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Specific determination of 20 primary aromatic amines in aqueous food simulants by liquid chromatography–electrospray ionization-tandem mass spectrometry

Sarah Kelly Mortensen, Xenia Thorsager Trier*, Annie Foverskov, Jens Højslev Petersen

Danish Institute for Food and Veterinary Research (DFVF), Mørkhøj Bygade 19, DK-2860 Søborg, Denmark

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Abstract

A multi-analyte method without any pre-treatment steps using reversed-phase liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) was developed and applied for the determination of 20 primary aromatic amines (PAA) associated with polyurethane (PUR) products or azo-colours. The method was validated in-house for water and 3% acetic acid food simulants using spiked migrates from plastic laminates. Detection limits ranged from 0.27 to 3 μg amine/L food simulants, and RSD values of within-laboratory reproducibility at the 2 μg PAA/L level ranged from 3.9 to 19%. PAA migration from plastic laminates and black nylon cooking utensils were determined with the method, and high levels of 4,4′-methylenedianiline and aniline were found in migrates from about half of the tested cooking utensils. The method fulfils present legislative demands in the EU for screening and verification of PAA migration from food contact materials.

Keywords: Azo-dyes; Food contact materials; Migration; LC–MS/MS; Primary aromatic amines; Polyurethane

1. Introduction

Primary aromatic amines (PAA) are from an industrial point of view very useful chemicals, which can be transformed into a multitude of products, such as pesticides, pharmaceuticals, explosives, rubber, epoxy polymers, azo-dyes, and aromatic polyurethane (PUR) products [1]. As such, PAA are rarely intended to be present in the final product, but can however be found as residuals from incomplete reactions, as isomeric impurities that did not react, as by-products, or as degradation products either from intermediate chemicals or final products [2–5]. Many PAA are toxic compounds and/or suspected human carcinogens [5–8]. The combination of a potentially high exposure and high toxicity explains why the use of these chemicals is extensively regulated, both in national and EU legislation [9,10].

According to Directive 2002/72/EC [9], food contact materials (FCM) may not release PAA (expressed as aniline) in a detectable quantity using an analytical method with a detection limit of 20 μg aniline equivalents/kg food or food simulant (analytical tolerance included). PAA have been shown to migrate from laminates, i.e. multi-layered plastic materials that contain residual amounts of unpolymerised aromatic isocyanates from PUR based adhesives [11–13], as PAA are formed when aromatic isocyanates react with water. PAA used to manufacture azo-colours, epoxies, and plastics in FCM can also migrate into foodstuffs [8,14–16].

PAA migration is expressed in terms of aniline equivalents (eq.) in Directive 2002/72/EC (the Plastics Directive) due to a spectrophotometric screening method, which is widely used by manufacturers of plastic laminates to test if PUR based adhesives are fully cured prior to their use. In this Marcali-based method [17], free PAA in aqueous migrates are diazotised and then coupled to form azo-dyes, which are measured spectrophotometrically against an aniline calibration curve. Although very sensitive (detection limits for the

* Corresponding author. Tel.: +45 72347471; fax: +45 72347448.
E-mail address: xtt@dfvf.dk (X.T. Trier).

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total concentration of PAA around 1 μg aniline eq./kg), the complexity of the diazo-coupling reactions results in a lack of robustness towards small changes in experimental conditions [18,19], in varying sensitivity to individual PAA, in the risk of positive or negative interferences, and the risk of producing false positives. From a legislative point of view, the use of such an unspecific method for compliance testing is unsatisfactory. Therefore, there is an obvious need for specific verification methods, which are able to detect PAA well below the sum limit of 20 μg aniline eq./kg and determine if the detected PAA species are on the FCM positive list or not.

In response to this demand, a task force in the FCM division of the European Committee for Standardization (CEN 194/SC1, WG2/TG 9) has worked for the past few years with the development and standardisation of methods for the enforcement of the PAA migration limits set in Directive 2002/72/EC. They recently suggested that the spectrophotometric method should only be used for screening purposes and that PAA sum levels above 2 μg aniline eq./kg should be verified with a specific method. This inspired the development of the present LC–MS/MS method, as well as a number of other analytical methods: A solid-phase extraction (SPE) HPLC-UV method for 10 PAA with an integrated pre-column back-flush procedure [20], and a SPE-GC–MS method in which eight PAA are derivatised using solid-phase analytical derivatisation with trifluoroacetic anhydride [21].

