



Artifacts in amine analysis from anodic oxidation of organic solvents upon electrospray ionization for mass spectrometry

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Different phenylenediamines were used to explore anodic oxidation in solution during electrospray ionization (ESI) mass spectrometry analysis. In our experiments, a series of unknown ionic species was detected in the phenylenediamine solutions. Our results propose that reactions of phenylenediamines with species formed by anodic oxidation of typical ESI solvents during the electrospray ionization process such as formaldehyde are producing these peaks. Identification of these compounds *inter alia* suggests formal alkylation, a reaction not reported so far as a result of electrolytic oxidation in the prospective organic solvents.

Keywords: electrochemistry, phenylenediamines, electrolytic oxidation, electrospray ionization, formal alkylation

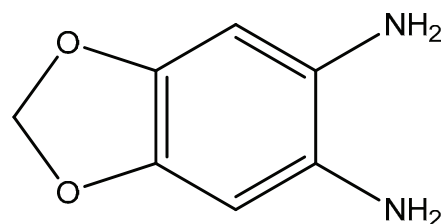
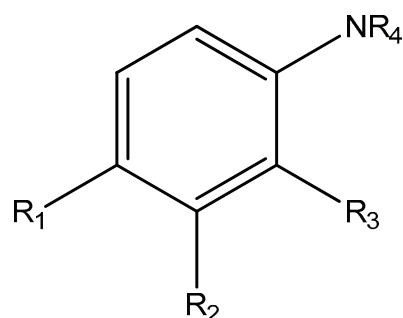
Introduction

Electrospray ionization (ESI) has nowadays become perhaps the most prominent soft ionization technique for mass spectrometry (MS). Blades *et al.*¹ were first to recognize the ESI source as a special-type electrolytic cell that was specified further by Van Berkel *et al.*² as a controlled-current electrolytic cell. Products of the electrochemical reaction leave the metal-solution interface for the solution inside the capillary to modify its composition as initially introduced into the ESI emitter. The electrolytic reaction may involve the solvent, i.e. the formation of H⁺ contributing to a pH decrease in the case of using water as the solvent³ and also the analyte itself.^{4,5}

Analyte oxidation during ESI has been observed for metalloporphyrins,⁶ metallocenes and polycyclic aromatic hydrocarbons,² hydroxybenzols,⁷ dihydropyridines⁸ and aromatic amines.^{9–11} Oxidation is usually initiated by formation of a (radical) ion by anodic removal of one or two electrons and, if appropriate, dehydrogenation of the respective organic compound. Insertion of oxygen from the aqueous solvent was also observed.¹² The susceptibility to anodic oxidation

of ferrocenes, for instance, enables their employment as derivatization reagents for ESI-MS that form “electrochemically ionizable” derivatives;¹³ another application is the online tagging of proteins by reaction with *in situ* formed benzochinones.⁷ Electrochemically formed imines were further shown to react by addition of R–OH¹⁴ and R–SH¹⁵ in the *ortho*-position to detect natural sulfhydryls or by hydrolysis to the corresponding quinone.^{9,10}

Formation of the radical cation was shown for the tertiary aromatic diamine *N,N,N',N'*-tetramethyl *para*-phenylenediamine (pNNTN) by electrochemical oxidation in the ESI needle¹⁶ and was thought to result from dissolving a charge-transfer complex in a solvent of high polarity requiring a proper compound-specific electron-donor/acceptor pair. This compound is, and always has been, attracting the interest of chemists because it forms a stable radical cation already in solution, the famous *Wurster's blue*.¹⁷ Protic and nucleophilic solvents such as water and methanol, that are commonly used with ESI, are expected to consume such radical cations



4,5-methylenedioxy-*o*-phenylenediamine
(45meth), 152 g/mol

R1	R2	R3	R4	Name	Abbreviation	Molecular mass [g/mol]
NH ₂	H	H	H ₂	<i>p</i> -phenylenediamine	pPD	108
N(CH ₃) ₂	H	H	(CH ₃) ₂	<i>N,N,N',N'</i> -tetramethyl- <i>p</i> -phenylenediamine	pNNNN	164
H	H	NH ₂	(CH ₃) ₂	<i>N,N</i> -dimethyl- <i>o</i> -phenylenediamine	oNN	136
H	H	NH ₂	H ₂	<i>o</i> -phenylenediamine	oPD	108
H	NO ₂	NH ₂	H ₂	3-nitro- <i>o</i> -phenylenediamine	3NO	153
NO ₂	H	NH ₂	H ₂	4-nitro- <i>o</i> -phenylenediamine	4NO	153
OCH ₃	H	NH ₂	H ₂	4-methoxy- <i>o</i> -phenylenediamine	4meox	138
F	H	NH ₂	H ₂	4-fluoro- <i>o</i> -phenylenediamine	4F	126
Cl	H	NH ₂	H ₂	4-chloro- <i>o</i> -phenylenediamine	4Cl	142

Figure 1. Structures of the used phenylenediamines. The general chemical structure is given and completed by a table listing the functional groups present at different sites of the molecule; names of the respective compounds and their abbreviation used in the text, and the corresponding molecular masses are provided.

by nucleophilic reactions, thereby decreasing the lifetime of radical (cation)s in solution.⁵ Another possibility is the reaction of the radicals with a second analyte molecule resulting in polymerization. Oligomerization of aromatic amines was another process observed to be enhanced by electro-oxidation with the electrospray process, for example, for *ortho*-phenylenediamine (oPD)⁹ and aniline.¹⁰

In addition to these organic compounds, common organic solvents used with ESI are also subject to anodic oxidation. Thus, oxidation of 2H₂O was shown to result in O₂ + 4H⁺ + 4e⁻.¹ Methanol is known to form different species during electrochemical oxidation, such as CO₂, CO, H-C•=O, formaldehyde, formic acid, H₂C•OH, CH₃-O-CH₂-O-CH₃ (dimethoxymethane) and the methoxylate radical.¹⁸⁻²¹ The anodic oxidation of methanol is initiated by chemisorption to the electrode surface in association with dehydrogenation that may finally result in formation of CO,²² but usually formaldehyde is one of the major products.²³ Organic compounds subjected to anodic oxidation in methanol as a solvent were found to be methoxylated.²⁴ Apart from the protonation by acidification due to the electrolysis of water, elec-

trolytic processes involving other solvents do not seem to have played a role in ESI-based analysis of organic compounds yet.

