in *positive*-mode, but strong matrix signal remains in the negative-ion spectrum (Fig. 1). Favorable thermodynamics for negative-ion matrix suppression are to be expected from matrices that are less proton donating in their photoreactions than DHB. While the acid–base behaviour of excited states is often much different from that of the ground state,[14] and the excited-state proton-transfer reactions of matrices are not currently understood, we nevertheless chose as candidate matrices those that have a ground-state of basic or neutral character.[15] In water, these matrices have the following pK_a values: 4-nitroaniline (4NA) 6.4; 2-amino 3-hydroxypyridine (AHP) 7.2; and 3-aminoquinoline (3AQ) 8.78.

For the matrix 3AQ, full matrix suppression was observed in negative ion mode, for both MOPS and HEPES. A representative mass spectrum is shown in Fig. 2 for 3AQ/ MOPS. The concentration where the effect appears is near M/A = 10, as would be predicted from the molecular sizes, in the manner noted in Ref. 1. As anticipated, and in analogy to matrix suppression in positive mode, abundant matrix is observed in the positive-ion spectrum. Also as expected, suppression is observed only in one polarity since protonation and deprotonation cannot both have a large driving force.

The intermediate case where neither the positive nor negative suppression reaction is

favoured appears to have been observed for the matrices 4NA and AHP. For these matrices, matrix suppression was not observed in either positive- or negative-ion modes. Representative results for 4NA/MOPS are shown in Fig. 3 at a high analyte concentration. It was expected from molecular sizes and the proximity requirement that suppression would appear when the matrix/analyte ratio was 10 or less, which is clearly not the case here. Nevertheless, the relative MOPS signal is stronger in negative mode.

Matrix suppression vs. matrix/analyte ratio

Matrix–analyte proximity or contact for all excited matrix molecules, and hence efficient ionization, is guaranteed when the analyte is sufficiently homogeneously mixed and concentrated, that the matrix forms no more than bilayers between the analyte molecules after drying and crystallization.1 As the analyte becomes less concentrated, full suppression cannot be achieved since some excited matrix aggregates will not be able to react with analyte. On the other hand, in the absence of optimal mixing, or if plume reactions are significant, then the analyte concentration dependence of matrix signal in the region near suppression should be informative.

The concentration dependence of the matrix-to-analyte signal ratio is particularly interesting for the 3AQ/MOPS pair. As seen in Fig. 4 there are three distinct regions in the curve. When the analyte is dilute (high M/A molar ratio, on the right of the Fig.), the trend is roughly linear, as would be expected for homogeneous mixing and uniform ionization probability. At an M/A ratio of 700–1000, there is a sudden decrease in matrix signal, indicating the onset of the matrix suppression effect. Between M/A = 700 and 50, there is a remarkable plateau where increasing the analyte concentration has little effect on signal ratios. Only below M/A = 50 does the final drop to full suppression occur. This structured curve should be compared with the behaviour we have observed for several analytes in the non-suppression polarity, which is linear and completely unremarkable.

The three part curve of Fig. 4 was found to be characteristic for matrix suppression in positive-ion mode as well. Figure 5 shows the concentration curve for valinomycin (MW = 1111) in DHB. This was one of the examples of strong matrix suppression in Ref. 1. Once again we see the linear region at high M/A, the drop to a plateau at M/A = 1000, and the

final step to full matrix suppression at low M/A.

Similar results were obtained for other biomolecules such as adenosine monophosphate (negative ions), substance P, human insulin, ubiquitin and cytochrome c (positive ions). Even in cases where full matrix suppression was not achieved (ubiquitin and cytochrome C), there was a distinct drop or plateau in relative matrix signal at low M/A. This is shown in Fig. 6 for cyctochrome C. The flatter plateau-like region extends to higher M/A (2000–4000) than for the other analytes. Near suppression is reached at M/A = 2000, where only quite small matrix signals were present. In this case the matrix signal increases again at yet lower M/A, and the reproducibility of the signal decreases. This is attributed to poor co-crystallization of matrix and analyte, which was also reflected in the strong variation in signal ratios with position of the laser on the sample. Failure to make the last step to full suppression is believed to be due to such mixing problems, as is discussed below.

If the proximity of excited matrix pairs and analyte is indeed a major precondition for matrix suppression, it follows that larger analytes will induce suppression at a higher M/A mole ratio because they are physically larger. A qualitative trend of this type has also been noted by other authors.[2,3] The effect should scale roughly with the surface area of the analyte, or the mass to the 2/3 power, assuming similar density for most analytes and treating them as generally spherical in shape.[17] The curve in Fig. 7 for analytes in DHB has a clear dependence on analyte mass, with approximately the expected power dependence. However, the best fit curve does not have a zero intercept, as expected from the simple model. Clearly, as molecular size decreases the molecules become less well described as spheres, and some offset of the curve is to be expected.

The plateau region

While the matrix/analyte proximity requirement seems to explain the analyte concentration where full matrix suppression is observed, it does not explain the drop to the plateau that is also observed at higher M/A ratios. That the plateau occurs at M/A as high as 1000–4000 is remarkable, since at this concentration many matrix molecules are quite far from analyte, on average. This suggests that a certain amount of diffusion and mixing occurs in the desorption plume, allowing initial matrix ions to be consumed by analyte rather than leading to matrix signal. If this begins at M/A = 1000, then the typical diffusion range in

the plume before collision-free conditions are reached is $(1000)^{1/3}$ = 10 neighbours, which does not seem unreasonable. This hypothesis is also consistent with the conclusions of other authors that reactions continue in the plume.[4,5,9–11]

Matrix suppression and sample crystallization

As noted above, a requirement for matrix suppression is good mixing of matrix and analyte in the solid phase. This can be strongly influenced by choice of solvent or drying method, and is currently part of the empirical MALDI 'art'.