PAA methods developed in related fields, such as the monitoring of environmental waters, which could potentially be used for testing aqueous FCM simulants include: (1) a comprehensive SPE-GC–MS method, in which 56 aromatic amines are derivatised into their iodobenzene equivalents, thus permitting detection levels in water of 0.5–8 μg/L [22]; (2) a solid-phase microextraction GC–MS method for 18 PAA derivatised by diazotation and iodination has also been used to detect 0.002–0.038 μg/L levels in water [23]; and (3) determination of nitroaromatic explosives and their degradation products in environmental water samples using cartridge SPE and LC–MS/MS [24].

In comparison to the above-mentioned methods, the present LC–MS/MS method has the advantage of directly injecting migrates without any pre-treatment steps, such as derivatisation or preconcentration. The method was validated for the determination of PAA in aqueous food simulants at concentration levels relevant for the enforcement of migration limits set in the Plastics Directive. Furthermore, the method has been used to detect PAA migration from plastic laminates as well as nylon (polyamide) cooking utensils made with black colorants.

2. Experimental

2.1. Chemicals and solvents

Analytical grade standards of the 20 PAA listed in Table 1 were obtained from Acros Organics (Geel, Belgium), TCI America (Portland, OR, USA), Chem Service (West Chester, PA, USA), Fluka (Buchs, Switzerland) and Riedel-de-Haën (Seelze, Germany). The purities of the standards are shown in Table 1. Stock solutions of each compound (500.0 μg/mL) were prepared in analytical grade ethanol from Merck (Darmstadt, Germany), except for 4,4′-DPE, which was dissolved in acetone from Rathburn Chemicals (Walkerburn, UK). Stock solutions were stored in a refrigerator for up to 1 year, except for *p*-PDA, which was only refrigerated for up to 1 month because of its instability. Working solution mixtures containing 5.0 μg/mL of 9–10 PAA were prepared by diluting stock solutions with Milli-Q water. These mixtures were stored in a refrigerator for up to 5 weeks, except for *p*-PDA that was prepared every 3 days. All solutions were protected from light by covering their containers with aluminium foil.

Analytical grade methanol was obtained from Fischer Scientific (Loughborough, Leicestershire, UK), glacial acetic acid was purchased from Merck, and 97% pentafluoropropionic acid (PFPA) was purchased from Acros Organics. Water was either glass distilled or purified using a Milli-Q purification system from Millipore (Billerica, MA, USA).

2.2. Samples

Four printed laminate samples collected from Danish manufacturers and importers of multi-layered plastic FCM between December and January 2002 were used to produce various matrices for method validation. These samples were sealed in a moisture-resistant laminate and stored in a freezer at −20 °C until use. In addition, the method was applied for the determination of PAA migration from 10 freshly made laminates collected from Danish manufacturers in August 2004 as well as for 11 different black nylon cooking utensils (i.e. ladles, spoons, skimmers and whisks) purchased at Danish retail stores on September 28, 2004.

2.3. Migration tests

Freshly produced laminates from August 2004 were permitted to cure at room temperature for a period of 3 days before being exposed to 3% acetic acid (w/v) in distilled water at 40 °C for 24 h as well as for 10 days. Single-sided migration tests were conducted for 7 of the 10 laminates by filling 100 mL of 3% acetic acid solution into heat-sealed pouches with an inner surface area of 2 dm^2 according to CEN standard EN 13130-1 [25]. PAA migration was determined in the remaining three laminates (nos. 5–7) by totally immersing a 10 cm × 10 cm piece of the sample in 100 mL of 3% acetic acid solution, as these laminates could not be heat-sealed. All of the tests were performed in triplicate, and a single blank test was also included by preparing a migration cell that only contained 100 mL 3% acetic acid.

