Quantitative determination of primary aromatic amines by cation-exchange solid-phase extraction and ultra-high-performance liquid chromatography

Margarita Aznar, Elena Canellas, Cristina Nerín*

Analytical Chemistry Department, GUIA Group, I3A, CPS

University of Zaragoza, Mª de Luna 3, 50018 Zaragoza, Spain

marga@unizar.es; elenac@unizar.es; cnerin@unizar.es

*Corresponding author. Tel.: +34 976761873; fax: +34 976762388

e-mail address: cnerin@unizar.es (C. Nerín)

Abstract

Primary aromatic amines (PAAs) have been broadly studied due to its high toxicity. In this work a method for the analysis of 22 PAAs in aqueous simulants has been developed. The method is based on a solid-phase extraction step using cation-exchange cartridges and the subsequent analysis of the extracts by ultra-high-performance liquid chromatography with mass spectrometry detection (MS). The recoveries obtained for all the amines analyzed ranged between 81 and 109 %, linear range was between 0 and 75 μg L^{-1}, with RSD values between 4.5 to 13.4% and an average value of 7.5% and limits of detection at μg L^{-1} level. The method has been applied to two real samples obtained from migration experiments of polyurethane based laminates to simulant B (aqueous 3% (w/v) acetic acid solution) which represents the worst case for migration of aromatic amines. The main amines found were methylenedianiline isomers, coming from the corresponding residual diisocyanate used during polyurethane adhesive polymerization. The total amines quantity found was 26 and 6.3 μg of aniline equivalents per kg of food simulant.

Keywords

PAAs, amines, SPE, cationexchange, ultra-high-performance liquid chromatography, migration, food packaging

1. Introduction

Primary aromatic amines (PAAs), which are suspected to be carcinogenic compounds [1], could be present in a wide variety of samples. For this reason, its presence in different matrix has been broadly studied, especially in those materials that have been in contact with food [2,3].

PAAs can be produced by decomposition of azo dyes used in printing inks, as the chromophoric azo group under certain conditions can be reduced to form aromatic PAAs. Since the azo dyes are extensively used in commercial articles such as textiles, cosmetics or plastics and also as food colorants [4,5], the determination of aromatic PAAs coming from these dyes has been carried out previously by different methods of analysis, such as by GC-MS after derivatization with isobutyl chloroformate in hair dyes samples [6], by HPLC with on-line post-column photochemical derivatization in industrial dyes [7], by HPLC with electrospray ionization mode (ESI)-MS/MS detection in industrial dyes [8] or by μ-HPLC with electrochemical detection [9] in some food colorants.

PAAs appear as well as neoformed compounds from polyurethane (PU) adhesives, commonly used for preparing multilayer laminates, which will be further used as food contact materials. PU adhesives are formed by the polymerization of polyols and diisocyanates monomers. In case the adhesive has not properly cured or if the ingredients have been mixed wrongly, the polymerization reaction will not be efficient and the remaining unpolymerized aromatic isocyanates will come into contact with water producing primary aromatic amines. Although this risk is real and well known, PU adhesives have been extensively used for different purposes. Apart from its use in laminates of food packagings, it has been used in the manufacture of biocompatible prosthesis and medical devices [10] or in household and filling materials (PU foams). As part of the ongoing investigations about the degradation of PU adhesives, several methods have been developed to isolate and chemically analyze their degradation products such as the primary aromatic amines. In biomaterials, the analysis have been

focused on toluenediamine (TDA) isomers, which have been carried out by HPLC-MS [11] and also by GC-MS after derivatization with pentafluoropropionic anhydride (PFPA) [12] [13]. For PU foams a comparison of different methods of analysis of aromatic amines coming from toluendiisocyanate (TDI)-based PU foams has been developed by Marand et al [14], that found a better sensitivity when the samples were derivatized with PFPA and analyzed by GC-MS than when the samples were analyzed by LC-MS. PU laminates used in food packagings are multilayered films cured with polyurethane adhesives. Previous papers about the analysis of amines in aqueous simulants in contact with food contact laminates have been previously published, using GC-MS with derivatization [15] and LC-MS [16].