The effect of poor crystallization on matrix suppression can vary from moderate to severe. In Fig. 8 we show the concentration dependence of the sinapinic acid (SA)/AMP signal ratio up to M/A = 3000, in negative-ion mode. The curve has a linear region, a possible plateau and a suppression region. Suppression is observed at low M/A, since AMP is a small molecule. In the M/A < 100 region, however, we find a significant dependence of the signal ratio on the sample solution used. If more concentrated AMP and matrix solutions are mixed on the sample tip, lower signal ratios are obtained than if less concentrated solutions are used. Apparently the solvent volume is modifying the crystallization process, perhaps via the rate of crystallization. This remains a minor effect, since it only appears at low M/A and leads to signal ratios differing by about a factor of two. The AMP/SA combination is perhaps predestined to show problems of this type, even at higher M/ A the scatter in the measurements is much higher than for MOPS in 3AQ, as shown in Fig. 4. In addition to the predominantly biological analytes discussed above, matrix suppression was also sought for analytes of a different nature, such as synthetic polymers. Materials tested included polyethyleneglycols (PEG) 1000 and 2000, cyclodextrins and dextran 1000. These were studied in DHB matrix, as they are preferentially observed as cations, especially alkali adducts.

With the usual dried droplet sample preparation methods, no suppression with DHB as matrix was observed for any of these compounds. The concentration curves also showed no clear signs of the usual approach to suppression. It is our experience that such materials are somewhat more difficult to analyse via MALDI than proteins of equivalent molecular weight. They often require a higher laser fluence, and the signal variability from sample to sample and from spot to spot can be quite high. These factors suggest that

homogenous co-crystallization of matrix and analyte is much more difficult to achieve than for other samples which easily show matrix suppression.

We were able to verify this for the case of PEGs by using an alternative deposition method to achieve better sample homogeneity. We prepared a normal dried-droplet MALDI PEG sample, which was placed in vacuum, and rapidly heated by passing a current through the support. The resulting thermally desorbed material was collected on another, cold MALDI tip, and then studied in the usual MALDI instrument. Using this method, full matrix suppression could sometimes be observed for PEGs as large as MW = 3500, clearly showing that intimate mixing in the solid state of matrix and analyte is a critical factor in enabling matrix suppression.

Matrix suppression using delayed extraction

It has been found that delayed extraction can lead to enhanced signal of some analytes, presumably as a result of more complete matrix-analyte reaction in the plume before ions are moved to the collision free region.[8] As noted above, the plateau region of the matrix suppression concentration curves also suggests that plume reactions can lead to reduced matrix signal. Therefore delayed extraction might be expected to extend the concentration range where suppression is observed.

This was tested for the case of DHB/valinomycin. As shown in Fig. 5 suppression under continuous extraction conditions is observed only at M/A < 10. Using delayed extraction with a delay time of 650 ns, full suppression was found at M/A up to 90. This suggests that plume reactions can indeed be significant even at near suppression concentrations. More importantly, it strongly suggests that the plume and prompt chemistry are quite similar.

UV vs IR MALDI ionization mechanisms

The model developed in Ref. 1 and used here pertains directly to UV MALDI, where electronic excitation of the matrix is achieved by single photon resonant absorption. Recently Niu *et al.*[18] have observed that IR and UV excitation of the same matrix yield similar mass spectra, which led them to suggest that IR and UV MALDI operate via a common mechanism. Given the substantial differences between IR and UV MALDI, such as the much larger threshold fluences in the IR, this conclusion could be open to criticism.

Even so, their results might be interpreted as calling into question the role of electronic excitations in UV MALDI, since they are *presumed* not to play a role in IR MALDI.

We find that this presumption is premature. We irradiated DHB and sinapinic acid MALDI samples on an IR-transparent glass slide with 1064 nm light from a Nd:YAG laser. Fluences were in the range 10^7–10^8 W/cm^2, where IR MALDI typically is performed. Both samples absorbed a substantial fraction of the laser light, as indicated by the reduction in emission from an IR-card behind the sample. From DHB we observed a strong blue emission, typical of the fluorescence which is found with UV excitation. For sinapinic acid, the emission was weaker, but still apparent to the naked eye. Again the fluorescence was comparable in intensity and colour to that found with tens of microjoules of UV excitation.

This experiment is not identical to that of Niu *et al.,* in that we used 1 μm rather than 2.9 μm excitation. This means that fewer photons are required to reach an electronic excited state. However, it should be recalled that at equivalent irradiance, photon density is inversely related to photon energy. In other words 3 μm light has about three times the photon density of 1 μm light at the same irradiance. Ladder-climbing resonant multiphoton excita- tion to high levels seems then quite possible, with subsequent crossing to electronically excited states. Also, it has yet to be established whether rapid crystal destruction plays a role in MALDI ionization.[19] If so, non-photoinduced electronic excitation is possible. Such triboluminescence has long been known,[20] the emission of methyl salicylate (mint flavour) in a sugar candy matrix is an example familiar to many. DHB is also a salicylate, specifically the 5-hydroxy derivative of salicylic acid.

Conclusions

The matrix suppression effect has been demonstrated in negative-as well as positive-ion MALDI mass spectra. The matrices tested show suppression in only one polarity or not at all. In those cases where suppression is observed, the matrix to analyte signal ratios follow a typical curve with increasing analyte concentration. The curve and the extended concentration range for suppression using delayed extraction suggest that gas-phase reactions continue in the desorption plume at high M/A. At low M/A the primary ions are depleted before significant plume expansion occurs. The analyte concentrations where suppression is observed are consistent with the proximity requirement of Ref. 1. Poor co-crystallization may be a common reason for lack of suppression, as was demonstrated for PEGs in DHB and AMP in sinapinic acid. Suppression may also be rare with certain matrices, such as 4NA and AHP, due to unfavour- able thermodynamics. The possibility that IR and UV MALDI rely on the same primary ions was demonstrated by observation of IR-induced fluorescence from DHB and sinapinic acid samples.

