Solvent Effect on Analyte Charge State, Signal Intensity, and Stability in Negative Ion Electrospray Mass Spectrometry; Implications for the Mechanism of Negative Ion Formation

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The effect of solvent composition on negative ion electrospray ionization (ESI) mass spectrometry was examined. The onset potentials for ESI of a series of chlorinated solvents and methanol were found to be within the range predicted by D. P. H. Smith, based on differences in the surface tension of the solvents used. The tendency toward electric discharge decreased with increasing percent weight of chlorine in the solvent. This effect has been attributed to an increasing propensity for electron capture for more highly chlorinated solvents. Addition of the electron scavenger gas SF_6 was even more effective at suppressing corona discharge phenomena. In a comparison of ultimate signal intensity obtainable for a test analyte in 10% methanol, the highest signal, which was stable over the widest range of temperatures, was exhibited by chloroform compared to dichloromethane, 1,2-dichloroethane, carbon tetrachloride, and methanol (100%). Chloroform, thus, is a recommended solvent for negative ion electrospray mass spectrometry (ES/MS) when solubility is not a limiting issue. Solvent polarity was shown to exhibit a profound influence on the distribution of charge states in negative ion ES/MS. For both chlorinated and nonchlorinated organic solvents, the higher the solution dielectric constant, the more the charge-state distribution is shifted toward higher charge states. These observations build on the "electrophoretic" mechanism of droplet charging. Solvents with high solution dielectric constants are considered to be most effective at stabilizing multiply charged ions (where charge separation is greatest), and they are likely to increase the level of droplet charging. Solvents with high basicities (gas phase and solution phase) and high proton affinities, yet low dielectric constants, favor lower charge states in ES mass spectra of lipid A and cardiolipin from *Escherichia coli*. This indicates that gas-phase processes and solvent basicity contribute much less toward ion formation than solution-phase solvation via preferred orientation of the solvent dipole. *(J Am Soc Mass Spectrom 1993, 4, 546–556)*

Use of electrospray mass spectrometry (ES/MS) has undergone tremendous growth in recent years [1, 2]. Development of the ES/MS technique, along with the concurrent introduction of matrix-assisted laser desorption mass spectrometry [3], has enlarged the possibilities for analyses of compounds of high molecular weight (e.g., > 10,000 Da) that were largely inaccessible by other mass spectrometric ionization techniques. This new capability for macromolecular analysis has spurred interest from many research disciplines, particularly those partaking in biological analyses. The scientific literature documents a widening array of compound types that have been successfully ionized via ES/MS. The unique ability of the ES process to readily produce ions of high charge states distinguishes the technique from other mass spectrometric ionization methods.

In addition to increasing the applicability of the method, major interest lies in achieving a better comprehension of fundamental aspects of the ES process. This includes improving the understanding of the mechanism of ionization as well as the influence of experimental parameters on the character and yields of ions that are produced. The ES process can be thought of as occurring in two steps, that is, charged-droplet formation and ion desorption into the gas phase. Droplet charging is initiated by the appearance of the Taylor cone [4] that is formed in response to the introduction of liquid into the imposed electric field. Under appropriate conditions of liquid flow and applied potential, the cone tip extends out to form a thin liquid "filament" [5–7], which breaks up to form individual charged droplets. Solution conductivity plays a major role in the electrostatic disruption of liquids.

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Solutions of intermediate conductivity can produce the most stable ES "jets." This is because sufficient free charges are available in the bulk liquid to allow electrostatic force to build up on the surface (not always possible with insulating liquids), yet conductivity is not so high that electric discharge sets in at higher potentials [6].

Several mechanisms of ion desorption from charged droplets have been proposed, starting with the "charged-residue" model of Dole et al. [8], wherein the ES process produces droplets containing single solvated solute macromolecules that accumulate charge as the final solvent molecules are evaporated. Another view of the process was set forth in the "ion evaporation" mechanism of Iribarne and Thomson [9, 10]. This model proposes that depletion of solvent from charged droplets via evaporation leads to Rayleigh instability of droplets with critically high ratios of surface charge density-to-droplet radius. The electrostatic energy associated with the charged droplet thus becomes large enough to desorb charged analyte ions into the gas phase.

Subsequent investigations have found inconsistencies with each model. Notably, Röllgen et al. [11] contend that the rate of ion evaporation during solvent evaporation is not sufficiently high to account for the totality of the ES ion current. Meng and Fenn [12] argue that their observations of significant effects of cosolutes on the distributions of cluster ions in the gas phase are inconsistent with the charged-residue model because ions formed according to this model would be expected to be relatively insensitive to certain properties of cosolutes. Clearly, the mechanism of ionization occurring during the ES process is still undergoing elucidation.

Recently, a few researchers have been successful in relating information found in ES mass spectra to solution-phase processes. Chait and co-workers [13] showed that changes in the conformation of small proteins can be reflected in the charge-state distribution of multiply charged species observed in the positive ion ES mass spectrum. The shift in charge-state distribution toward higher values for low-pH solutions was attributed to an increased ability to protonate denatured (unfolded) proteins. It was argued that a "tight" protein conformation can accept far fewer protons. Smith and coworkers [14] observed a rough correlation between the number of basic residues on a polypeptide and the maximum number of positive charges observed in the ES mass spectrum. Later, negative ion ES/MS work by the same group [15] showed the charge-state distribution of certain analytes in aqueous systems to be dependent on solution basicity. Meng and Fenn [12] deduced that the amount of clustering in various solvents increases as the solubility of the analyte in the solvent decreases.

Reports of negative ion applications of ES/MS have so far been the exception rather than the rule. Negative ion ES experiments pose certain problems that are less severe for positive ion experiments. A major limitation is that negative ion ES is more susceptible to electric (corona) discharge phenomena than are positive ion experiments. This increased propensity toward electric discharge arises from electrons that emanate from the sharp edges of the ES capillary (where the charge density is highest and the work function is lowest), or from the drop surface itself (for conducting liquids because the surface is at nearly the same potential as the metal) [6]. A practical outcome of this increased tendency toward corona discharge is that stable ES currents are more difficult to achieve.