According to the Directive of food contact materials (Commission Directive 2002/72/EC [17] and Directive 19/2007/EC [18]), the limit of PAAs released by the packaging material (expressed as aniline) must be below 10 μg aniline equivalents/kg of food or food simulant (10ppb). The German Federal Institute of Consumers Health Protection and Veterinary Medicine (BgVV) established some years ago a spectrophotometric method to determine the total content of PAAs in food packaging materials. ("Photometric determination of Primary Aromatic Amines in Food Simulants" CEN/TC194/SC1/TG9). In this method the primary aromatic amines are derivatized to an azo dye and quantified as aniline hydrochloride equivalents. Although this colorimetric method reaches the required sensitivity, it has important drawbacks, the most critical one is the absence of selectivity that does not allow the individual quantification of the amine. This lack of selectivity can also lead to false positive results in the presence of other colorants likely present in the sample. For this reason it was considered interesting to develop a method with low detection limits and a good selectivity in order to be able to determine which individual PAAs are migrating from the packaging to the foodstuff and in which concentrations. The method proposed in this paper combines the solid-phase extraction (SPE) of the sample with the analysis of the extract by ultra-high-performance liquid chromatography -MS. The SPE step allows the concentration of the analytes and the elimination of residual matrix components that could induce to ionic suppression effects during the MS detection. Advantages of ultra-high-performance liquid chromatography in terms of resolution and speed have been broadly proved [19-21]. The combination of the SPE extraction with the ultra-high-

performance liquid chromatography technology coupled to MS spectrometry, offers a sensitive and accurate method for PAAs quantification.

2. Material and methods

2.1. Reagents and solutions

DSC-SCX cation-exchange cartridges (500 mg/ 3 mL) were purchased from Supelco (Bellefonte, PA, USA) and the manifold system was from Waters (Milford, MA, USA).

PAAs were bought to Sigma-Aldrich (St Louis, MO, USA) and all of them had analytical quality.

Supergradient HPLC-grade methanol, HPLC-grade acetic acid and ammonia (solution 32%) were purchased from Scharlau Chemie (Sentmenat, Spain), HPLC-grade acetone was acquired from Merk (Darmstadt, Germany) and water purified with a Milli-Q 185 Plus system (Millipore, Bedford, MA, USA) was used..

The PAAs selected for this study are shown in table 1. Individual solutions of around 1000 mg L^{-1} were prepared in methanol, except 4,4-DPE that was prepared in acetone. The solutions of *p*-PDA, 4MmPDA, 2,6-TDA and 1,5-DAN were prepared monthly because of possible amines degradation processes.

2.2. Ultra-high-performance liquid chromatography *separation*

Chromatography was carried out in an Acquity system using an Acquity UPLC BEH C18 column of 1.7 μm particle size (2.1 x 100 mm), both from Waters (Milford, MA, USA).

Ultra-high-performance liquid chromatography conditions were optimized in order to achieve a good chromatographic resolution and sensitivity. Several parameters were tested such as the mobile phase composition or the possibility of a post column addition. Chromatography was carried out at 0.3 mL min^{-1} column flow and 45ºC column temperature. Methanol and water were used as mobile phases and the ultra-high-performance liquid chromatography gradient is shown in table 2.

119

120 *2.3. Mass spectrometry detection*

121 A Quattro micro atmospheric pressure ionization (API) tandem quadrupole mass
122 spectrometer with an electrospray probe in positive mode (ESI+) from Micromass
123 (Beverly, MA, USA) was used. Acquisitions were carried out in SIR (selected ion
124 recording) and MRM (multiple reaction monitoring) modes.

125 For SIR detection, [MH^+] ions were monitored. Cone voltages were optimized between
126 20 and 50 V. For MRM detection, transitions to daughter ions were monitored, collision
127 energies were optimized from 15 to 30 V. Voltages optimization was carried out by the
128 direct perfusion into the MS at 10 μL min^{-1} of individual solutions of 10 mg L^{-1} of each
129 amine. Table 1 shows [MH^+] ions, daughter ions, cone voltages and collision energies
130 selected for each amine.