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Figures

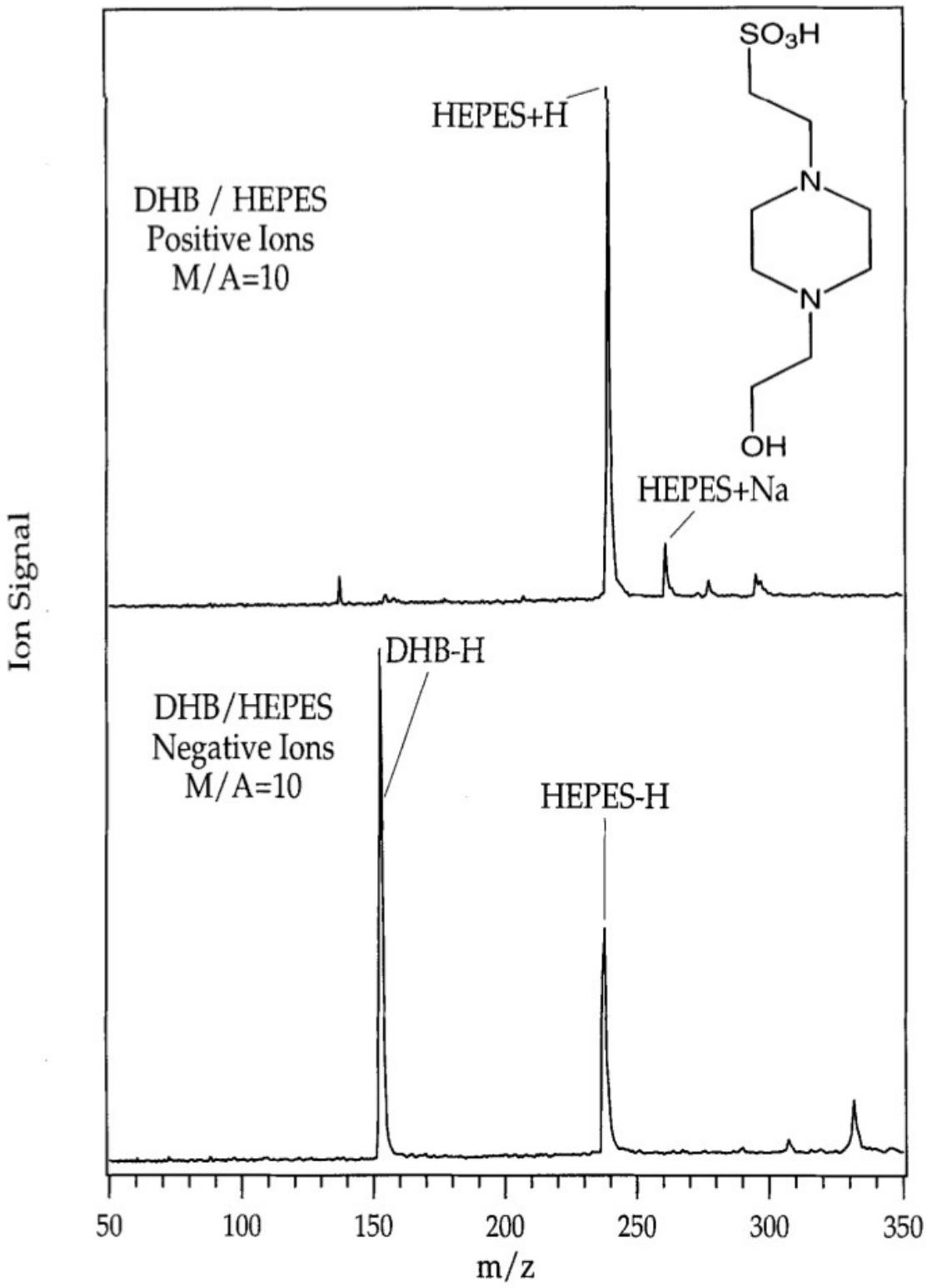

Figure 1. Matrix suppression by HEPES in DHB. Although HEPES is normally observed more strongly as a negative ion, it only suppresses DHB matrix signals in positive-ion mode. The polarity in which matrix suppression occurs is thus determined both by the matrix and the analyte. The structure of HEPES is shown in the inset.