To combat the instability created by corona discharges, researchers have modified experimental conditions. One approach used in early negative ion ES/MS experiments by Yamashita and Fenn [16] was to introduce O_2 gas to act as an electron scavenger and thus suppress the corona discharge phenomenon. Kebarle and co-workers [17] demonstrated an improved electric discharge suppression capability by using SF_6, a gas having a higher electron affinity than oxygen. A different approach to reducing the corona discharge problem was taken by Chowdhury and Chait [18] who used sharpened ES capillary tips to reduce the onset potential for positive ion ES of aqueous solutions. If these specially modified ES capillaries were not used, the high conductivity and high surface tension of the aqueous solutions led to corona discharge before stable ES currents could be achieved.

In the present work, several series of related solvents were used to gain insight into how solvent properties influence fundamental aspects of the ES process in the negative ion mode. Specific investigation was made into relative onset potentials for a series of chlorinated solvents, in accordance with the predictions of Smith [5]. In addition, the onset of discharge was compared for this same series of solvents. The effect of temperature on the signal intensity and stability of a test analyte as a function of solvent was also evaluated. Last, the influence of solvents, both chlorinated and nonchlorinated, in determining the predominate charge state of test analytes was evaluated and rationalized. Implications from this latter study pertaining to the mechanism of ion formation were examined. These studies build directly on our first report of solvent effects in negative ion ES/MS [19].

Experimental

All mass spectrometry experiments were performed on a Vestec 201 ES mass spectrometer (Vestec Corporation, Houston, TX). The instrument ion source was described in detail in a previous publication [20]. For each series of experiments, solutions containing identical quantities of the test compounds were infused at a constant flow rate of 2 μL/min using a Sage syringe pump (Orion Research, Boston, MA). To minimize concentration differences between samples and to ensure complete dissolution of all samples, test analytes

were initially dissolved in 1.0-mg/mL quantities in suitable solvents (i.e., methanol for the signal stability and signal intensity studies; chloroform for the charge-state studies). An aliquot (one part) of these initially prepared solutions was diluted with nine parts of the particular solvent under investigation, making the final concentration of all solutions 0.10 mg/mL.

For each solvent series tested, experimental conditions were held rigorously constant. This includes ES chamber temperature (excluding the signal intensity study), the distance between the ES capillary (needle) and the converging nozzle (counterelectrode), as well as the nozzle, skimmer, and collimator voltages. Only the voltage applied to the ES capillary was varied slightly from run to run, to maintain the most stable, intense signal possible.

Literature values for the surface tensions of various solvents were obtained from ref 21. Dielectric constants at various temperatures, reported as ϵ (°C), were calculated for each solvent by using literature values and equations found in ref 22. The gas-phase basicities and proton affinities (PA), both in kilojoules per mole, were found in a compendium entitled "Evaluated Gas Phase Basicities and Proton Affinities of Molecules; Heats of Formation of Protonated Molecules" [23].

Dipalmitoyl-L-α-phosphatidic acid, diphosphoryl lipid A, and cardiolipin were purchased from Sigma Chemical Co. (St. Louis, MO). High-performance liquid chromatography-grade solvents were obtained from EM Science (Gibbstown, NJ). Sulfur hexafluoride was purchased from Air Products (Tamaqua, PA).

Results and Discussion

Signal Stability

The investigation of solvent effects in negative ion ES/MS begins with a comparison of the voltages required for the onset of electrospray. Smith [5] offered a qualitative model to describe the form of the dependence of the onset potential (V_0):

$$V_0 \alpha \left[\frac{2Tr_c \cos \theta_0}{e_0}\right]^{1/2} \ln\left(\frac{4h}{r_c}\right) \quad (1)$$

where T is the surface tension, r_c the ES capillary radius, θ_0 the half-angle of the Taylor cone, e_0 the permittivity of free space, and h the capillary-to-counterelectrode distance. The applicability of eq 1 was tested by Kebarle and co-workers [17] for methanol and water, and it was found to be quite accurate in predicting the onset voltages for these two solvents.

According to eq 1, if the ES capillary radius and the distance between the capillary and the counterelectrode are held constant, the voltage required to achieve onset of ES should be proportional to the square root of the surface tension of the solution passing through the capillary. Solutions consisted of a test analyte, phosphatidic acid, dissolved in 10% chloroform, and 90% of one of the solvents in the following series: methanol, 1,2-dichloroethane, dichloromethane, chloroform, and carbon tetrachloride. The "onset of electrospray" was defined as the first appearance of $[M - H]^-$ (m/z 648) above the noise background (signal-to-noise ratio ≥ 3); obtained onset voltage values are plotted in Figure 1a. The difference in onset voltages would be expected to be largest for pure solvents. Of the solvents listed above, the highest surface tensions are those of chloroform and carbon tetrachloride (each 27.0 dyne/cm, 20 °C), whereas the lowest is that of methanol (22.61 dyne/cm, 20 °C) [21]. This implies that under constant experimental conditions, according to eq 1, the ratio of onset voltages between chloroform and methanol, or carbon tetrachloride and methanol,