131 For the optimization of the tune MS parameters, 4 different desolvation gas
132 temperatures ranging from 300 to 450ºC and 4 different desolvation gas flows ranging
133 from 450 to 700 L h^{-1} were tested. Optimization was carried out by the injection of a
134 mixture of 2 mg L^{-1} of the 23 amines. Final MS parameters are shown in table 2.

135

136 *2.4. SPE extraction*

137 For the SPE extraction, the loading and the elution volumes were optimized. The
138 loading solution contained around 100 μg L^{-1} of the 22 selected PAAs and it was
139 prepared in purified water 3% (w/v) acetic acid. Following the cartridge indications, the
140 elution solvent was methanol 5% (v/v) NH_3. For the optimization of the loading and the
141 elution volumes, sample volumes ranging from 20 to 100 mL were passed through the
142 cartridges, and then, the cartridges were eluted. Consecutive 1 mL aliquots of the
143 elution solvent were collected and analyzed by ultra-high-performance liquid
144 chromatography -MS. The water dilution factor of the extract collected was also
145 optimized.

146 SPE final extraction protocol was as follows: The cartridges were conditioned with 2
147 mL of methanol and equilibrated with 2 ml of purified water containing 3% (w/v) acetic
148 acid. Afterwards, 80 mL of sample solution were passed through the cartridges at

around 1 to 1.5 mL min^{-1}. The cartridges were cleaned with 2 mL of purified water with 3% (w/v) acetic acid and dried with vacuum. Finally, they were eluted with methanol containing 5% (v/v) NH_3. The first milliliter of the eluate was discarded and the second one was collected for the ultra-high-performance liquid chromatography -MS analysis. Before the analysis, the SPE extract collected (1 mL) was diluted 1/1.6 with water, and a 15 µL aliquot was injected into the ultra-high-performance liquid chromatography system.

To check the reproducibility of the system, 4-aminoazobenzol was added as internal standard to the extract before the analysis.

2.5. Calibration curves and analytical parameters

For building the calibration curves, solutions of the 22 PAAs with concentrations ranging between 0 and 70 µg L^{-1} were prepared in purified water with 3% (w/v) acetic acid. Solutions were extracted following the SPE extraction protocol and the extracts were analyzed by ultra-high-performance liquid chromatography -MS under the conditions previously optimized.

To calculate the reproducibility of the method, 3 solutions with the same concentration (around 30 µg L^{-1}) were analyzed in different days and the results were compared.

Recovery of the method was checked by comparing the results obtained when a 30 µg L^{-1} PAAs solution was passed through the SPE cartridge to those obtained when the equivalent solution was directly analyzed by ultra-high-performance liquid chromatography -MS.

2.6. Sample analysis

Two different laminates used in food packaging were analyzed. Materials used for laminates elaboration are shown in table 4. The adhesive used for laminates was polyurethane solvent based. The samples were collected and extracted just after its manufacturing.

178 A 10 x 10 cm piece of laminate was immersed in a 100 mL simulant solution, then the
179 ratio of 2 dm^2 in contact with 100 ml of simulant was used. Simulant B (purified water
180 3% acetic acid) was chosen because it represents the worst case for migration of
181 aromatic amines. These solutions were kept at 70ºC during 2 hours.

182 Due to the possible variability inside the samples, experiments were carried out by
183 triplicate. Afterwards, an aliquot of 80 mL was SPE extracted and analyzed by ultra-
184 high-performance liquid chromatography -MS with the conditions of the method
185 proposed.

186

187 *2.7. Softwares*

188 Toxicity was evaluated with the software Toxtree v1.51 (Ideaconsult , Sofia , Bulgary).

189

190 **3. Results and discussion**

191 The 24 PAAs used for this study are shown in table 1. They were selected on the basis
192 of their origin, both PU adhesives and azo dyes, used in food packagings.