In contrast, in this report we present data providing evidence of analyte-solvent chemical reactions initiated by anodic oxidation processes under standard ESI conditions. We used different phenylenediamines to explore their behavior using ESI-MS analysis in nucleophilic solvents as commonly applied, i.e. methanol, acetonitrile and water; the compound structures are depicted in Figure 1. Non-substituted phenylenediamines differed in the position of the two amino groups to each other, i.e. *ortho* and *para*; for comparison, the corresponding tertiary amines were also included. *N,N*-dimethyl *ortho*-phenylenediamine (oNN) formed a structural analog of *Wurster's red*, the less stable cation of *N,N*-dimethyl *para*-phenylenediamine. Different substituents of *ortho*-phenylenediamines at the 4-position of the ring were selected to provide different electron-donor/electron-acceptor capabilities. In addition to the oxidation products of phenylenediamines already described, i.e. the radical molecular ion for tertiary amines and polymerization products, we identified other

species in ESI-MS analysis of the different phenylenediamine solutions as reaction products with species formed from the solvent using the electrospray process. To the best of our knowledge, this is the first time these reaction products of phenylenediamines have been characterized by ESI-MS; their appearance has not yet been reported amongst the prospective electrochemical reaction products.

Materials and methods

Chemicals

The following chemicals were purchased from Sigma Aldrich, Taufkirchen, Germany: 4,5-methylenedioxy-*o*-phenylenediamine dihydrochloride [45meth]; 4-methoxy-*o*-phenylenediamine dihydrochloride [4meox]; *o*-phenylenediamine [oPD]; *p*-phenylenediamine [pPD]; 4-fluoro-*o*-phenylenediamine [4F]; 4-chloro-*o*-phenylenediamine [4Cl]; 3-nitro-*o*-phenylenediamine [3NO]; 4-nitro-*o*-phenylenediamine [4NO]; N,N,N',N'-tetramethyl-*p*-phenylenediamine [pNNNN]; N,N-dimethyl-*o*-phenylenediamine [oNN], ethyl acetate, CD₃OD. Methanol, acetonitrile and other solvents (HPLC grade) were ordered from VWR, Darmstadt, Germany. ESI tuning mix for Fourier transform ion cyclotron resonance (FT-ICR) mass calibration was obtained from Agilent, Waldbronn, Germany. For nano-ESI analyses, ChemSolute methanol p.a. (Th. Geyer GmbH, Renningen, Germany) was used.

ESI flow injection full scan and tandem-MS analyses

For assessment of relative signal intensities among the different phenylenediamines, stock solutions were prepared in methanol at a constant molar concentration of 16.3 mM. For every analysis, a fresh dilution was prepared. Relative signal intensity in ESI-MS was acquired at the same molar concentration for all compounds. For this, 5 μ L of each stock solution was diluted with 30% methanol to obtain a final concentration of 27 μ M, i.e. $2.7 \cdot 10^{-5}$ M.

In order to test the influence of solvents on signal intensity, 1 mg 4meox was dissolved in 10 mL methanol, ethyl acetate, acetonitrile, hexane, dichloromethane and water, respectively, diluted (1:300) and injected.

For low resolution tandem mass spectrometry (MS/MS), the target mass was set to the precursor mass and the scan range to m/z 50 to target m/z +10 u. The precursor mass was isolated and subjected to collision-induced dissociation MS/MS analysis, 20,000 target ions were collected at a maximum accumulation time of 200 ms. A 27 μ M solution of 4meox was analyzed in methanol, ethyl acetate, acetonitrile and water as described above.

Accurate mass by ESI-FT-ICR-MS

Accurate m/z values of the unknown species were determined using a 7 Tesla Bruker FT-ICR ESI-MS BioApex II with XMass 7.0.2 using a syringe pump at a flow rate of 2 μ L min⁻¹. Nitrogen was used as the nebulizer (30 psi) and dry gas (10 psi at 200°C). Full scan mass spectra were acquired in the m/z

100–2000 range. 120 μ M 4meox in 30% methanol was used; for confirmation, 4meox was re-analyzed in pure methanol, water and acetonitrile. For ethyl acetate and acetonitrile, detection limits were too low.

Accurate MS/MS by ESI-Orbitrap collision-induced dissociation

High-resolution MS and MS/MS spectra were acquired using a chip-based nanoelectrospray ion source, TriVersa NanoMate (Advion, Ithaca, NY, USA) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The spray voltage was set to 1.5 kV and N₂ delivery gas at a pressure of 0.3 psi. Solutions of 10–100 μ M 4meox, 4F, oNN and oPD in methanol and 4meox in acetonitrile and ethyl acetate were infused using a flow of approximately 150 nL min⁻¹. Full scan spectra (m/z range 50–300) were acquired in ESI positive mode with a mass resolution of 100,000 at m/z 400 (equivalent to \sim 200,000 at m/z 200). Collision-induced dissociation (CID) MS/MS scans were accumulated and averaged over 1 min with a scan speed of 0.5 Hz (\sim 30 scans). For high resolution/high accuracy CID-multi-stage mass spectrometry (MSⁿ)-measurements, 5×10^5 ions were accumulated and CID-fragments were created in the linear ion trap using a normalized CID energy of 35–60 (arbitrary units) dependent on precursor stability. The resulting fragment ions were detected in the Orbitrap within an m/z range of 50–250.

Data evaluation, generation of peak lists and calculation of chemical sum formulas of the product and fragment ions matching the detected m/z with a mass deviation less than 10 ppm were conducted with the software Xcalibur 2.1 or Bruker Data Analysis 3.3.

Results and discussion

General appearance of phenylenediamines ESI mass spectra displays oxidation products

The general behavior of phenylenediamines under ESI conditions was surveyed. Typical mass spectra using different solvents, namely methanol, acetonitrile, ethyl acetate and water, are shown in Figure 2(a) with 4meox as the example. Although M+H⁺ at m/z 139 is the base peak, other peaks of minor intensities are also displayed. Along with a mostly minor NH₃ loss due to nozzle-skimmer fragmentation (NS-CID) [$-17 \text{ u} = m/z$ 122], other low abundant species were observed, such as an m/z series exhibiting a mass difference of 14 u starting from [M+10+H⁺]: M+10+H⁺ (m/z 149), M+24+H⁺ (m/z 163), M+38+H⁺ (m/z 177) and M+52+H⁺ (m/z 191), M+66+H⁺ (m/z 205) and M+80+H⁺ (m/z 219). These peaks were confirmed as not being present in blank solutions of the solvents with different instruments confirming the instruments' purity. It seemed rather unlikely that all instruments and all solvents would feature the same impurities. Also, peaks were present with all tested primary *ortho*-diamine compounds providing evidence that, very likely, they are not raw material contaminations; again it seems implausible that all used raw materials would contain the same contaminations resulting in these series.