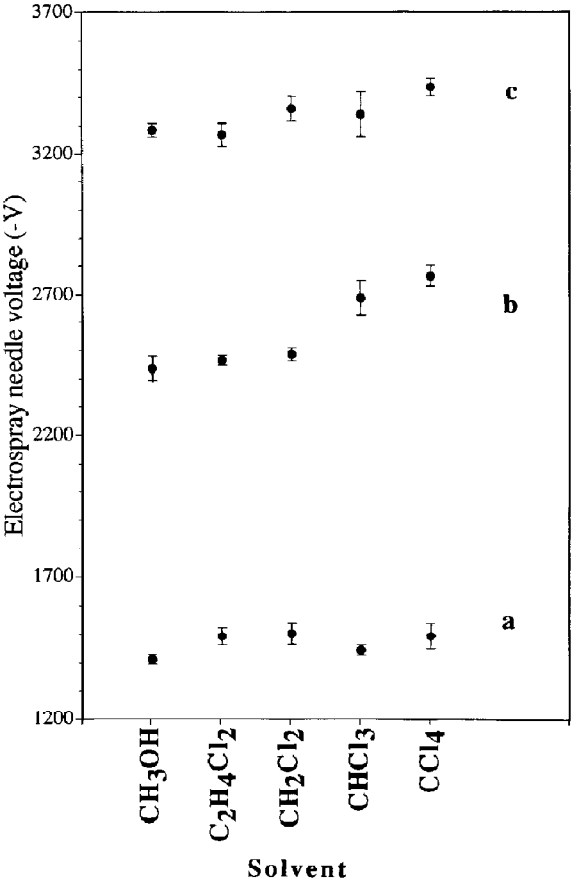


Figure 1. For dipalmitoyl-L-α-phosphatidic acid in 10% methanol and 90% of each solvent listed on the abscissa, ES capillary voltage versus (**a**) onset voltage, (**b**) corona discharge onset, and (**c**) corona discharge onset in the presence of SF_6. At 20 °C the surface tension (T) of methanol is 22.6 dyne/cm; for dichloromethane, T = 26.0 dyne/cm; for 1,2-dichloroethane, T(35°C) = 23.4 dyne/cm; for chloroform, T = 27.0 dyne/cm; and for carbon tetrachloride, T = 27.0 dyne/cm [21].

should be equal to the square root of the surface tension ratio, $(27.0/22.6)^{1/2} = 1.09$. Because each solution contained 10% chloroform, the onset voltages for all solvents are expected to fall within 9% of one another. Indeed, the range of observed onset voltages was from −1413 to −1504 V (i.e., 6.4% difference between the highest and lowest value). This small difference in onset voltages for the solutions under investigation is consistent with what would be predicted from Smith's description [5] of the dependence of onset voltage on surface tension. The relative standard deviation for each onset voltage determination was less than 3.1%.

Using the same series of solutions, we made a comparison of the voltage corresponding to the onset of discharge. This "onset of discharge" was arbitrarily defined as the minimum voltage that yields a total current of 2000 nA at the converging nozzle counterelectrode (actually at this current, discharge is quite prominent). The data in Figure 1b indicate a clear trend, showing an increase in the voltage leading to corona-discharge conditions with increasing chlorine content (e.g., percent weight Cl) of the solvent. The effect is most dramatic for carbon tetrachloride and chloroform. These data can be rationalized on the basis of the high electron-capturing ability of chlorine atoms owing to their high electron affinity relative to the other elements comprising the solvents. A higher field strength at the ES capillary exit leads to higher total ES currents [17]. Higher analyte signal intensities can also be achieved as a result of higher field strengths if adverse space-charge effects, which occur as a result of corona discharge processes, are reduced. This implies that the more highly chlorinated solvents can potentially offer higher analyte signal strengths. In addition, a lower propensity toward gas-phase breakdown via corona discharge for these same highly chlorinated solvents will offer increased local field uniformity to maintain a stable Taylor cone and thus offer greater constancy to generated ion signals.

An alternative means to suppress electric discharge is by the addition of gases with high electron affinities to the "bath gas" of the ES chamber. This approach was used in early negative ion ES/MS experiments [16], where oxygen gas was reported to provide increased stability relative to ambient air. More recent ES/MS experiments [17] document the use of SF_6 gas as a more efficient electron scavenger to suppress discharges in positive ion ES/MS. To test the efficacy of this latter approach in negative ion experiments, SF_6 was added at approximately 40 mL/min to the bath gas. The same sample solutions used to generate the data in Figure 1a and b were run again in the presence of SF_6. The onset of discharge was measured in a manner analogous to that used in generating Figure 1b; results are plotted in Figure 1c. The large increase in ES capillary voltage, which can be reached prior to the onset of discharge when SF_6 is added to the bath gas, serves to reinforce the notion brought forth by Kebarle and co-workers [17] that addition of this gas can improve stability in ES operation. A more subtle increase in the onset of discharge voltage appears to still be present in moving from left to right in Figure 1c, indicating a somewhat masked contribution to discharge suppression from the chlorinated solvents, even in the presence of SF_6. The random error associated with the measurements prohibits a more definite statement in this regard.

Signal Intensity

To test the actual sensitivity obtainable for the test analyte, phosphatidic acid, the absolute abundance of the $[M - H]^-$ ion (m/z 648) was monitored as a function of ES chamber temperature (as indicated by thermocouple reading) for the same series of chlorinated solvents (actually, 9:1 v/v, chlorinated solvent/methanol) and methanol (100%). The temperature was varied by adjusting the water flow rate through the ES chamber cooling jacket. All other experimental parameters were held constant except for the ES capillary voltage, which was optimized in each case. A maximum $[M - H]^-$ abundance was observed in the range 45–50 °C for each solvent (Figure 2).

Chloroform repeatedly yielded the highest analyte $[M - H]^-$ current at the detector. In addition, even though sensitivity was observed to drop off above 50 °C, the excellent ES stability afforded by the chloroform solvent allowed reliable observation of signal at the detector, even at chamber temperatures as high as 75 °C. None of the other solvents exhibited comparable levels of sensitivity or stability. Use of dichloromethane did afford relatively high signals, although the maximum ion abundance appeared at the lowest temperature of any solvent used. Moreover, it was the least stable solvent at higher temperatures, and beyond 65