193 First of all, toxicity of the PAAs studied was evaluated following Cramer rules [22]
194 with the software Toxtree. Cramer rules classify compounds in three groups depending
195 on their toxicity: low toxicity (class I), intermediate toxicity (class II) and high toxicity
196 (class III). Toxicity is estimated from the molecule structure. All the PAAs studied
197 belonged to class III, it meant a high toxicity. The presence of a nitro and a chlorine
198 substituent in the amines 5-N-*o*-T and 4-CA, classified them directly in class III. BNZ,
199 4,4-DPE, 4,4-MDA, 3,3-DMB, 4-4-Md*o*T, 4-ABP, *o*-diASD, 4,4-thioANL, 1,5-DAN,
200 2-NA, 4-AAB were classified in class III for having one aromatic ring not readily
201 hydrolyzed. The amines *p*-PDA, *m*-PDA, 2,6-TDA, 4-*m*-M-PDA, 2,4-TDA, ANL, *o*-
202 ASD, *o*-T, 22,4-DMA, 2,6-DMA, 2 (2-M-5-M-A were classified also in class III for
203 having an aromatic ring with complex substituents (amine group).

204 According to the Directive 2007/19/CE [18] of food contact materials, the limit of
205 PAAs released (expressed as aniline) must be below 10µg aniline equivalents/kg food

simulant. The aim of the work was to design a method of analysis for these amines with high sensitivity and also the possibility of knowing the origin of the amines detected.

The method was designed for simulant B, purified water 3% (w/v) acetic acid, since this simulant was considered the most restrictive, that means the worst case, for food packaging migration of amines. The direct analysis of the simulant by ultra-high-performance liquid chromatography -MS did not provide enough sensitivity, as the sample amount injected into the system was very low. For this reason the SPE experiments were set out.

3.1. LC separation

The first step was to get the efficient separation of all the amines in only one run using the Electrospray (ESI)-MS as detector .

Previous experiments had been carried out in the laboratory with an HPLC-MS system for the analysis of 8 PAAs. In these experiments, a conventional HPLC on C18 column of 4.6 mm of internal diameter and 3.5 μm of particulate diameter was used. In these analysis a volume of 50 μL was injected and a gradient of methanol/water was used. It was observed that a post-column addition of a formic acid solution of pH=3 (0.3 mL min^{-1}) drove to a clear increase of the amines sensitivity. This was related with a decrease of the mobile phase pH that enhanced the PAAs ionization in the mass spectrometer. Even though the sensitivity was improved achieving detection limits at the low ppb, it was not enough. In addition, to increase the number of amines from 8 to 23 would imply too long time analysis. In order to get a faster separation and better resolution, a ultra-high-performance liquid chromatography system was proposed.

For the ultra-high-performance liquid chromatography separation, methanol and acetonitrile were tested as organic phases. Both showed a similar separation ability but methanol provided a slight better sensitivity versus acetonitrile in the mass spectrometry detection. Therefore methanol was selected as organic phase. Different pH values of the aqueous phase were also checked (basic and acidic) but no improvements were observed. It could be because at neutral pH all the amines were already in their neutral form and in more acidic pH they protonized and retention in the column decreased. On the other hand, a more basic pH made more difficult the ionization in the mass

spectrometer and sensitivity decreased. The final gradient used is shown in table 2, and this gradient allowed the separation of the 23 amines in less than 9 minutes. It was checked if the post column addition of a formic acid solution of pH=3 in the ultra-high-performance liquid chromatography -MS system could improve the sensitivity of the analysis, but in this case, the flow addition caused a broadening of the amines peak bases. Even though different post-column flows and "T" addition systems were checked, it was not possible to avoid the peak base broadening, probably because the flow used in ultra-high-performance liquid chromatography was lower than that used in HPLC (0.3 vs. 0.6 mL min^{-1}) and for this reason, column flow was distorted by the post column addition flow. The post-column addition only improved the aniline signal that showed very low sensitivity in standard conditions, probably because it was unlikely to be ionized.

3.2. Mass spectrometry detection

All the PAAs, except 2,6-TDA, showed a maximum sensitivity at 450ºC of desolvation gas temperature and 700 L h^{-1} of desolvation gas flow. Even though this amine did not reach the maximum sensitivity at these conditions, it showed a good sensitivity too, with just a decrease of around 8% below the maximum, achieved at 350ºC of desolvation gas temperature.