M+H⁺ signal intensities and *m/z* patterns of the unknown peaks generated in ESI of 4meox solutions proved to depend on the solvent used for analysis. In Figure 2(b), the M+H⁺ signal intensities and sum of the areas of the unknown peaks in the different solvents are illustrated. The best M+H⁺ signal intensities were achieved in methanol when compared to water, acetonitrile and ethyl acetate. It has been suggested that sensitivity of the protonated analyte with ESI follows the degree at which the compound can become protonated in solution;²⁵ while a higher number of available protons in solution can be expected from the pK_a values of methanol and water as solvents, the overall *m/z* signal response for the M+H⁺ of 4meox in the organic solvents followed the reverse pattern of solvent proton affinities of 754 kJ mol⁻¹ (methanol), 779 kJ mol⁻¹ (acetonitrile) and 835 kJ mol⁻¹ (ethyl acetate)²⁶ eventually, due to competition for charge with the neutral solvent molecules. The relative extent of compound protonation among the organic solvents could be expected to be highest in methanol which exhibited the lowest proton affinity, while effects originating from surface tension and lower volatility could result in better performance compared to water which featured a commensurately low proton affinity (691 kJ mol⁻¹) as well.²⁶ However, the detailed interplay involving all impact parameters of ionization efficiency in electrospray is considered to be more complex.

Concerning the unknown species, in agreement with M+H⁺ signal intensities, acetonitrile produced slightly lower signal intensities compared to methanol. Among the tested solvents, ethyl acetate was the only solvent that resulted in a higher sum of peak intensities from the unknown species compared to the protonated molecule; the spectra exhibited a longer range +14 u series in which *m/z* 191 was particularly abundant. However, the relative intensity of the investigated unknown species with respect to the protonated molecular ion was particularly variable here. In water, mainly M+H⁺ and the NS-CID fragment M+H⁺-17 were observed along with *m/z* 241 and a cluster between *m/z* 273–281 (the latter all had minor intensities); in addition, *m/z* 224, as a potential NS-CID product of *m/z* 241-NH₃, was observed. Hence, the presence of *m/z* 273 and *m/z* 241 suggest residual water in the organic solvents. With hexane and dichloromethane, no reasonable signal intensities were achieved.

A very minor "M"-peak (i.e. *m/z* value corresponding to the molecular mass of the compound) was also detected for most of the compounds matching either the radical cation or the desaminated (hydrolyzed) phenylenediamine. Abundant radical cations in ESI are observed if the oxidation potential of the analyte is far below E_{1/2(ox)}=1V vs the saturated calomel electrode.²⁷ As resonance stabilization improves with planar structures, the stability of the radical decreases with sterical hindrance, i.e. if substituents "disturb" each other, as with oNN compared to pNN.²⁸ In conclusion, the *p*-phenylenediamines are excellent candidates to generate the M^{•+}; for pNNNN in particular, with an oxidation potential of 0.1–0.2V,²⁷ identity of this species was confirmed.¹⁶ Indeed, for pNNNN, M+H⁺ was not the base peak, but M^{•+}. However, since the electron transfer in solution was shown to occur primarily between

the radical and the free amine, even if protonated species were also present,²⁹ the extent of protonation vs M^{•+} should not depend on each other. No further peaks indicating the presence of species formed from the original compound other than *m/z* 150 (M+H⁺-15) were detected for pNNNN. M+H⁺-15 is supposed to be a NS-CID fragment¹⁶ indicating that the N-bound methyl groups cleave rather easily. The capability of stabilizing unpaired electron species was suggested to be maintained in the gas-phase, so that such compounds can cleave radical fragments in low-energy CID as an exception to the "even-electron rule".²⁷

While appearance of the desaminated molecule should be enhanced with electron-donating substitution of the phenylenediamine, 45meth, oNN and 4meox would have been good candidates to form this product with standard conditions, but the corresponding peak was too small for a reasonable quantitative comparison. In nano-ESI analysis, accurate masses of the oNN and 4meox "M"-peak matched the radical molecular ion. Apart from the radical cation, dimers of phenylenediamines [2M-4H+H⁺] (corresponding to *m/z* 273 for 4meox) and M+102+H⁺=[2M-substituent at 4-position-6H+H⁺] (corresponding to *m/z* 241 for 4meox) were detected as products of electrolytic oxidation, as was observed earlier.^{9,10} However, the +14 u series that we found with all substituents of the *ortho*-phenylenediamines at different extents, curiously starting from M+10+H⁺ (illustrated for the example of 4meox in Figure 2 and Table 1, *m/z* 149–163–177–191–205–219), has not yet been described.

Phenylenediamine oxidation was provoked by the electrospray ionization process

In nano-ESI experiments, higher relative peak intensity for the unknown *m/z* was observed (Figure 3) which seemed dependent on phenylenediamine concentration in the sample. Thus, for concentrated solutions of oPD and oNN, for instance, the molecular ion M+H⁺ was in low abundance compared to the oxidation products (not shown). Such effects are common for electrolytic processes that depend on the mass transport of the analyte.^{3,30} In particular, instead of *m/z* 191 and *m/z* 205, the ions with *m/z* 193 and *m/z* 207 containing one more oxygen than the original molecule were much more abundant. In time, intensity of different peaks decreased or increased and concentration of the species with potentially higher oxygen content, as deduced from accurate mass analysis, seemed to increase with the analysis time in nano-ESI applications.

Further experiments were conducted to confirm that these species are indeed formed by reaction of the phenylenediamines with the ESI solvent. We obtained confirmation of this from the direct infusion measurements of 4meox dissolved in CD₃OD shortly before analysis. The obtained mass spectrum is illustrated in Figure 3. The peaks are annotated with the corresponding mass differences to the spectrum using non-labeled methanol. Considering that the charge in electrospray would now be provided by the deuteron instead of a proton, each ionic species would feature an increase of at least 1 u, but more if a reaction product with D-labeled methanol was present or rapid H/D exchange