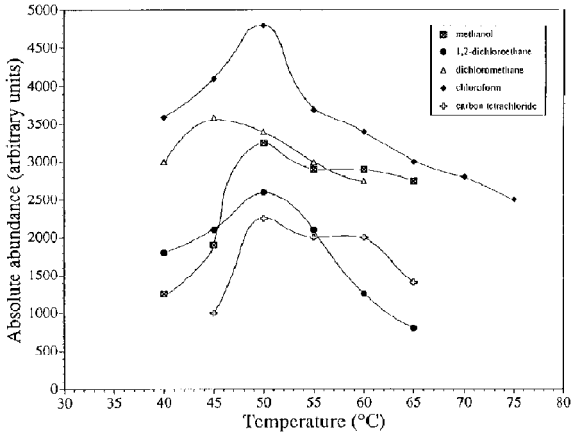
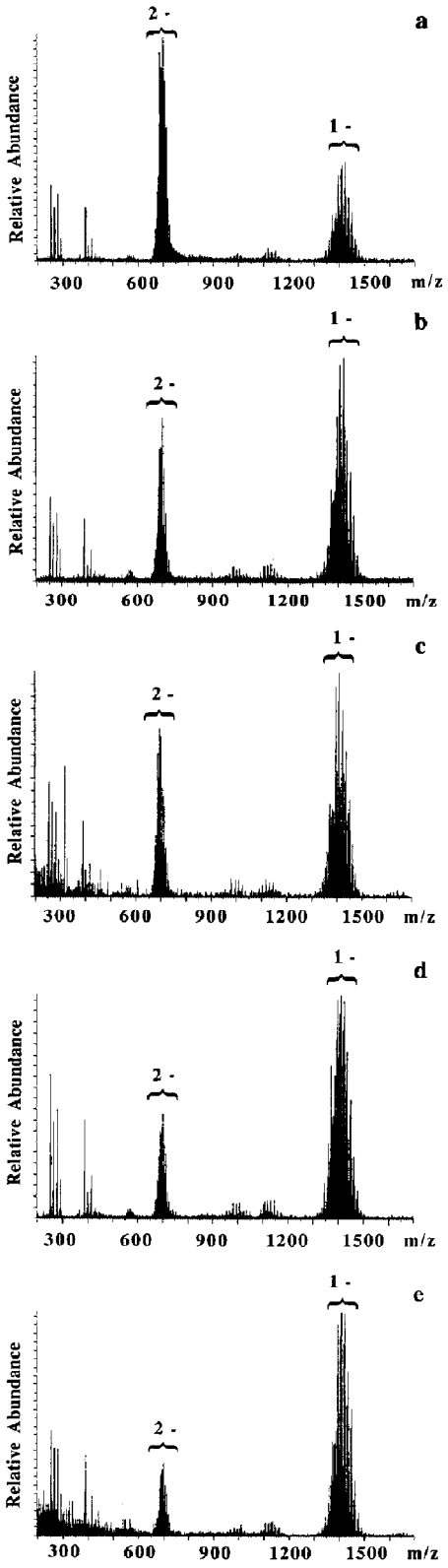


Figure 2. Signal intensity versus ES chamber temperature for $[M - H]^-$ (m/z 648) of dipalmitoyl-L-α-phosphatidic acid in 10% methanol and 90% of each listed solvent. Lines linking the points are meant only to serve as a visual guide; no implication concerning graphic fine structure is intended.



°C reproducible signals could not be obtained. The latter two phenomena can be rationalized by considering that dichloromethane exhibits the lowest boiling point (40 °C) of any of the solvents. As the chamber temperature was raised above the boiling point, the solvent may have begun to evaporate prior to reaching the tip of the stainless steel capillary, leading to a loss in ionization efficiency and ultimately in a complete breakdown of ES stability.

The remaining solvents exhibited even lower ultimate signals, decreasing in order of methanol, 1,2-dichloroethane, and, last, carbon tetrachloride. Each of these three solvents exhibited a maximum $[M - H]^-$ signal at a chamber temperature near 50 °C. Methanol exhibited very good stability up to 65 °C, as did carbon tetrachloride. Compared with these two solvents, 1,2-dichloroethane sensitivity was found to drop off rather precipitously above 50 °C, although stability at 65 °C was still adequate to detect a low-level signal. Of the solvents used, 90% chloroform with 10% methanol repeatedly exhibited the best sensitivity and stability characteristics for the test analyte. It is recommended for use as a solvent for negative ion ES/MS when analyte solubility is sufficient.

Distribution of Charge States

To investigate the influence that the solvent exerts on the charge state of anionic analytes in negative ion ES/MS, another test compound was used. The sodium salt form of cardiolipin from *Escherichia coli* was chosen owing to its excellent solubility in chloroform and because mass spectra were anticipated to be uncomplicated and readily interpretable because the compound contains two easily ionizable phosphate groups. To one part chloroform solution was added nine parts of each solvent in the same series. In the negative ion mass spectra (Figure 3a–e), abundant peaks corresponding to doubly and singly charged ions were observed. The cardiolipin, in fact, was found to consist of a mixture of compounds, each having the general form shown in Figure 4. A distribution of chain lengths existed for the ester-linked side chains (R_1, R_2, R_3, and R_4), however, resulting in the appearance of symmetric distributions of ions spaced 14 mass-to-charge ratio units apart (singly charged ions) and 7 mass-to-charge ratio units apart (doubly charged ions).

For a given charge state, the distribution of intact cardiolipin ions was quite similar for all solvents tested. The ratio of doubly to singly charged species, however, was observed to change dramatically depending on the solvent used. Because the cardiolipin has a variety of chain lengths, this feature was exploited to allow improved precision in estimating the ratios of

Figure 3. Negative ion ES mass spectra (54 °C) of the disodium salt of cardiolipin dissolved in 10% chloroform and 90% of (a) methanol, (b) dichloromethane, (c) 1,2-dichloroethane, (d) chloroform, and (e) carbon tetrachloride.

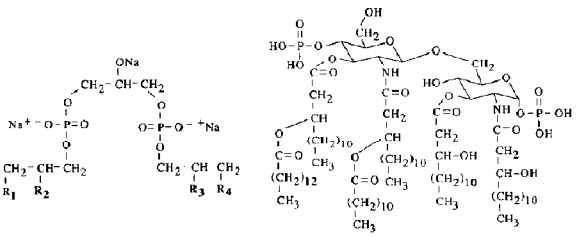


Figure 4. Structure of test compounds: cardiolipin (*left*) and diphosphoryl lipid A (*right*) from *E. coli*. The side chains (R_1, R_2, R_3, and R_4) on cardiolipin represent ester-linked long-chain fatty acids of varying length. Typically, each side chain will contain approximately 16 carbons.

doubly to singly charged ions. Because the distributions of ions of a given charge state arising from all solvents were so similar, any differences in solubility due to slightly different chain length (e.g., 17 instead of 16 $—CH_2—$ units on one of four long hydrophobic chains) were considered to have a negligible effect on the charge-state distribution. In each case, the particular variety of cardiolipin producing the most abundant singly charged ion also exhibited the most abundant doubly charged species. The ratio of abundance of doubly charged ions to that of singly charged ions was calculated for this pair, as well as for the pairs corresponding to homologous species deficient and excessive in one and two $—CH_2—$ units, resulting in a total of five measurements. The average ratio of doubly to singly charged species was calculated along with standard deviations for the five measurements; results are plotted in Figure 5.