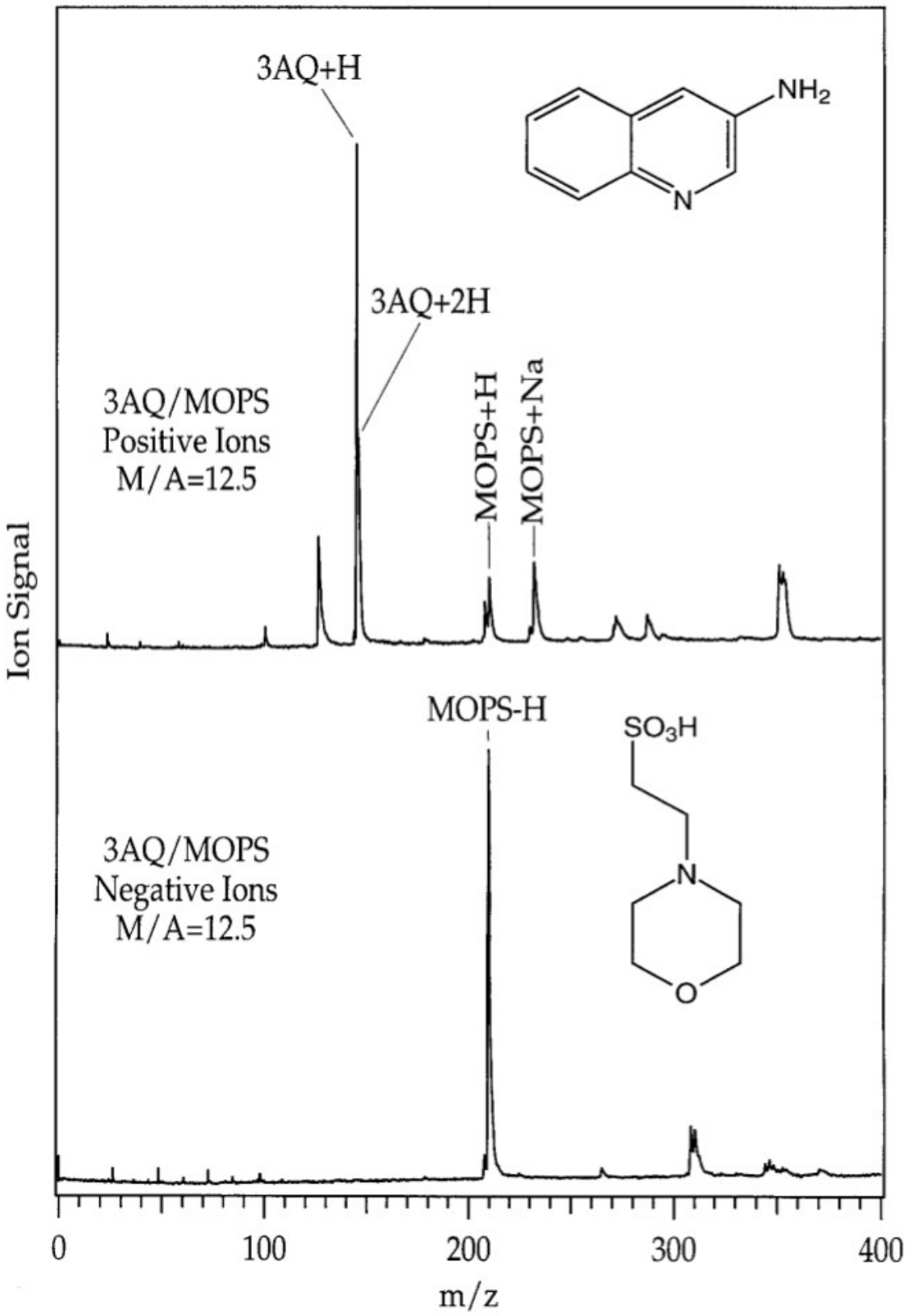

Figure 2. Positive and negative MALDI mass spectra of MOPS in 3-aminoquinoline, MW = 209. Full matrix suppression is observed in negative polarity, but strong matrix signals are found in the positive-ion spectrum. The upper structure is 3-aminoquinoline. The lower structure is MOPS.