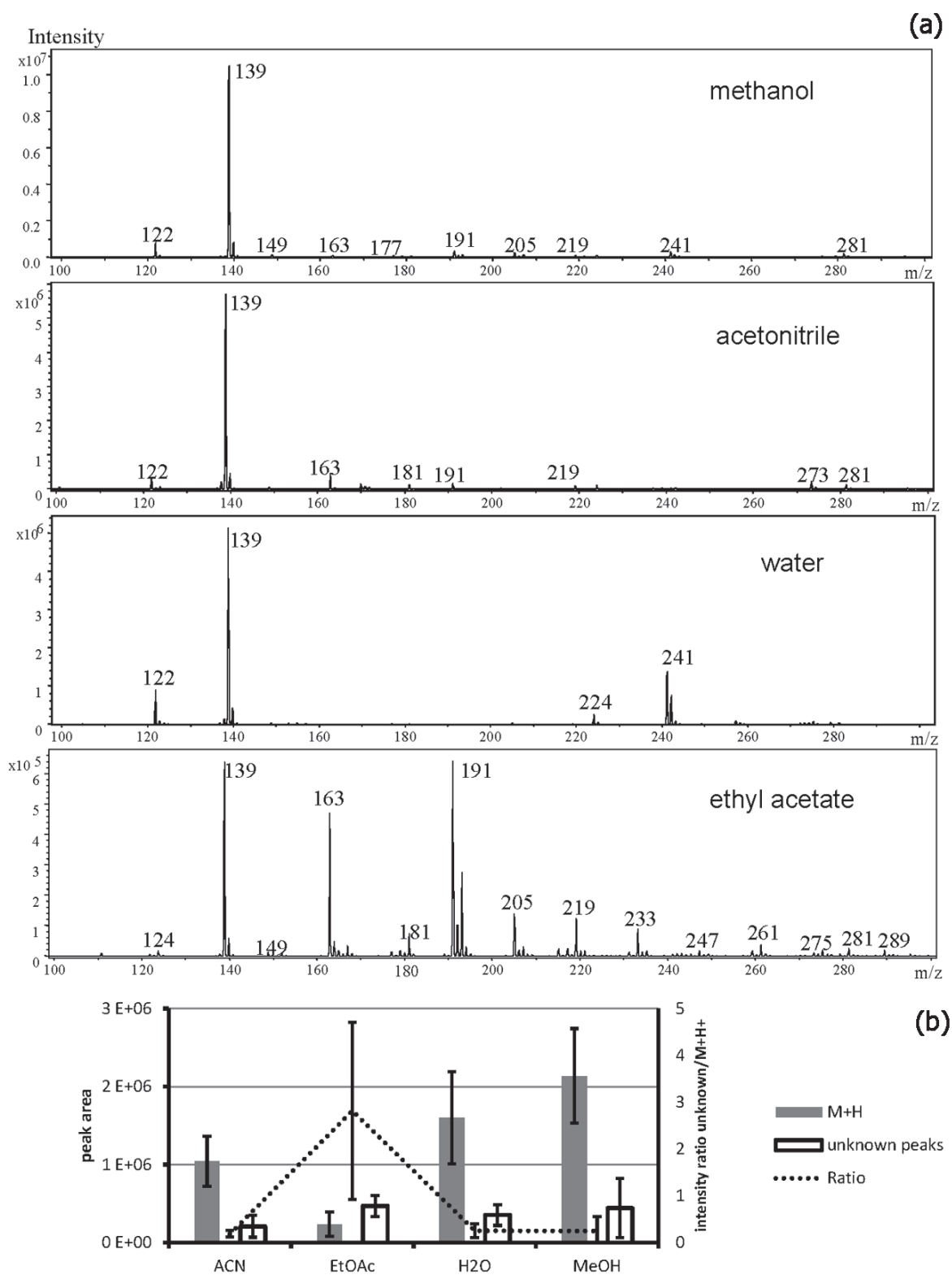


Figure 2. Influence of solvent composition on 4meox signal intensities in ESI-MS. (a) ESI full scan mass spectra of 4meox (direct infusion) using four different common ESI solvents: methanol, acetonitrile, ethyl acetate, and water. (b) Signal intensity of the protonated molecule ($M+H^+$, black columns) compared with the sum of signal intensities for ions potentially formed from 4meox involving anodic oxidation processes (unknown peaks, white columns) in different ESI solvents using the same molar concentration and flow rate. Samples were analyzed in triplicate. The ratio of unknown peaks/ $M+H^+$ intensities is given on the second y-axis in the form of a line diagram.

Table 1. Accurate m/z values obtained from ESI-FTICR analyses, their deviation from calculated sum formula proposals and CID fragments (quadrupole ion trap and orbitrap) for selected peaks in ESI-MS analysis of 4meox in methanol.

Species	Acc. m/z	Sum formula	Ex. m/z	Δ ppm	CID fragments ^a	
M-17+H ⁺	122.0601	C ₇ H ₈ N ₁ O ₁	122.0600	0.8	95	-HCN
M+H ⁺	139.0867	C ₇ H ₁₁ N ₂ O ₁	139.0866	0.7	122	-NH ₃
M+10+H ⁺	149.0711	C ₈ H ₉ N ₂ O ₁	149.0709	0.9	134/122/121/106	-•CH₃ , - HCN, -CO, •C₂H₃O
M+12+H ⁺	151.0865	C ₈ H ₁₁ N ₂ O ₁	151.0866	0.3		
M+24+H ⁺	163.0867	C ₉ H ₁₁ N ₂ O ₁	163.0866	0.6	148/133/122/120	-•CH₃ , -CH ₂ O, -•C₂H₃N , -•C₂H₃O
M+38+H ⁺	177.1021	C ₁₀ H ₁₃ N ₂ O ₁	177.1022	0.6	162/149/147/146/145/134/121/107	-•CH₃ , -C ₂ H ₄ , -CH ₂ O /-C ₂ H ₆ , -•CH₃O , -CH ₃ OH, -•C₂H₃O , -C ₂ H ₄ N ₂ , -•C₂H₄OCN /-C ₂ H ₂ N ₂ O
M+52+H ⁺	191.1182	C ₁₁ H ₁₅ N ₂ O ₁	191.1179	0.8	176/163/161/149/148/147/ 134/121	-•CH₃ , -C₂H₄ , -C ₂ H ₆ , - C ₃ H ₆ , -•C₃H₇ / -•C₂H₃O , -C ₂ H ₄ O, -•C₃H₅O
M+66+H ⁺	205.1339	C ₁₂ H ₁₇ N ₂ O ₁	205.1335	1.6	190/177/163/149/138/122	-•CH₃ , -C ₂ H ₄ , -C ₃ H ₆ , - C ₄ H ₈
M+80+H ⁺	219.1494	C ₁₃ H ₁₉ N ₂ O ₁	219.1492	0.9	204/163/148	
2M-36+H ⁺	241.1086	C ₁₃ H ₁₃ N ₄ O ₁	241.1084	0.8	226/221/198/137	
2M-4+H ⁺	273.1351	C ₁₄ H ₁₇ N ₄ O ₂	273.1346	1.8	258/256/242/241/138	
2M+4+H ⁺	281.1397	C ₁₆ H ₁₇ N ₄ O ₁	281.1397	0.04	266/251/205/149	

^aBold numbers indicate abundant peaks. Identity of fragments written in italic was confirmed by accurate mass measurement.

took place. Indeed, the m/z of the deuterated molecular ion provided the highest abundance for +2u instead of 1 (with the m/z 141 instead of m/z 139); here, H/D exchange through the semiquinonic state seems likely. The NS-CID fragment featuring m/z 122/123 supports this hypothesis; H/D exchange would primarily happen at the N and, thus, the molecule would be able to cleave ND₂H (-19 u) or NDH₂ (-18 u), respectively.

Even more interesting is the inspection of the +14u series starting from m/z 149. Although for detailed interpretation we would like to refer to the discussion about the identity of the species, very clearly deuterium was incorporated in these

species more than necessary for ionization of the compounds (m/z 150-166-183-199-215-231). Dilution of the CD₃OD stock solution with non-labeled methanol instead of CD₃OD again provided the original series of m/z 149, m/z 163, m/z 177 and m/z 191. In conclusion, we suggest that the species corresponding to these signals are indeed a result of reactions with the solvent as a consequence of electrolytic oxidation processes during the electrospray ionization. (Note: For the two *para*-phenylenediamines, this series was not detected, which may be influenced by the fact that these provide better mesomerisms stabilizing the cation and making it less reactive compared to their *ortho*-analogs.)