A clear trend revealing a decreased tendency toward formation of ions having the higher (2 −) charge state with decreasing solvent polarity is evident. The solvents listed on the abscissa in Figure 5 are arranged in decreasing order of polarity, as given by the solvent dielectric constant. These results are consistent with earlier results [19], which first established that the choice of solvent could affect the charge-state distribution of analyte species. In the previously described experiment monitoring the deprotonation of acidic sites on diphosphoryl lipid A from *E. coli* (Figure 4), the ratio of charge states (2 −/1 −) using dichloromethane was slightly inferior to that obtained using 1,2-dichloroethane. In the current example, the two are virtually indistinguishable (Figures 3 and 5). The observed trend for chlorinated solvents correlating higher charge states with higher solvent polarity may be rationalized on the basis of charge stabilization arguments, which follow. Such chlorinated solvents, however, cannot offer significant stabilization to positive counterions via lone-pair donation. Stabilization of ions must arise via preferred orientation of solvent.

To investigate whether a similar trend could be observed for nonchlorinated solvents that bear electron-donating groups, three solvents listed in order of decreasing dielectric constant (i.e., acetonitrile, ethanol,

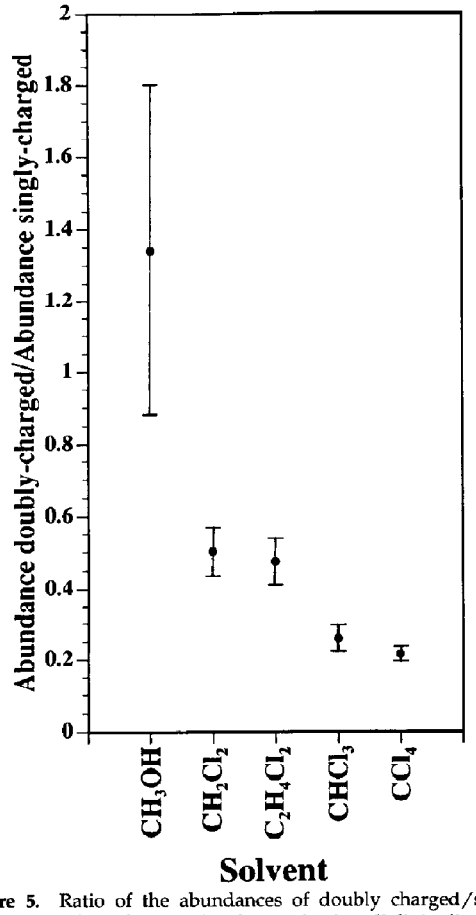


Figure 5. Ratio of the abundances of doubly charged/singly charged ions (54 °C) of the disodium salt of cardiolipin dissolved in 10% chloroform and 90% of: methanol ϵ (54 °C) = 27.3; dichloromethane ϵ (20 °C) = 9.08; 1,2-dichloroethane ϵ (54 °C) = 8.85; chloroform ϵ (54 °C) = 4.24; and carbon tetrachloride ϵ (54 °C) = 2.17. The solvents on the abscissa are listed in order of decreasing dielectric constant, as given by ϵ (T °C) [22]. Spacing on the abscissa is arbitrarily uniform.

and methyl formate) were chosen for evaluation. These solvents have virtually identical gas-phase basicities and proton affinities. Thus, large differences in the distribution of charge states are unlikely to result from Brönsted-type proton (or cation) transfer processes in the gas phase. The same test compounds [i.e., diphosphoryl lipid A from *E. coli* (proton counterion) and cardiolipin (Na^+ counterion) (see Figure 4)], were again used to evaluate the ratio of doubly to singly charged species.

Appearing in Figure 6 are the negative ion ES mass spectra obtained for diphosphoryl lipid A dissolved in one part chloroform and nine parts of each of the three solvents. The ratio of doubly to singly charged ions decreases dramatically with decreasing polarity, as expressed by the dielectric constant. The peak at m/z 1717, observed in all three mass spectra, arises from an impurity (i.e., the singly charged monophosphoryl lipid