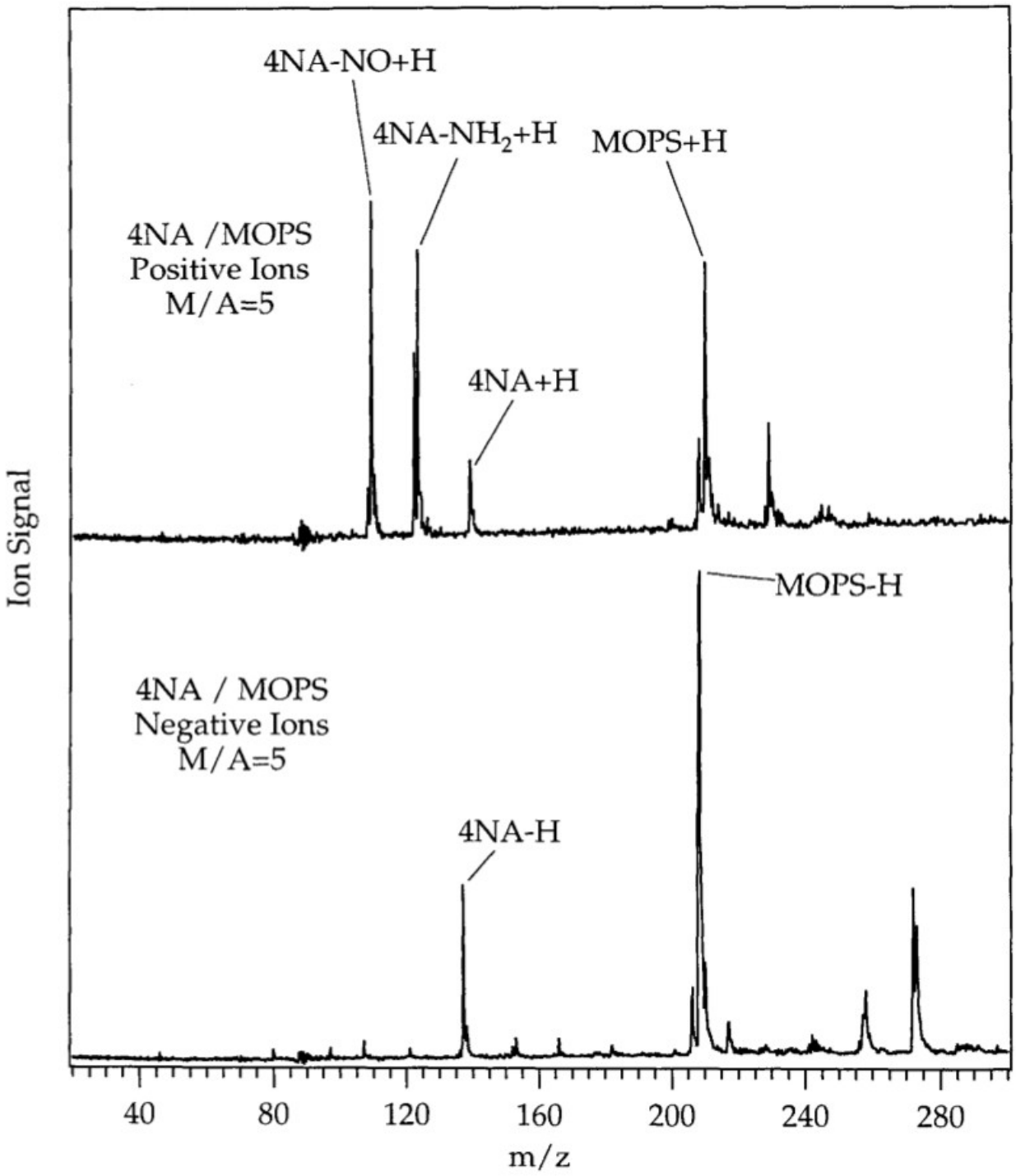

Figure 3. Positive and negative MALDI mass spectra of MOPS in 4-nitroaniline. At this matrix/analyte ratio of 5, matrix suppression would be expected, if it is thermodynamically possible. Instead, matrix signals are substantial in both polarities.

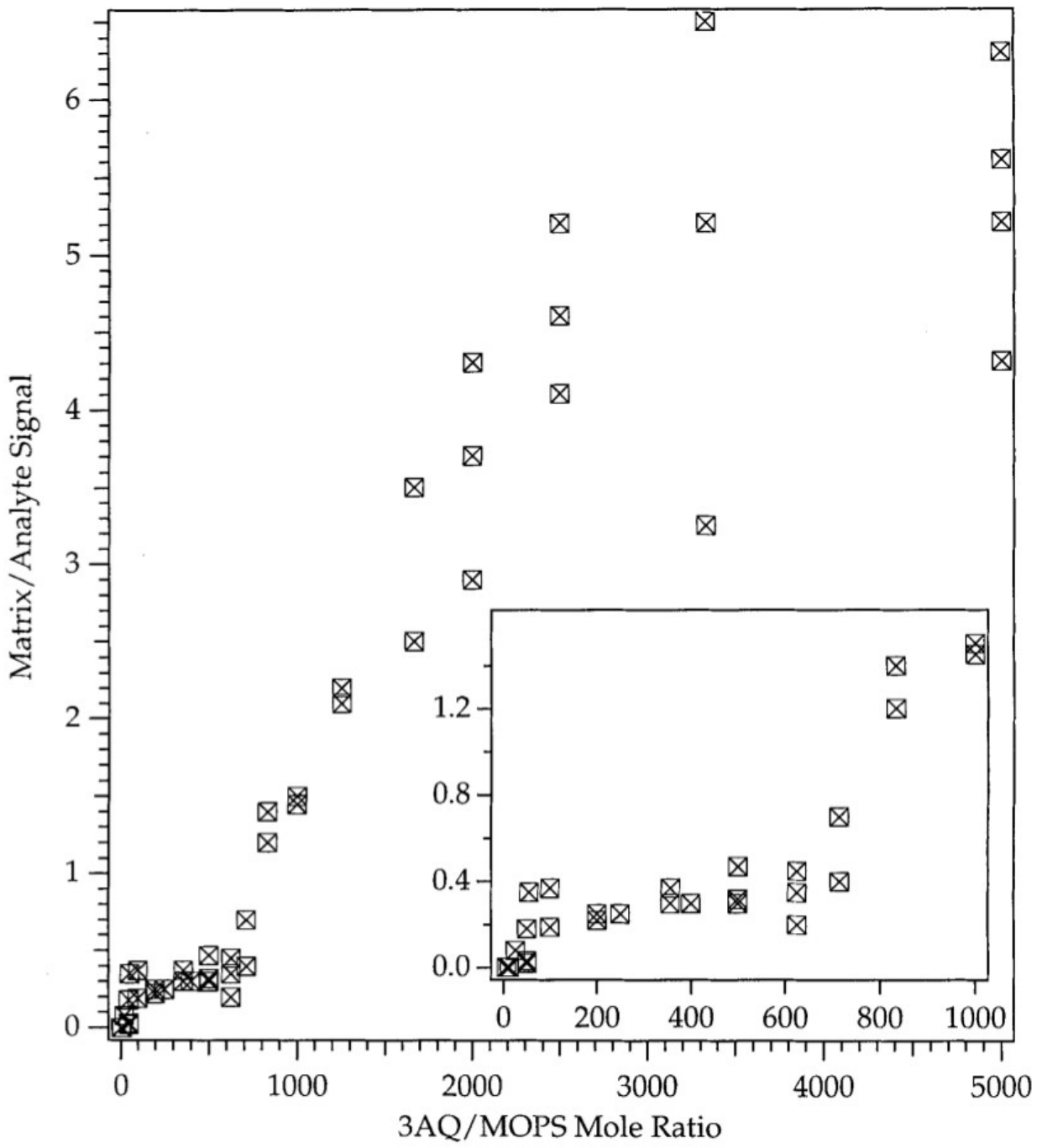

Figure 4. Matrix to analyte ion intensity ratios (negative ions) versus the matrix to analyte mole ratio in the sample. The matrix was 3-aminoquinoline and the analyte was MOPS. The prominent drop at M/ A = 1000 to a plateau is followed at M/A < 50 by a final drop to full matrix suppression. Each point represents a single measurement. The inset shows the low M/A region with an expanded scale.