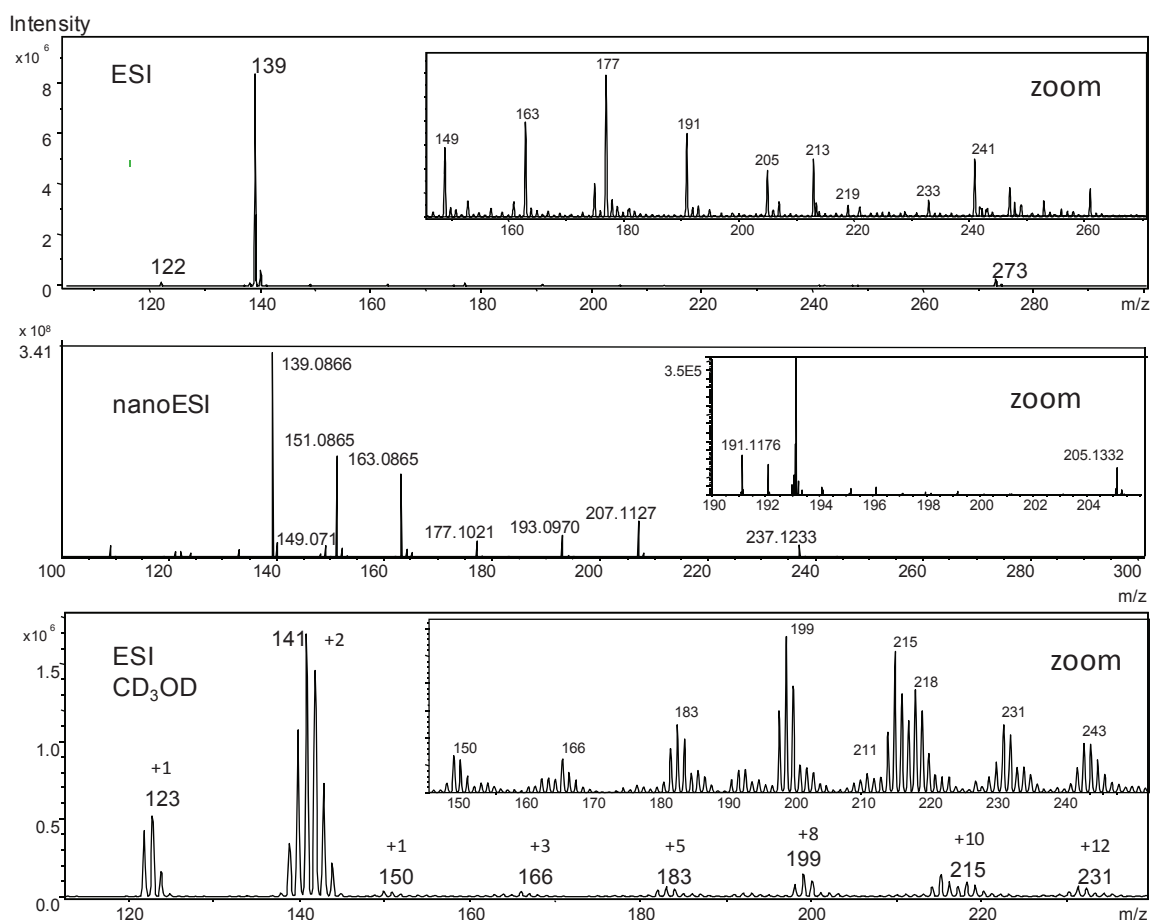


Figure 3. Evidence for anodic oxidation during electrospray ionization of 4meox in methanol. Fresh solutions of 4meox in methanol subjected to ESI and nano-ESI in methanol and, alternatively, CD_3OD . Oxidized species are easily detected under standard ESI conditions and their intensity enhanced in nano-ESI. Here, species with m/z 193 and m/z 207 (containing additional oxygen) had a significantly higher intensity compared with m/z 191 and m/z 205 that were detected at much lower intensities. Analysis in CD_3OD revealed incorporation of CD_2 from the solvent and supported particular structural isomers in front of others deduced from the theoretical sum formula calculation. The peaks are annotated with the corresponding mass differences to the spectrum obtained with non-labeled methanol.

Identification of the reaction products with solvent-born species

For structure elucidation of the unknown species, a solution of 4meox in methanol was analyzed by accurate ESI-FT-ICR-MS and ESI-Orbitrap-MS/MS. Table 1 presents the measured accurate m/z values for the methanol solution along with calculated sum formula proposals corresponding to those peaks and theoretical m/z values. Taking into consideration the expected mass accuracy of the instrument (<10 ppm threshold) and the expected presence of elements (nC, nH, 2-max. 4 N, nO), there were no alternative sum formula proposals to those listed in Table 1. All species (m/z 149, m/z 163, m/z 177, m/z 191, m/z 205, m/z 241, m/z 273) featured even electron configurations according to the nitrogen rule.

CID experiments were conducted to obtain further structure information (Table 1). CID fragments of the protonated phenylenediamine molecule indicated loss of ammonia

(–17 u).⁹ Interestingly, most of the unknown species in 4meox solutions showed an $\text{M} + \text{H}^+ - 15$ loss in MS/MS analysis. This fragment was not observed after CID of the $\text{M} + \text{H}^+$ and the first two species of the corresponding series from other phenylenediamines such as 4F (which generally produced a very poor fragmentation) or oPD. The mass difference was confirmed by accurate MS/MS as the loss of a methyl radical being an exception to the *even-electron rule* for fragmentations of even-electron species.²⁷ We suggest that the methoxy group featuring the only free methyl group in the compound is still present in these species and is responsible for this fragment. Cleavage of the methyl group from a heteroatom in the aromatic diamines was also suggested in ESI-MS of pNNNN for the CID product with m/z 150.¹⁶ In addition, there were no other $\text{M} + \text{H}^+ - 17$ losses observed except for the protonated phenylenediamine (m/z 139) and for the species with m/z 273, indicating that the remaining species had no primary amino-groups so that the amino groups are actually the site of modification.