Figure 1 shows the behavior of 2-M-5-M-A intensity at different desolvation gas temperatures and gas flows. All of the PAAs, except 2,6-TDA, followed the same pattern. It was observed that both the increase of the temperature and the increase of the gas flow, enhanced amines sensitivity. The increase of the signal between the minimal tested conditions of gas flow and temperature (300ºC and 450 L/h) and the maximum conditions (450ºC and 700 L/h) reached values of 450%, with an average value of increase of 200%. For 2,6-TDA the sensitivity was maximum at 350ºC gas flow temperature, nevertheless the decrease showed at 450ºC was just a 7% of the maximum signal.

It was not possible to detect the 5-N-*o*-T neither in positive or negative electrospray mode and finally it was not included in the development of the method

3.3. SPE extraction

As the direct analysis of the simulant by ultra-high-performance liquid chromatography -MS did not provide the required sensitivity for all the amines, SPE experiments were set out.

Due to the positive nature of the PAAs in acidic solutions, cation-exchange cartridges were used for the SPE extraction. The cartridges were benzene sulfonic acid polymerically bonded. The sulfonic acid provided a selective extraction of cationic compounds and the benzene group some hydrophobic interactions. For the elution, methanol with 5% (v/v) NH_3 was used, as this solution had a basic character that neutralized the PAAs allowing their elution, and due to the methanol, it had also an elution effect over the possible hydrophobic interactions.

For all the sample volumes loaded in the SPE optimization, over a 98% of each amine was collected in the second fraction. Therefore, it was decided to discard the first milliliter of the eluate and collect the second one for the ultra-high-performance liquid chromatography analysis. Results of the loading volume optimization for some representative PAAs are shown on Figure 2. Figure 2 shows for 7 PAAs, their relative area at the different loading volumes related to the area found at the minimum loading volume tested (20 mL). Results showed that as long as the loading volume increased, there was an almost linear increase of the signal that did not lay down for none of the amines. A final loading volume of 80 mL was selected thinking in real sample extractions, since the volume usually fixed in migration experiments was 100 mL.

As it has been described, the elution solvent used for the SPE was methanol with 5% ammonia (v/v). Since a C18 column was used for the chromatography, the resolution obtained injecting the extract directly was quite poor. For this reason, water was added in different proportions to the SPE extract. Figure 3 shows the difference between the analysis of the extract (3a) and the extract diluted 1:1 with water (3b) for a loading solution of 8 representative PAAs. It was observed a clear improvement of the signal without decreasing the sensitivity. In order to checked if higher water volumes would improve the chromatography the extract was also diluted 1:2, 1:3 and 1:4. For 1:3 and 1:4 dilutions a clear decrease of the signal was observed. Dilution 1:2 showed better results for the first peaks but no clear improvements for the rest of the peaks. Finally SPE extracts (1 mL) were diluted with 1.6 mL of water

300

301 *3.4. Calibration curves and analytical parameters*

302 Table 3 shows the results of the calibration curves and the analytical parameters of the
303 method. Good regression coefficients were obtained, reaching values over 0.992 for all
304 the amines. Linear range varied from 0-40 to 0-75 ppb depending on the amine. This
305 range was appropriate since it was not expected to have concentrations over these
306 values in real samples.

307 Analysis were carried out in SIR and MRM mode. Table 3 shows the limits of detection
308 (LODs) of the 22 amines in SIR mode. Very low limits of detection were obtained, 15
309 out of the 22 amines had detection limits below 0.06 μg L^{-1} and all of them below or
310 equal to 1 μg L^{-1}, except 4-CA that had a detection limit of 2.4 μg L^{-1}. The lowest
311 detection limit was obtained for 4,4-DPE (0.003 μg L^{-1}). When MRM mode was used,
312 only 5 amines decreased their detection limit with respect to SIR detection limits in a
313 considerable mode: 1,5-DAN (LOD_{MRM}=0.05 μg L^{-1}), 4,4-MDA (LOD_{MRM}=0.002 μg L^{-1}), 2-M-5-MA (LOD_{MRM}=0.01 μg L^{-1}), 4,4'-MD*o*T (LOD_{MRM}=0.003 μg L^{-1}) and 4-ABP (LOD_{MRM}=0.004 μg L^{-1}). Finally SIR mode was selected because better results
316 were obtained for most of the amines. Table 3 shows the limits of detection for the 22
317 PAAs analyzed expressed as μg of amine per liter of simulant solution and also
318 expressed as μg of aniline equivalents per kg of food simulant. For calculating the μg
319 of aniline equivalents per kg of food simulant next equation was used, taken into
320 account the proportion of laminate/food simulant used in our experiments (2 dm^2
321 laminate/ 100 mL food simulant) and the proportion established in the Directive
322 2007/19/CE (6 dm^2 laminate/ 1kg food simulant):