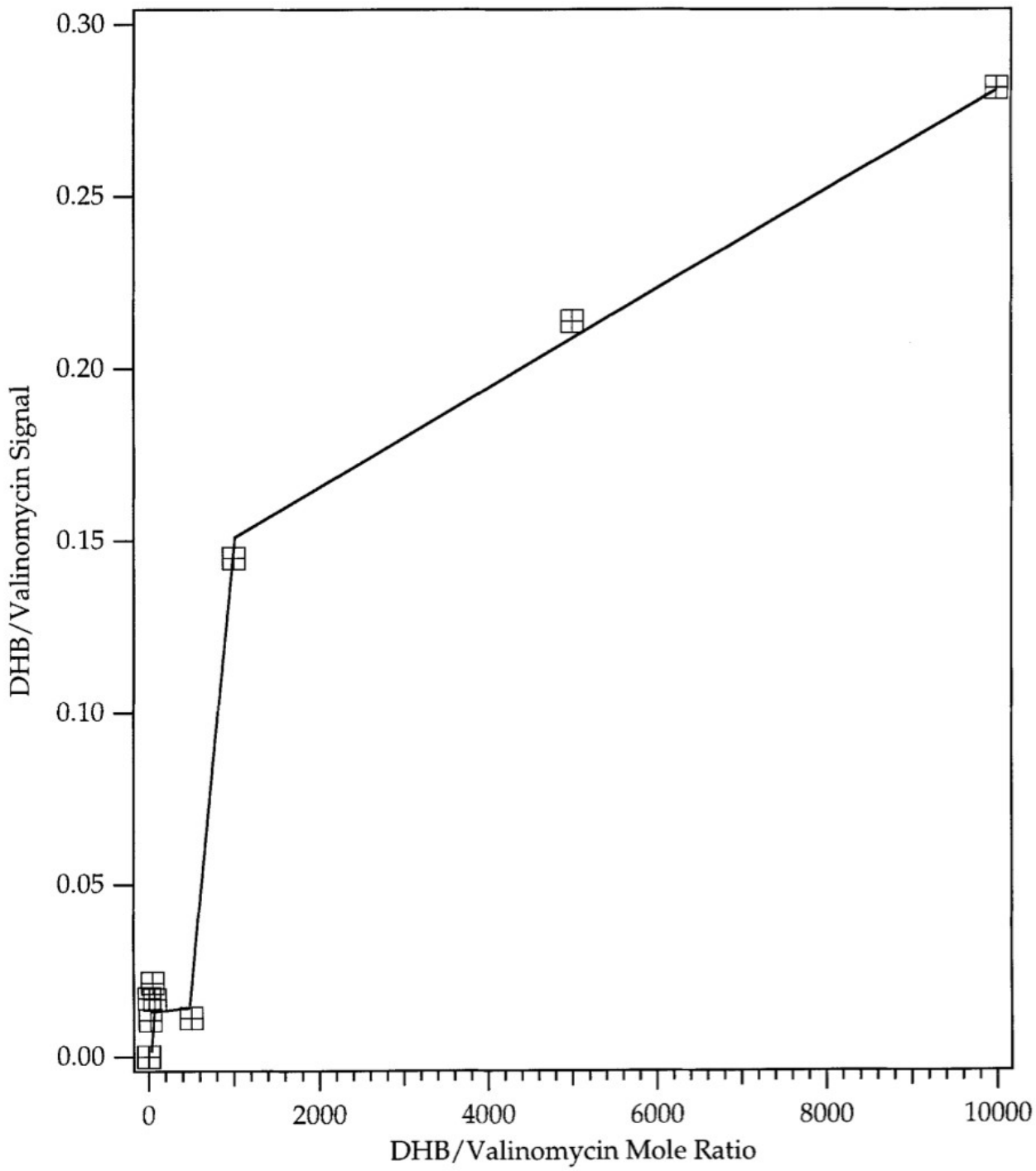

Figure 5. Matrix to analyte ion intensity ratios (positive ions) versus the matrix to analyte mole ratio in the sample. The matrix was DHB and the analyte was valinomycin. This system also shows a drop to a plateau by a final decrease to full matrix suppression. Each point represents the average of 3 measurements. The lines are drawn to guide the eye.