Figure 4(a) presents structure proposals derived from sum formula calculations according to the determined accurate m/z values and in conclusion of the obtained CID spectra. With our analyses, we could not differentiate between isomeric species comprising different structures for the same sum formula so that few different structural isomers are exemplarily illustrated. Identity of the species with m/z 273 as one of the two dimers (**12**, **13**) according to the corresponding species observed earlier for oPD ($[2M - 4H + H^+]$ and $[2M - 6H - \text{substituent at 4-position} + H^+]$) was confirmed by our results. For the second dimer corresponding to m/z 241 (**10**, **11**), only minor intensities were obtained; interestingly, $M + H^+ - 17$ was not detected in MS/MS, although species (**10**) should contain two primary amino groups.⁹ We therefore suggest that m/z 241 could be obtained from the azo-isomer (**11**), or at least a mixture of the structural isomers. The $M + H^+ - 43$ loss (m/z 198 in the CID spectrum) could correspond to a $\text{CH}_2 = \text{CH} - \text{NH}_2$ loss.

Our results suggested that the +14 u series was obtained from reaction products with solvent species formed during electrolytic oxidation. In water, m/z 149, m/z 163 and m/z 177 from the +14 u series were not detected, confirming that the reaction partner for producing these species is evidently the organic solvent (water does not contain carbon). However, a designated reaction partner to form these species is difficult to identify. Electrochemical processes were shown to generate the phenylenediamine radical cation that may exchange H radicals with the solvent. Radical hydrogen exchange of phenylene compounds has been extensively studied and takes place mainly with the C-bound H of, for example, methanol; while reactions of radical cations with alcohols R-OH also lead to the alkoxy-derivatives.^{14,31,32} (Note: peaks matching alkoxy-derivatives with methanol for the species detected at m/z 163, m/z 177 and m/z 207 were observed at m/z 193, m/z 207 and m/z 237 in accurate nano-ESI analysis, Figure 3.) The occurrence of the CH_2 -“building block” in formation of the species detected as +14 u series seems very unusual; we found formal $+\text{CH}_2$ -incorporation and even $+\text{C} - 2\text{H}$ in the case of the ion with m/z 149 with hints of modification at the amino groups. Usually, radical reactions in solution happen by hydrogen exchange or $-\text{C}-\text{C}$ rather than by $-\text{N}-\text{C}$ linking as suggested by our results.

We therefore propose that the reaction with formaldehyde formed by anodic electrolytic oxidation of methanol might be involved at least in formation of the first two species by the known addition/elimination mechanism for the reaction between amines and carbonyl compounds [Figure 4(b)]. Alternatively, the radical cation could be formed at the anode and react there with neutral solvent molecules. However, the species corresponding to m/z 191 and m/z 205 cannot be explained solely by formaldehyde involvement and anodic H-abstraction. Since we observed oxidation products for all primary amines with three different organic solvents, the designated reaction partners had to be present or formed in all these solvents, which have a methyl group bond to either-OH (methanol), $-\text{CN}$ (acetonitrile) or $-\text{COOEt}$ (ethyl acetate). Alternative involvement of the ether ethyl group in ethyl acetate could be responsible for the different pattern of additional

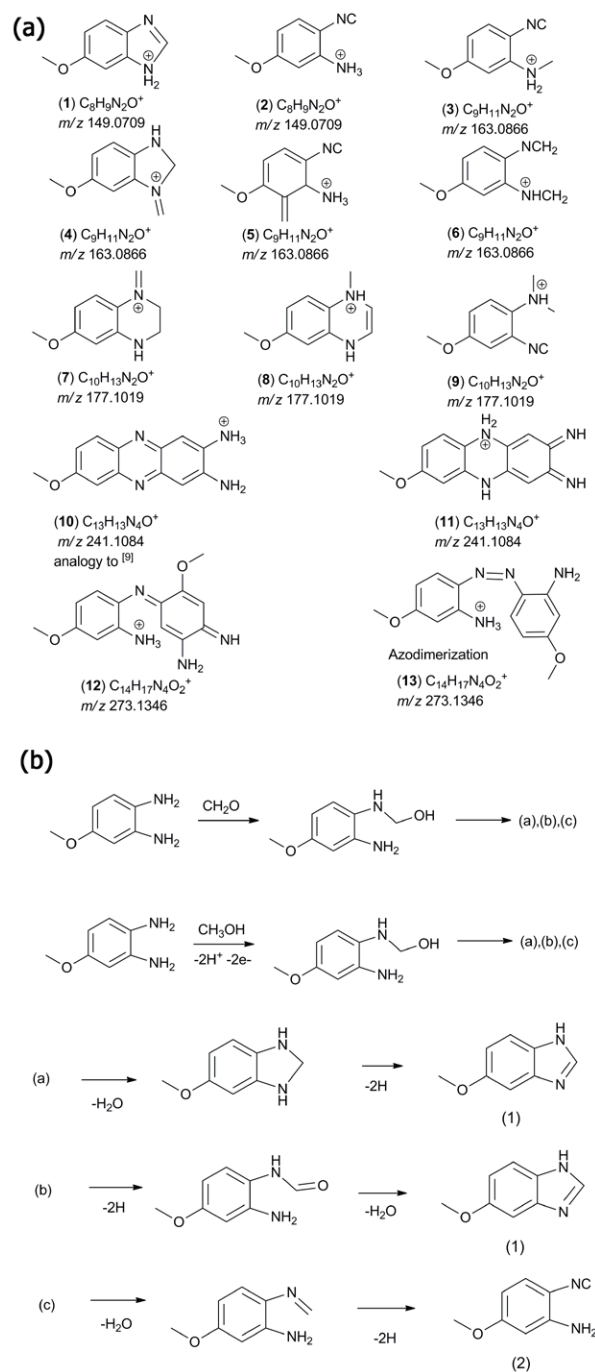


Figure 4. Potential products of anodic oxidation during electrospray ionization in methanolic solution of 4meox.

(a) Structure proposals for ions observed in ESI-MS analysis of 4meox in methanol that may originate from oxidation processes. Proposals incorporate results from calculated sum formulas corresponding to the accurate masses of these species and considering the results from MS/MS analysis and full scan analysis in deuterated methanol.

(b) Two alternative reaction mechanisms of 4meox and methanol leading to an ion with m/z 149, first assuming the *in situ* formation of formaldehyde by anodic oxidation subsequently reacting with the analyte in solution, second starts from anodic oxidation of the analyte itself and subsequent reaction with methanol at the anode.

species there, where the species featuring +2 and +4 carbons [with m/z 163 and m/z 191] are relatively more abundant than with the other solvents [Figure 2(a)].

Thus, reaction with formaldehyde could result in the imine [presence in solution was confirmed by accurate mass and was relatively more abundant with nano-ESI, Figure 3] that would be further oxidized to the isonitrile (**2**) or, alternatively, form the cyclic imine (**1**) to obtain m/z 149. The MS/MS fragment $M+H^+-HCN$ could be obtained from both structures and, although the cyclic imine would be expected to be more stable in solution, oxidation of imines to nitriles is often observed in electrochemistry; in return, the absence of $M+H^+-17$ would support the imine structure. However, with oNN featuring only one primary amine but the other tertiary, $M+10+H^+$ [m/z 147] was particularly abundant (not shown). Here, the isonitrile structure may be evident; but HCN was not cleaved in CID. For other *ortho*-diamines, both isomers may be present.