323 $$\frac{\mu\text{g of aniline equivalents}}{\text{kg}} = \frac{\mu\text{g of amine}}{L} x \frac{\text{Mw}_{\text{aniline}}}{\text{Mw}_{\text{amine}}} x \frac{6\,\text{dm}^2}{1\,\text{kg}} x \frac{0.1\,\text{L}}{2\,\text{dm}^2}$$

324 The LOD values for each amine expressed as μg of aniline equivalents per kg of food
325 simulant were added, obtaining a total LOD of 1.56 μg/kg of food simulant. Therefore,
326 the detection limit of the method was much lower than the quantity allowed by the
327 Directive 2007/19/CE of food contact materials (10 μg/kg), that proved the suitability of
328 the method for the migration study of PAAs to food.

Good reproducibilities of the method were also obtained, ranging from 4.5 to 13.4 with an average value of 7.5%.

The values obtained for the recovery of the extraction were very satisfactory, as all the amines reached values over 80% and 18 out of 22 were over 90%.

3.5. Sample analysis

Table 5 shows the amines found in laminates migration experiments and their concentrations. As expected, the main amines found were methylenedianiline (MDA) isomers, coming from residual MDI used during PU adhesive polymerization. The highest concentration of MDA isomers was found in laminate 1, releasing a total MDA concentration of 184.6 μg L^{-1}, corresponding to 26.0 μg of aniline equivalents/kg of food simulant, after applying a 6 dm^2 to 1 kg food simulant conversion factor. The value was higher than the allowed limits (10 μg /kg). On the other hand, laminate 2 showed a total MDA concentration of 6.3 μg L^{-1}, corresponding to 0.96 μg of aniline equivalents/kg of food simulant, in this case, below the allowed limits (10μg /kg). These results had sense since in laminate 1 polyethylene was used as support material, and polyethylene has been described as a polymer in which the diffusion coefficients of organic compounds are very high, what means that the amines can cross the polyethylene barrier very quickly.

4. Conclusions

A method based on the selectivity and capacity for concentration of SPE and the advantages of ultra-high-performance liquid chromatography -MS technology has been designed for the analysis of PAAs in aqueous simulants. Very low detection limits, good linearity, reproducibility and high recoveries were obtained for the analysis of 22 primary aromatic amines and the application of the method to real samples was successful.

Acknowledgements

This work has been supported by the Spanish Ministry of Education and Science in the frame of PETRI projects (PET2006_0512_00). E. Canellas acknowledges her grant to Gobierno de Aragón.

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406 Captions List
407
408 Figure 1: Intensity of the signal for 2-M-5-M-A at different desolvation gas
409 temperatures (ºC) (x axis) and desolvation gas flows (L h^{-1}) (y axis). Experiments
410 carried out with the same desolvation gas temperature are circled.

411 Figure 2: Evolution of the relative area (%) of 7 amines for different loading volumes
412 (mL) related to the area at the minimum loading volume: "●" m-PDA, "◆" 4,4-MDA,
413 "■" 2-NA, "▲" 1,5-DAN, "□" o-ASD, "+" 4-4-DPE, "×" 2,4-TDA

414 Figure 3: Chromatogram of the SPE extract of 8 amines at different conditions: a-
415 Injection of the pure extract, b-injection of the extract diluted 1:1 with water

416