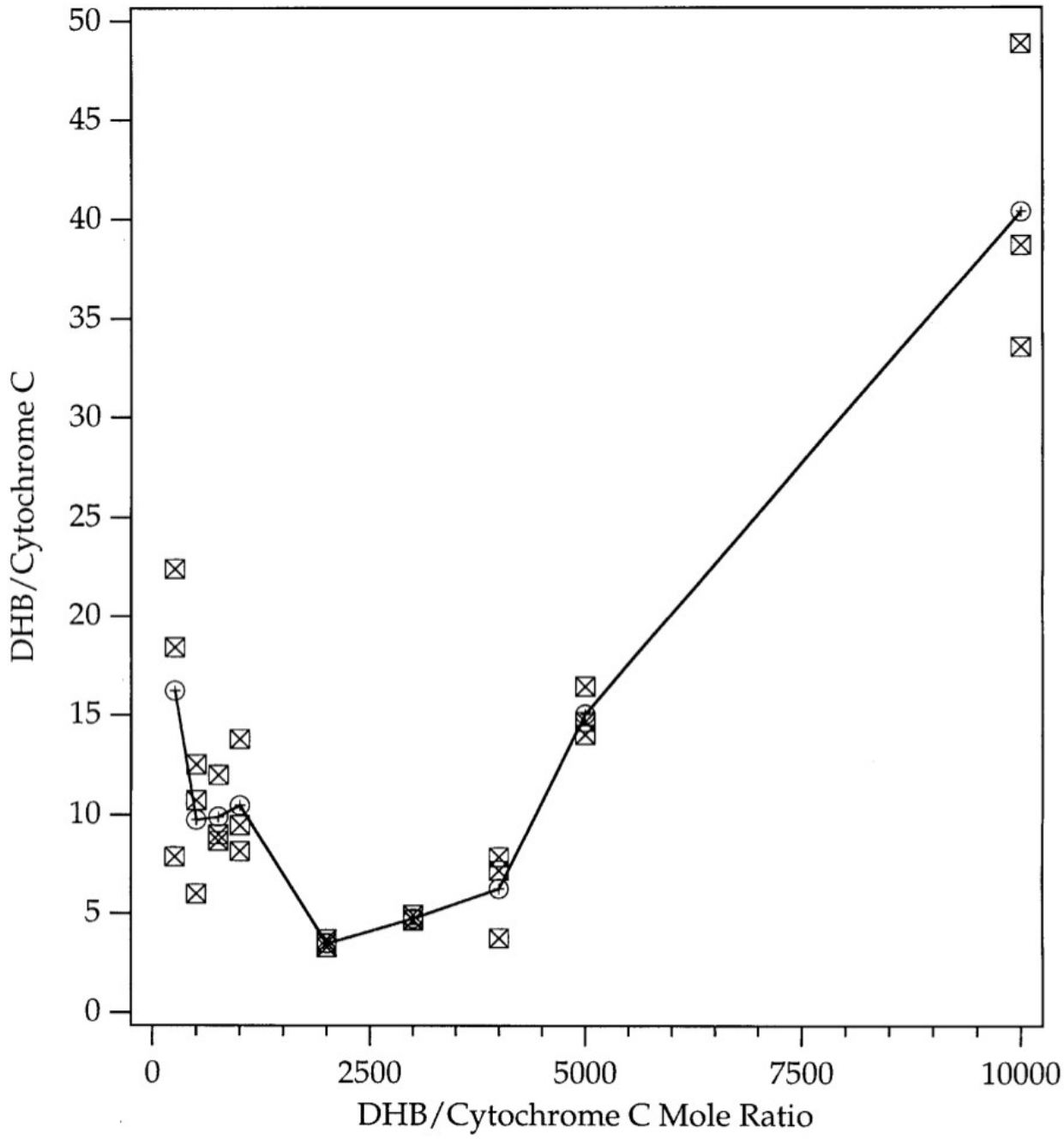

Figure 6. Matrix to analyte ion intensity ratios (positive ions) versus the matrix to analyte mole ratio in the sample. The matrix was DHB and the analyte was cytochrome C. The averages of the individual measurements are connected with lines to guide the eye, otherwise the points are single measurements. There is a strong decrease in relative matrix signal between M/A = 2000 and 4000, but full suppression was not observed. The increase in matrix signal and data scatter at low M/A is attributed to poor co-crystallization of matrix and analyte.