The ion with m/z 163 again could be formed by two subsequent addition/elimination reactions with formaldehyde (**3**, **4**, **6**). For that, the proposed intermediate in Figure 4(b) could be the origin. This time, the corresponding species of oNN [m/z 161] was detected only with nano-ESI, indicating that rapid formation of the major ion with $M+24+H^+$ under standard conditions requires the availability of both primary amine functions. Other possible isomeric structures such as (**5**) would involve one primary amine group only but with the ring instead as the alkylation site; this was deduced from the appearance of the corresponding species m/z 161 in nano-ESI analysis of oNN featuring one primary amino group only; however, this species cleaved an imine group [CH_2N^+] in CID. Removal of hydrogen from the ion with m/z 163 would result in a species with m/z 161 [a mixed imine–isonitrile], which was also detected in analysis of 4meox (not shown).

The ion with the accurate m/z value of 177.1019 matches the tentative sum formula of $C_{10}H_{13}N_2O$ (**7–9**) that corresponds to a $+CH_2-$ sum formula of the suggested structure for m/z 163. It was barely detected using acetonitrile as a solvent. CID analysis of the species with m/z 177 suggested cleavage of ethene and, therefore, the potential presence of two carbons between the two nitrogen atoms (for example **7**). Further, in the MS/MS analysis of the corresponding series from oPD [$m/z=4meox-30u$], this was the first species clearly cleaving a methyl group, indicating a methyl-substituted N might be present in this species (**8**, **9**). In contrast to m/z 163, the corresponding species of oNN with m/z 175 had formed under standard conditions but barely with nano-ESI, again suggesting that different isomers might be the major ion with each of the two conditions. Thus, the ion observed at m/z 177 may not require both amino groups to be primary *per se*; this assumption would lead to additional structure proposals where carbon residues could be located at either one of the N atoms (**9**) or at the ring. Considering further the cleavage of a fragment with $C_2H_4N_2$, we found no single structure that would simultaneously meet all these requirements; therefore, the presence of a mixture of isomeric species producing different fragments appears plausible.

The species with a higher m/z from 177 on cannot be solely formed by the addition/elimination reaction with *in situ* produced formaldehyde. Intensities of the +14 u series from m/z 191 have become comparably low. Accurate values of m/z 191 actually match an ion with the sum formula of $C_{11}H_{15}N_2O_1^+$. This species appears to have four more carbon atoms compared to the original molecule; the number of heteroatoms does not change, resulting in a structure with just two more double-bound equivalents. The corresponding species of 4F, i.e. m/z 179, was the only one with an $M+H^+-15$ CID fragment for the 4F series and the corresponding species for oPD, i.e. m/z 161, cleaved methyl as well indicating a methyl-substituted N might be present in this species. $M+H^+-42$ (propene as confirmed by accurate MS/MS) was very abundant. There is evidence from the low resolution ethyl acetate MS/MS spectra that, in fact, two different species in the two solvents might be formed. In comparison with the MS/MS spectrum in methanol, the ethyl acetate (LR-) CID spectrum of the precursor ion with m/z 191 featured an abundant m/z 151. In the CID spectrum in methanol, an abundant $-28u$ was detected (Table 1) suggesting that this compound loses an ethylene group as confirmed by accurate MS/MS. $M+H^+-28$ was barely detected with 4F (S/N ~7) but was abundant for oPD. Similar to m/z 177, it was hard to find a suitable structure proposal here and more isomers are possible. In the subsequent +14 u ions, i.e. m/z 205 and m/z 219, according to the accurate mass, one and two, respectively, more CH_2 -units are formally introduced to the core body (Table 1).

At this point, the results of the analysis using CD_3OD (Figure 3) are more detailed to confirm the sum formulas that were obtained from accurate mass analysis and evaluate the structure proposals in Figure 4. Ions with m/z 123 and m/z 141 as the deuterated molecular ion and its NS-CID product were already interpreted in "Phenylenediamine oxidation was provoked by the electrospray ionization process" in the "Results and Discussion" section. Apart from the D^+ required for ionization, the ion with m/z 150 shows no D-incorporation from the solvent, while the appearance of m/z 166 implies the incorporation of 2 D, m/z 182/183/184 of 4–6 D and m/z 198/199 6–7 D compared to the corresponding species in methanol. This means results from this analysis support the isonitrile structure for the ion detected at m/z 149 (**2**); but since the $M+1$ peak is higher than would be expected for the isotopic pattern, we suggest occurrence of the other proposed isomeric structure (**1**) to be possible. However, m/z 151 could also be explained by an ion formed after quick H/D exchange on the primary amino group at (**2**). As far as it concerns m/z 166, (**5**) would match, (**3**) would result in an ion with m/z 167 but could match if no D^+ was required, i.e. if the N-bound hydrogen was retained for charging the molecule, while (**4**) and (**6**) would result in a peak with m/z 168. The ions featuring m/z 182/183/184 are interesting; with structure (**8**) 5 D or 4 D would be incorporated if no D^+ was required, i.e. if the N-bound hydrogen was retained for charging; with structure (**7**) 6 and (**9**) 5–6 D can be expected. The ions with the m/z 198/199/200 (and m/z 214/215/216) again confirm that indeed three CD_2 units were formally transferred from the solvent to the molecule.