Laminate samples from 2002 were likewise exposed to distilled water and 3% acetic acid in single-sided migration tests using heat-sealed pouches with the same volume to area ratio as described above. Exposure conditions for the

Table 1
Compound specific parameters for the 20 PAA included in the method [7,9–10]

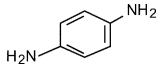
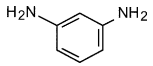
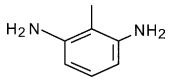
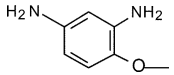
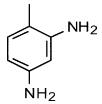
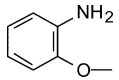
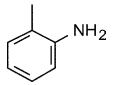
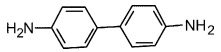
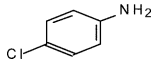
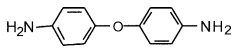
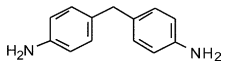
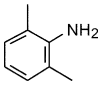
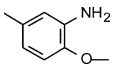
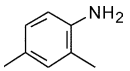
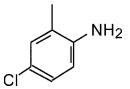
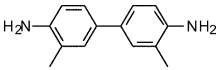
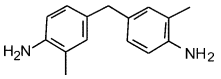
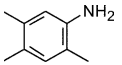
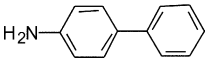
Name (abbreviation)	CAS no.	Structure	Purity (%)	IARC Group[a]	Directive 2002/61/EC[b]	Directive 2002/72/EC[c]	Time segment (min)	Retention time (min)	MRM traces (*m*/*z*)[d]	Collision energy (eV)[f]
p-Phenylenediamine (*p*-PDA)	106-50-3		>99	3	–	–	1 (1.5–4.8)	2.44	109.1 → 92.2 (109.1 → 65.4)	22
m-Phenylenediamine (*m*-PDA)	108-45-2		>99	3	–	20 μg/kg		2.72	109.1 → 92.2 (109.1 → 65.4)	22
2,6-Toluenediamine (2,6-TDA)	823-40-5		99.9	–	–	+		3.00	123.1 → 108.3 (123.1 → 106.2)	18
4-Methoxy-*m*-phenylenediamine[e] (4-M-*m*PDA)	615-05-4		–	2B	+	–		3.47	139.1 → 124.1 (139.1 → 107.2)	14
2,4-Toluenediamine (2,4-TDA)	95-80-7		>99	2B	+	+		3.47	123.1 → 108.3 (123.1 → 106.2)	18
Aniline[e] (ANL)	62-53-3		99	3	–	+		3.71	94.0 → 77.2 (94.0 → 51.3)	18 (30)
o-Anisidine (*o*-ASD)	90-04-0		>99	2B	+	–	2 (4.8–6.3)	5.03	124.1 → 109.2 (124.1 → 92.3)	14
o-Toluidine (*o*-T)	95-53-4		>99.5	2A	+	–		5.09	108.1 → 91.4 (108.1 → 93.2)	18
Benzidine (BNZ)	92-87-5		99.5	1	+	–		5.11	184.1 → 156.0 (184.1 → 166.0)	30
4-Chloro-aniline (4-CA)	106-47-8		98	2B	+	–		5.34	128.0 → 93.1 (128.0 → 111.2)	18
4,4′-Diaminodiphenylether (4,4′-DPE)	101-80-4		98	2B	+	+		5.45	201.1 → 108.3 (201.1 → 184.0)	18
4,4′-Methylenedianiline (4,4′-MDA)	101-77-9		97	2B	+	+		6.05	199.1 → 106.2 (199.1 → 77.2)	22 (50)

Table 1 (*Continued*)

Name (abbreviation)	CAS no.	Structure	Purity (%)	IARC Group[a]	Directive 2002/61/EC[b]	Directive 2002/72/EC[c]	Time segment (min)	Retention time (min)	MRM traces (*m*/*z*)[d]	Collision energy (eV)[f]
2,6-Dimethylaniline (2,6-DMA)	87-62-7		99	2B	–	–	3 (6.2–7.0)	6.40	122.1 → 105.3 (122.1 → 107.2)	18
2-Methoxy-5-methylaniline (2-M-5-MA)	120-71-8		95	2B	+	–		6.59	138.1 → 123.2 (138.1 → 106.2)	14
2,4-Dimethylaniline (2,4-DMA)	95-68-1		99	3	–	–		6.66	122.1 → 107.2 (122.1 → 105.2)	14
4-Chloro-*o*-toluidine (4-C*o*T)	95-69-2		98	2A	+	–		6.74	142.0 → 107.2 (142.0 → 125.2)	18
3,3′-Dimethylbenzidine[e] (3,3′-DMB)	119-93-7		99	2B	+	+		6.77	212.1 → 196.0 (212.1 → 211.1)	25
4,4′-Methylenedi-*o*-toluidine (4,4′-MD*o*T)	838-88-0		>95	2B	+	–	4 (6.9–9.0)	7.03	227.2 → 120.2 (227.2 → 178.2)	22
2,4,5-Trimethylaniline (2,4,5-TMA)	137-17-7		99	3	+	–		7.31	136.1 → 121.3 (136.1 → 119.3)	18
4-Aminobiphenyl (4-ABP)	92-67-1		98.4	1	+	–		7.57	170.1 → 153.2 (170.1 → 152.1)	18