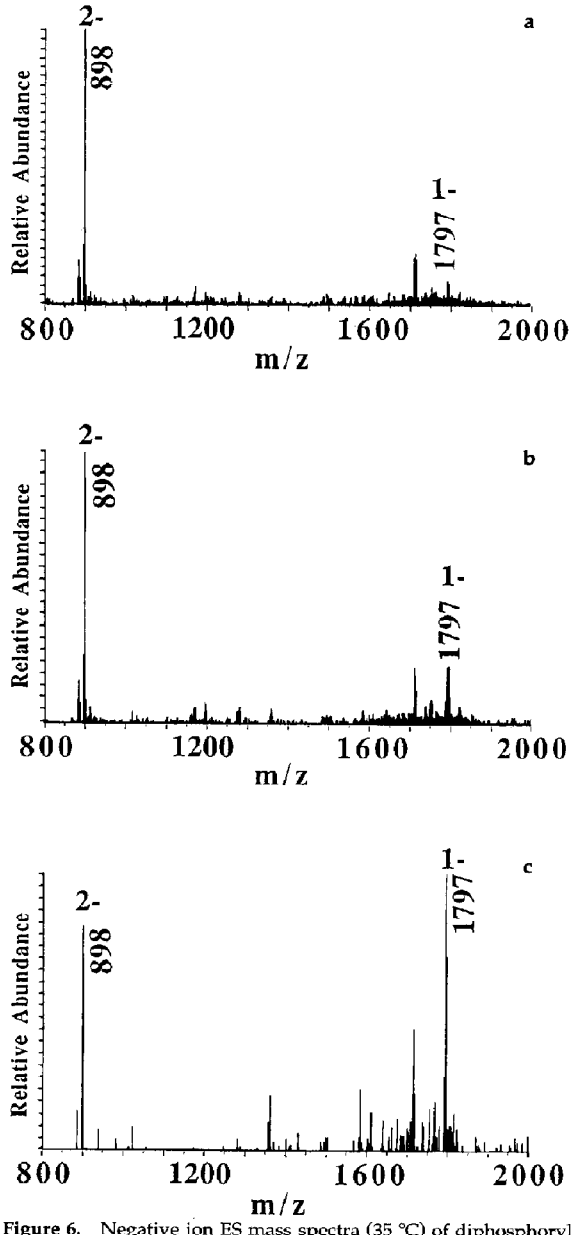


Figure 6. Negative ion ES mass spectra (35 °C) of diphosphoryl lipid A from *E. coli* initially dissolved in one part chloroform to which was added nine parts of (a) acetonitrile, (b) ethanol, and (c) methyl formate.

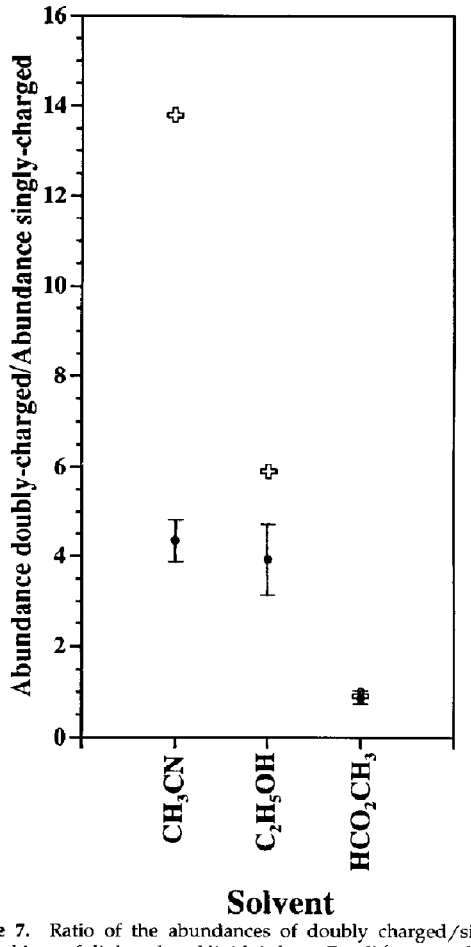


Figure 7. Ratio of the abundances of doubly charged/singly charged ions of diphosphoryl lipid A from *E. coli* (*cross marks*, 35 °C) and cardiolipin (*circles*, 35 °C) dissolved in 10% chloroform and 90% of: acetonitrile, ϵ (35 °C) = 35.1, GB = 755.6, PA = 788.3; ethanol, ϵ (35 °C) = 22.8, GB = 754.0, PA = 787.8; and methyl formate, ϵ (35 °C) = 7.75, GB = 757.3, PA = 790.4. The solvents on the abscissa are listed in order of decreasing dielectric constant, as given by ϵ (T °C) [22]. Spacing on this axis is arbitrarily uniform. GB is the gas-phase basicity in kilojoules per mole and PA the proton affinity in kilojoules per mole [23].

A species) [24]. Plotted in Figure 7 are the ratios of doubly to singly charged species for the two test compounds. Although less dramatic in magnitude, a similar trend showing increased propensity toward forming singly charged species with decreasing polarity was evident for cardiolipin. The existence of species bearing different chain lengths in the cardiolipin sample mixture again allowed data to be presented with error bars. These data affirm that solvent polarity contributes heavily to the determination of charge state in negative ion ES/MS. This result can be explained on the basis of an increased stabilization of multiply charged ions by those solvents possessing high dielectric constants. The gas-phase contribution (if any) toward ion production in this series should be equivalent for all of the solvents because the gas-phase basicity is virtually the same for each solvent.

To further assess the contribution of solvent basicity and proton affinity toward the determination of charge state in negative ion ES/MS, a series of alcohols listed in order of decreasing dielectric constant (methanol, ethanol, 1-propanol, 2-propanol, and 1-butanol) was

chosen to evaluate the tendency toward formation of doubly charged versus singly charged species for the same two test compounds. Another feature of these solvents is that the order of decreasing dielectric constant is also the order of increasing proton affinities and gas-phase basicities, with 2-propanol and 1-butanol (proton affinities and gas-phase basicities virtually equal) exhibiting the only anomaly to this latter ranking. Solution-phase basicities have also been measured by titration in glacial acetic acid [25, 26], indicating an order of basicity (methanol < ethanol < 2-propanol) that parallels the gas-phase data. The series could then provide insight into the predominating processes responsible for ion formation; results are plotted in Figure 8.

A clear trend displaying a decreased tendency toward formation of ions of higher charge state from solvents having low dielectric constants is evident. The fact that the gas-phase basicity and the proton affinity of the solvents are increasing from left to right (except for 1-butanol < 2-propanol) serves to indicate clearly that gas-phase factors are minor, perhaps negligible, contributors to the removal of cations from analyte molecules. In other words, even though 2-propanol and 1-butanol had the highest affinity for protons in the gas phase, they exhibited the weakest ability to remove the second proton from diphosphoryl lipid A. Significantly, a similar statement can be made diminishing the importance of solution-phase basicity in determining the predominant charge state. In examining methanol, ethanol, and 2-propanol, the higher charge state became less prominent for solutions of increasing basicity. Solution polarity thus appears to be the dominant factor that dictates the charge state of the analyte in obtained negative ion mass spectra. As was the case in Figure 7, the magnitude of the observed differences in charge state was much more pronounced for diphosphoryl lipid A than for cardiolipin.