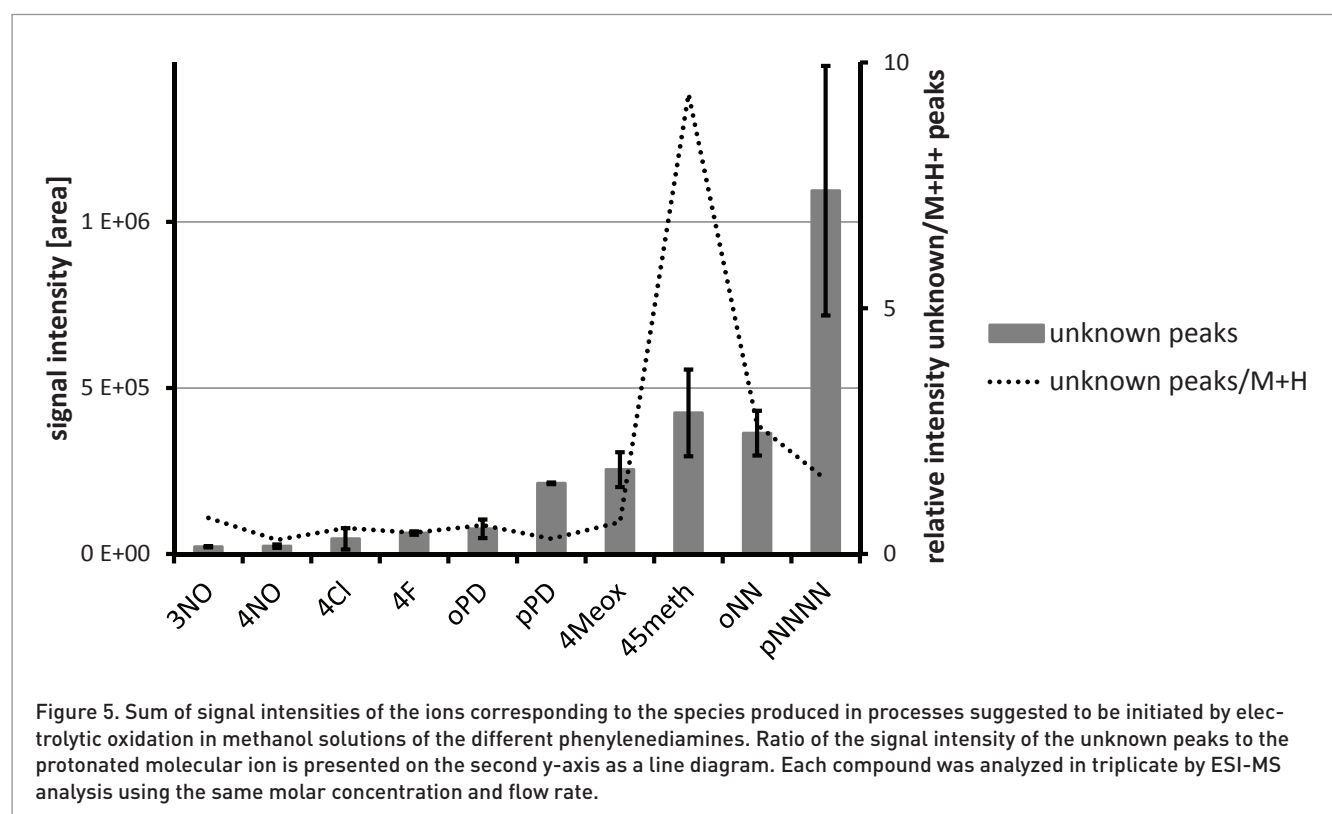
The distribution of the D-labeled species in the spectrum further approves that more than one isomer might be present at a time.

Taking these results together, we ascribe the appearance of the unknown species ($m/z < 241$) to reactions with reactive species in the solvent formed by electrolytic oxidation. Apart from the reaction with the *in situ*-formed formaldehyde, a common reaction mechanism that would lead to CH_2 incorporation from methanol under the given conditions is not described in the literature. Moreover, to our knowledge, our report is the first report on the *in situ* reaction of analytes with common solvents involving anodic oxidation under normal ESI conditions. Although Van Berkel *et al.*¹⁶ speculated that the ion with m/z 195 in analysis of pNNNN was a reaction product of the radical cation with the solvent (+30 u suggests methoxylation), the authors did not comment any further on its identity or whether this species may be formed in solution already or during the ionization process itself. However, one documented example may be the reaction of nickel (II) octaethylporphyrine with chlorine radicals formed by anodic oxidation of the solvent dichloromethane under nano-ESI conditions suggested by Van Berkel and co-workers.³⁰

Oxidation was enhanced by electron-donating substituents

The conjugation of phenylenediamines facilitates their susceptibility to oxidation and the heteroatoms with their lone pair electrons enable mesomerism stabilizing possible

intermediates. Oxidation of phenylenediamines is known to be enhanced with electron-donating substituents; substitution of these molecules with, for example, $-\text{OH}$, $-\text{OCH}_3$, $-\text{N}(\text{CH}_3)_2$ and CH_3 , aids in intermediate stabilization.³³ Planarity is further expected to support reactivity towards oxidation by reducing the sterical hindrance for mesomeric effects.²⁸ Thus, the number and intensities of the additional peaks would be expected to vary from compound to compound: However, the intensity of the unknown peaks predominantly followed the intensity of the protonated molecular ion; the highest relative increase was observed for 45meth followed by oNN (Figure 5). For the *N*-methyl substituted compounds pNNNN and oNN and the *para*-phenylenediamines, intensity of M^{*+} significantly contributed to the abundance of oxidized species. Since their radical cations are fairly well stabilized in solution, their reaction with other species might be less pronounced. With 45meth, a higher reactivity through planarity was confirmed than would be brought about by the second substituent only; the methylenedioxy group, instead of two methoxy groups, forces the molecule in a more planar configuration. In contrast, comparing oPD and pPD planarity seemed to stabilize the ion against further reaction; pPD takes up a more planar configuration in which the two amino groups do not interact and mesomerism is enhanced;³⁴ as a result, the extent of oxidation (mainly the radical cation) was slightly lower than in a solution of oPD with the same molarity that exhibited the series of oxidation products described herein instead of the radical molecular ion.



Conclusions

As well as the formation of the radical molecular cation with tertiary amines, primary phenylenediamines are subject to oxidation during the electrospray process. Tertiary aromatic diamines appeared well oxidizable in solvents such as methanol and a stable radical molecular ion was detected by ESI-MS. In comparison, radicals of primary aromatic *ortho*-diamines are less stable in solution and were barely detected; instead, these compounds appeared to react with constituents of typical ESI solvents such as methanol to form a series of formal CH₂-addition that has not yet been detected with ESI-MS. The appearance of this series is highly interesting with respect to the nature of this reaction. We provide evidence that reaction of the *ortho*-phenylenediamines with formaldehyde from anodic oxidation of the solvent may be partially responsible for these species.

In direct conclusion to our results, potential electrolytic oxidation of all solution constituents in the ESI needle implicitly needs to be considered, in particular for investigations on easy oxidizable compounds.²⁷ The potential reaction of amines with electrolytically formed formaldehyde in methanol, which is enhanced with low flow rates and narrow capillaries as with nano-ESI on the one hand and high analyte concentrations on the other, may be of specific importance, for instance, in bioanalytics. Thus, we found evidence for arginine oxidation to the imine during ESI in methanol solution by formal C addition corresponding to the addition of formaldehyde with subsequent water cleavage (not shown).

Also, for investigations on reaction products of phenylenediamines with other compounds using ESI, i.e. when using phenylenediamines as reaction partners in organic chemistry, or other easy oxidizable compounds, potential reactions provoked by the described intrinsic electrochemical processes, rather than by the anticipated chemical reaction, need to be considered. Since analysis of phenylenediamines may be hampered by the observed phenomena, redox buffers could be employed to reduce formation of these species during the ionization process if required.⁴ Other means to potentially reduce the extent of interferences would be low analyte concentrations and high flow rates. On the other hand, phenylenediamines could potentially serve as indicators for certain solvent species formed during electrolytic processes such as formaldehyde or for residual water. Future investigations on the appearances presented here are expected to further the understanding of the processes resulting in the observed species.

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Supplementary information

The main raw data the manuscript is based on are provided as a supplement to this manuscript accessible from the journal website.

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