[a] IARC classification groups: 1 = carcinogenic to humans; 2A = probably carcinogenic to humans; 2B = possibly carcinogenic to humans; 3 = not classifiable as carcinogenic to humans.
[b] List of PAA in the 19th amendment to Directive 76/769/EEC, which restricts the use of azo-dyes that can be reduced to any of the 22 listed aromatic amines.
[c] List of PAA which are either listed as an approved monomer (SML value) or can be derived from the approved aromatic diisocyanates (marked +) listed in Directive 2002/72/EC.
[d] Primary MRM traces used for quantitative purposes are listed first, and secondary MRM traces for verification are in parentheses.
[e] Standards were made from the hydrochloride or dihydrochloride salt of the compound, but calculations were done with reference to the pure compound.
[f] Optimal collision energies were determined for the secondary MRM traces of ANL and 4,4′-MDA and are shown in parentheses.

Table 2
Types of samples used for method validation: Migrates were produced from contact between food simulants and food packaging laminates

Type	Matrix	Exposure time (h)	Temperature (°C)
1	Distilled water migrate	24	20
2	Distilled water migrate	0.5	90
3	Milli-Q water	–	–
4	3% acetic acid migrate	24	40
5	3% acetic acid	–	–

Exposure times and temperatures were chosen according to the intended use of the laminates. A dash (–) denotes that the matrix was not exposed to any laminate samples.

migration tests performed with these laminates were chosen according to the intended use of the food packaging material and are listed in Table 2.

Nylon cooking utensils were also tested for PAA migration using 3% acetic acid as a food simulant. All the cooking utensils were intended for contact with warm food, and they were washed prior to migration testing. Migration tests for cooking utensils were performed by immersing the surface area that is intended for food contact for 2 h at 100 °C. For the purpose of investigating the long-term migration kinetics of PAA, the samples were kept immersed at 95 °C in new portions of the food simulant for 2 × 5 days alternating with shorter test periods of 2 h and 10 min, respectively, at 100 °C.

2.4. LC–MS/MS analysis

HPLC separation was performed on an Alliance 2695 separation module from Waters (Milford, MA, USA) equipped with a high-pressure gradient pump and a column heater. The amines were separated on a Zorbax SB-C3 column (2.1 mm × 150 mm, 5 μm) from Agilent (Palo Alto, CA, USA) at 40 °C. The sample volume injected was 3 μL, and a 0.5 μm stainless steel pre-column filter was used to protect the column from particulates. Optimum separation was achieved using a gradient composed of solution A (4.7 mM PFPA in methanol) and solution B (4.7 mM PFPA in Milli-Q water). The gradient elution program was as follows: 0–3 min, 20% A at a flow of 200 μL/min; 3–7.5 min, a linear increase from 20 to 80% A and from 200 to 500 μL/min; 7.5–8 min, 80% A at 500 μL/min; 8–11 min, 5% A at 500 μL/min; 11–15 min, 5% A at 200 μL/min.

A Quattro Ultima triple quadropole instrument from 1999 (Micromass, Manchester, UK) with Masslynx v. 4.0 software (Micromass) was used for data acquisition and processing. Ionisation of the analytes was achieved using an electrospray interface in the positive ion mode (ESI^+), and ionisation source parameters were as follows: capillary voltage (V_{cap}), 1.0 kV; cone voltage (V_{cone}), 20 V; Hex 1 voltage (V_{Hex1}), 20 V; desolvation temperature, 400 °C; source temperature, 130 °C. Nitrogen was used as nebulising gas (maximum flow), desolvation gas (flow-rate of 780 L/h), and cone gas (flow-rate of 40 L/h). Argon was used as collision gas at a pressure of 2×10^{-3} mbar. Data acquisition was performed in the multiple reaction monitoring (MRM) mode using either MH^+ or $M^{\bullet+}$ as the precursor ion. MRM transitions and collision energies for the 20 PAA are listed in Table 1. Each time segment had three to six MRM traces. The dwell times in time segments containing three to four ion traces were set to 0.5 s, and time segments with five to six ion traces had dwell times of 0.2 s.