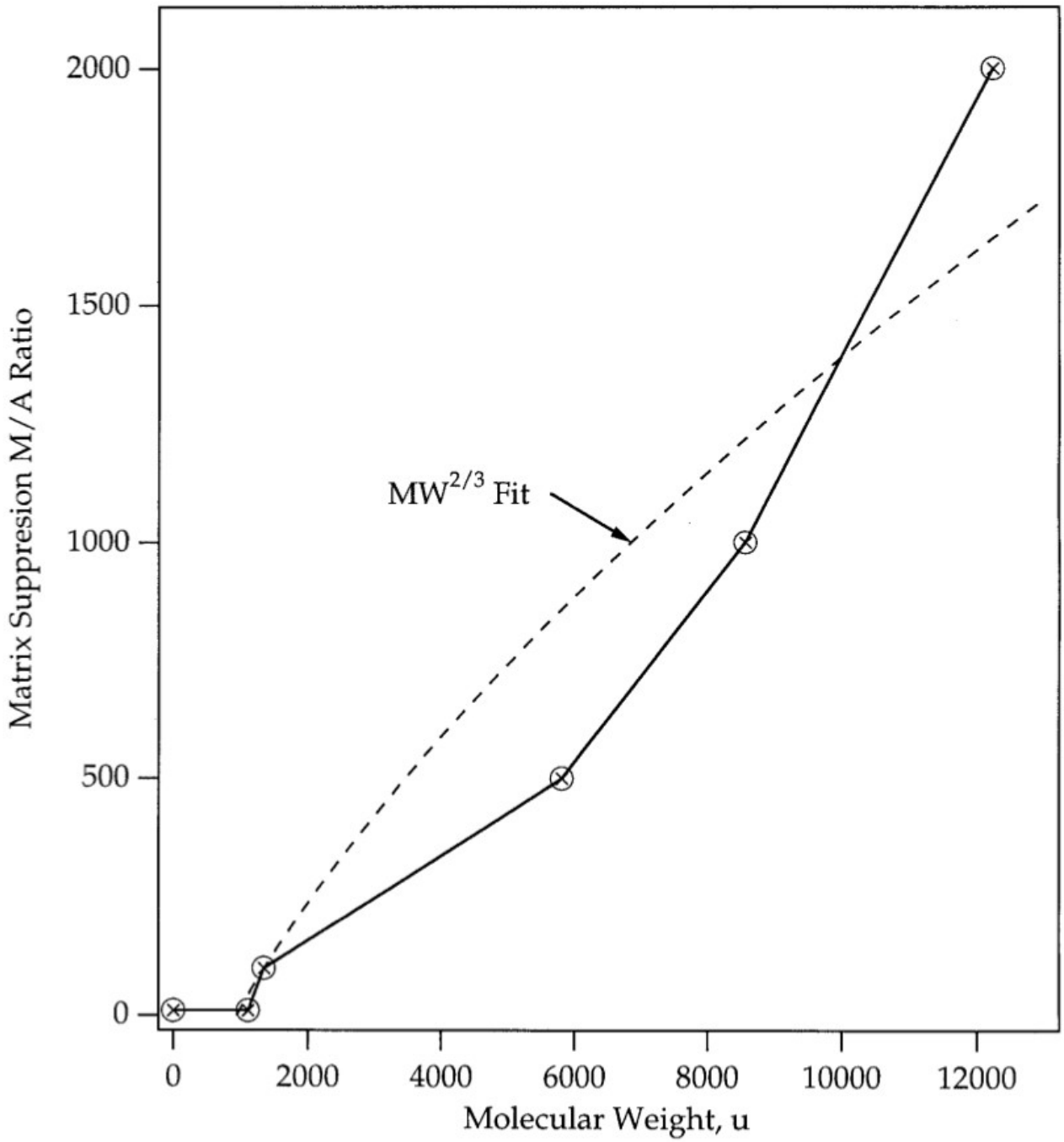

Figure 7. Positive ion M/A ratios for either minimum matrix signal or appearance of full matrix suppression, versus analyte mass. The matrix was DHB and the analytes were HEPES, valinomycin, substance P, human insulin, ubiquitin and cytochrome C.

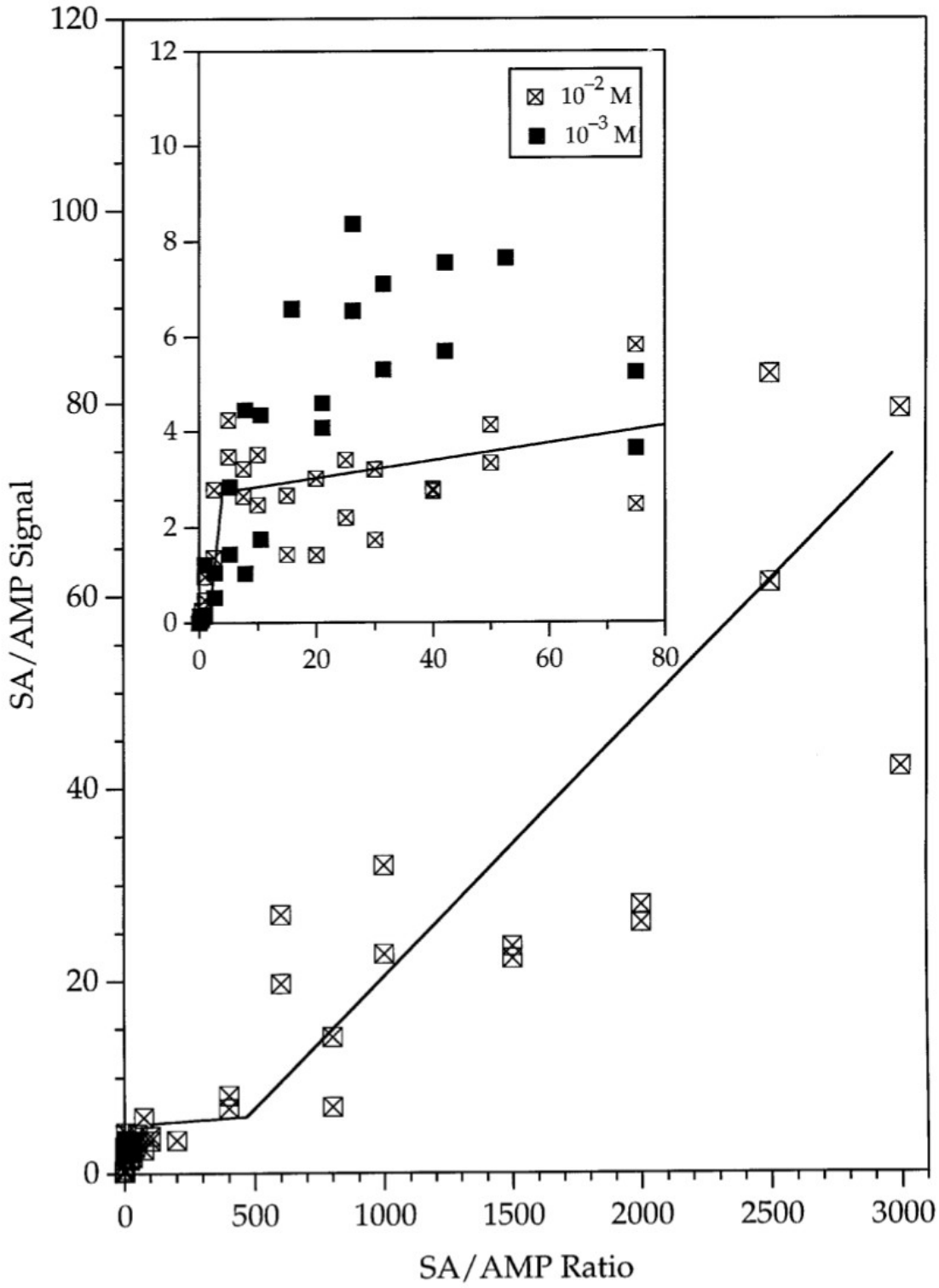

Figure 8. Matrix to analyte ion intensity ratios (negative ions) versus the matrix to analyte mole ratio in the sample. The matrix was sinapinic acid and the analyte was AMP. Each point represents a single measurement. The inset shows the low M/A region with an expanded scale, for two measurement series that differed only in the sample preparation methods. These series differed in the concentration of the matrix solution, as indicated. The analyte solutions were also adjusted in concentration, so as to use similar volumes in each case. The lines are drawn as an approximate fit, to guide the eye.