As a "control" experiment, three solvents (dimethyl formamide, acetonitrile, and methanol) with rather similar dielectric constants were chosen to perform the same types of experiments using the same two test compounds. If, indeed, the dielectric constant is responsible for determining the charge state of analyte species, we would expect the $(2-/1-)$ charge-state ratio to be similar for all compounds because the difference in the magnitude of the dielectric constant is small for these three solvents. Shown in Figure 9 are the results of the experiment, where the solvents on the abscissa are listed in order of decreasing dielectric constant. For cardiolipin, the ratios of doubly to singly charged species are virtually indistinguishable for the three solvents. For diphosphoryl lipid A, however, a slight downward trend from left to right is observed. Because the charge-state distribution falls within a small range for all solvents (compared to much larger differences in charge state observed in Figures 5, 7, and 8), this experiment supports the view that the dielectric constant plays the predominant role in dictating the charge-state distribution in negative ion ES/MS.

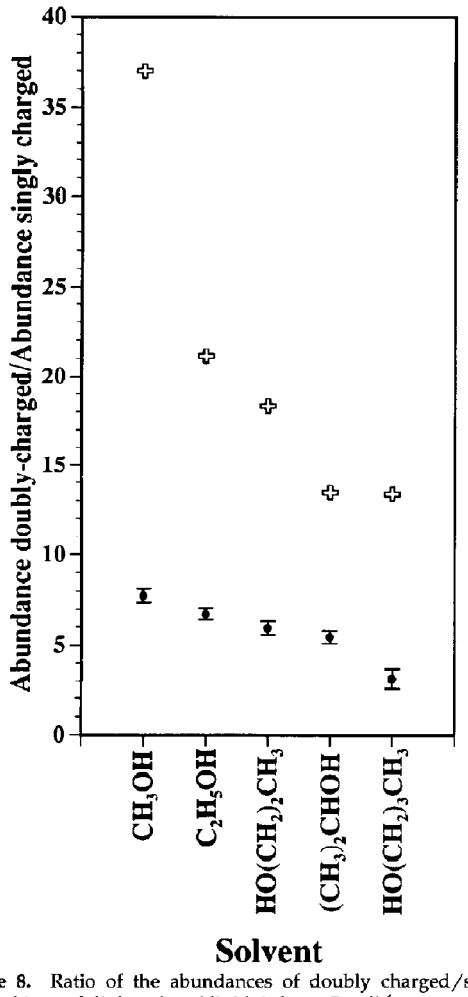


Figure 8. Ratio of the abundances of doubly charged/singly charged ions of diphosphoryl lipid A from *E. coli* (*cross marks*, 34 °C) and cardiolipin (*circles*, 34 °C) dissolved in 10% chloroform and 90% of: methanol, ϵ (34 °C) = 30.9, GB = 728.4, PA = 761.1; ethanol, ϵ (34 °C) = 23.0, GB = 754.0, PA = 787.8; 1-propanol, ϵ (34 °C) = 18.9, GB = 765.7, PA = 798.3; 2-propanol, ϵ (34 °C) = 17.2, GB = 767.3, PA = 800.0; and 1-butanol, ϵ (34 °C) = 16.0, GB = 766.9, PA = 799.6. The solvents on the abscissa are listed in order of decreasing dielectric constant as given by ϵ (T °C) [22]. Spacing on this axis is arbitrarily uniform. GB is the gas-phase basicity in kilojoules per mole, and PA the proton affinity in kilojoules per mole [23].

The fact that the charge-state distribution of diphosphoryl lipid A is detectably affected by a small difference in solvent dielectric constant, whereas that of cardiolipin is not noticeably affected (Figure 9), is consistent with data from Figures 7 and 8. In both Figures 7 and 8, the magnitude of the shift in charge

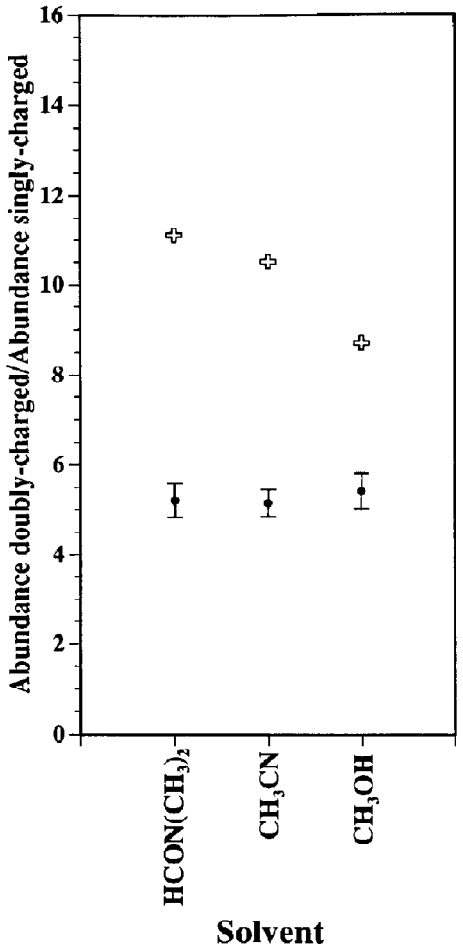


Figure 9. Ratio of the abundances of doubly charged/singly charged ions of diphosphoryl lipid A from *E. coli* (*cross marks*, 34 °C) and cardiolipin (*circles*, 45 °C) dissolved in 10% chloroform and 90% of: dimethylformamide, ϵ (25 °C) = 36.7; acetonitrile, ϵ (34 °C) = 35.1 and ϵ (45 °C) = 33.5; and methanol, ϵ (34 °C) = 30.9 and ϵ (45 °C) = 28.9. The solvents on the abscissa are listed in order of decreasing dielectric constant as given by ϵ (T °C) [22], although the interval between values is relatively small. Spacing on this axis is arbitrarily uniform.

state as a function of changing solvent is more pronounced (i.e., data points exhibit a steeper dependence on dielectric constant) for diphosphoryl lipid A. This indicates that the tendency to form ions of different charge state is more critically dependent on the solution polarity for diphosphoryl lipid A (H^+ counterion) than for cardiolipin (Na^+ counterion). The pronounced difference in the degree of dependence of the charge state toward solvent polarity for the two compounds could be at least partially attributed to the differing nature of the cation present in each case. For cardiolipin, the Na^+ (larger radius) counterion experiences a smaller degree of stabilization upon solvation compared with the H^+ (smaller radius) counterion of diphosphoryl lipid A. This may contribute to the explanation as to why the ability to remove the second positively charged counterion (i.e., to form the dianion) increases more dramatically with increasing dielectric constant for diphosphoryl lipid A than for cardiolipin.

The general tendency for the diphosphoryl lipid A to exhibit a higher charge state than the cardiolipin in Figures 7–9 (i.e., for a given solvent, the cross marks always mark a larger ordinate value than the circles) can be explained by the larger distance between the fixed phosphoryl charge sites for the former compound (see Figure 4). This implies that the energy required to remove the second proton from diphosphoryl lipid A is relatively close to that required to remove the first, regardless of which proton leaves first. For cardiolipin, it is likely that the negative charge created after loss of the first Na^+ serves to bind the second Na^+ counter-ion more tightly because the sites are sterically proximal. This would tend to diminish the intensity of the doubly charged peak in all mass spectra.

Implications for the Mechanism of Negative Ion Formation

Attributing observed ES mass spectral characteristics to individual solvent parameters, of course, is complicated by the inability to independently vary a single solvent parameter without simultaneously altering others. Competing forces due to different solution properties can surely influence the appearance of the mass spectrum to different degrees. Certain solvent characteristics, such as specific conductance, surface tension, and viscosity, which are known to influence ES performance in a variety of ways, exhibited no obvious correlation to the observed charge-state distributions. Rather, each experiment in this study has pointed to the predominance of solution polarity in determining the preferred ES/MS charge state.

In relating our observations to the process of charged-droplet formation, solvents with higher dielectric constants can offer increased stabilization to highly charge-separated species (i.e., multiply charged ions). Implicit in this conclusion is that ion formation in negative ion ES/MS is highly dependent on solution-phase separation of cations from anionic functional groups located on analyte molecules. This interpretation builds on the "electrophoretic" [6, 7, 27] mechanism of droplet charging. According to this mechanism, the imposed electric field induces the separation of positive and negative charge carriers present in solution. Outside the ES capillary exit, negative ions are (electrically) migrating away from the ES capillary (toward the counterelectrode), and positive ions are migrating back to the capillary tip (away from the counterelectrode). This results in a net buildup of negative charge in the solution leaving the ES capillary. The outcome is that the highly charged droplets

formed at the tip of the liquid filament extending from the Taylor cone each contain excess negative ions.

The dielectric constant of the solvent determines its ability to disperse the attractive forces between formed ions and their parting counterions. Our results suggest that solvents with higher dielectric constants are capable of increasing the degree of solution-phase charge separation and ion solvation in the steps leading up to charged-droplet formation, most likely resulting in increased droplet charging. Furthermore, it is reasonable to expect that if the solvent dielectric constant is extremely low, even singly charged species will be formed in low yields because minimal dissociation of ion pairs will occur in solution. This is likely to be a factor contributing to the low test analyte signal observed when the carbon tetrachloride solvent was used (Figure 2), despite the ability to use higher ES field strengths with this highly chlorinated solvent.

Analyte solubility can also exert an influence on the characteristics of ES mass spectra [12]. Solubility affects the Gibbs free energy of an ion in solution, which can alter, in turn, the magnitude of the local electrostatic field required to lift an ion off a droplet surface into the gas phase, as professed in the "ion evaporation" mechanism. As one referee of this report pointed out, this factor could exert an influence on the observed charge-state distribution. Solubility parameters, such as Hildebrand's δ [28], often parallel the behavior of other solvent polarity scales when relative solvent rankings are established. The δ-parameter, however, is normally used to predict the solubility of rather nonpolar nonelectrolytes in low-polarity solvents [28], which limits its applicability here. Nevertheless, analyte charge-state distributions consistently shifted to lower values with decreasing δ for the homologous series of chlorinated solvents (Figure 5) and alcohols (Figure 8). Certain data points in Figures 7 and 9, however, lie in opposition to this trend. Direct evidence to diminish the contribution of solubility factors to the observed differences in analyte charge-state distributions was obtained via qualitative turbidity measurements on solutions of cardiolipin present in increasing concentration in each of the three solvents used to generate Figure 9. Large differences in cardiolipin solubility were observed (i.e., solubility decreased in the following order: methanol $\gg$ dimethyl formamide > acetonitrile). If analyte solubility played a major role in determining the $(2-/1-)$ charge-state ratio, then the ordinate values for cardiolipin in Figure 9 should not have been constant.

Additional evidence corroborating the dominance of the dielectric constant in determining analyte charge state was provided by an experiment wherein cardiolipin was dissolved in 9:1 methanol/chloroform at 1.0-, 0.2-, and 0.05-mg/mL concentration levels. Under constant conditions at 49 °C ES chamber temperature, the ratios of $(2-/1-)$ charge states were observed to be 10.7, 10.2, and 11.5, respectively. The relative standard deviation (0.061) is within the random error limits for these determinations, indicating that the charge-state distribution is not dependent on analyte concentration in this concentration range. If individual analyte molecules can be considered to be relatively isolated systems at all three concentration levels, then the influence of the dielectric constant in determining analyte charge state should be the same in all three cases. The observation of no detectable link between analyte concentration and the observed $(2-/1-)$ ratios is consistent with this notion.

For the nonaqueous systems studied here, evidence was provided that basic functional groups do not significantly augment cation dissociation. Solution-phase stabilization of multiply charged species in negative ion ES/MS is thus highly dependent on induced specific orientation of the solvent dipole. High gas-phase basicities and high proton affinities are shown to play comparatively insignificant roles in determining the final level of multiple charging observed in the mass spectrum. Gas-phase separation and solvation of positive and negative charge centers are thus inferred to be, at most, minor processes.

Acknowledgments

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