External calibration standards were used to quantify PAA levels in migrates from laminate samples. Calibration curves were constructed with standards containing all 20 PAA in either Milli-Q water or 3% acetic acid at concentrations of 0.5, 2, 5, 10, 15, 25, and 50 μg/L, and the response of each calibration standard was determined at least twice. Linear regression of calibration data was calculated using a weighted least squares method (weight $1/x$) in the Quanlynx software.

3. Results and discussion

3.1. Method development

Originally the method was intended to include 28 PAA that were either known degradation products of the approved diisocyanates in Directive 2002/72/EC, azo-colour degradation products listed in Directive 2002/61/EC, known isomeric impurities of the approved diisocyanates [2], or monomers approved for plastic FCM (Table 1). However, eight PAA were left out for different reasons: 2,2′- and 2,4′-methylenedianiline were not commercially available, 2-naphthylamine and 4,4′-thiodianiline were exceptionally expensive, 5-nitro-*o*-toluidine gave no signal despite many attempts (also with negative ionisation), 1,5-diaminonaphthalene and *o*-dianisidine and gave irreproducible results, and 4-aminodiphenylamine gave inconsistent split peaks at the pH employed. Preliminary tests to establish the range of elution while checking the separation of the isomers were done on three PAA: *p*-PDA and *m*-PDA, which are low-weight, mono-amine isomers and 3,3′-DMB, a high-weight, double-ringed diamine.

Different mobile phase compositions were tested for the chromatographic separation of the PAA. Pentafluoropropionic acid (PFPA) was utilized as a mobile phase modifier at a concentration of 9 mM in a 20:80 methanol (MeOH)/water mixture. Initial problems with high noise led to the investigation of mobile phases without any PFPA modifier. A signal was obtained without the use of PFPA, confirming that a pH below the pK_a of the analytes is not necessary to detect basic analytes with ESI^+. Previous studies have also shown that protonated analytes can be detected with ESI^+ in neutral or basic solutions, where $pH > pK_a$ [26,27]. However, eliminating the acid modifier compromised the chromatography of 3,3′-DMB, which showed split peaks. Ultimately, a PFPA concentration of 4.7 mM was chosen to avoid problems with split peaks, as the analytes would be predominantly in their

protonated forms at pH 2. A mobile phase gradient composed of acetonitrile (AcN)/water with PFPA was also tested, but the HPLC gradient using MeOH/water with PFPA generally gave better ion intensities with ESI^+. As there was good baseline separation between the late eluting peaks, the flow was increased at the end of the run. No significant differences were observed between analyte responses at 200 μL/min versus 500 μL/min, which indicates that the PAA are still sufficiently ionised at the higher flow rate. Satisfactory ionisation at 500 μL/min is possibly due to the low oxidation potentials of the PAA, the neutral ion-pairs being concentrated on the surface of the droplets, or the rather low PAA concentrations. Separation of the 20 PAA using the final HPLC gradient program composed of MeOH/water with 4.7 mM PFPA is shown in Fig. 1.

Full scan mass spectra from 50 to 550 *m/z* (some up to 800 *m/z*) were acquired for all PAAs at $V_{Hex1} = 5$ V and $V_{cap} = 2$ kV. Typical ions were MH^+, $[MH + H_2O]^+$ and $[MH + solvent]^+$, the solvent being either MeOH (Fig. 2) or AcN. Small clusters of $M_2{}^+$ ions with H-losses, e.g. $[M_2 - 3H]^+$, $[M_2 - 2H]^+$, and $[M_2 - H^+]$ were also seen for about half of the PAA, and no K^+, Na^+ or Ca^{2+} adducts were observed. In addition, the beginning of an ion series that increased by a constant number of *m/z* units was observed for some of the PAA, indicating that polymerisation is possible under these oxidative conditions. Some of the PAA (e.g. *p*-PDA, 2,4-TDA, BNZ, and 3,3′-DMB) produced both MH^+ as well as $M^{\bullet+}$ ions (Fig. 2). The formation of $M^{\bullet+}$ radicals was likely the result of the oxidative conditions caused by the high capillary voltage [28]. The MH^+ or $M^{\bullet+}$ ions generally had the highest S/N and were therefore chosen as mother ions.

In general, V_{Hex1} had a significant effect on the response of MH^+ and $M^{\bullet+}$ as well as the presence of solvent adducts. At a $V_{Hex1} = 20$ V, hardly any solvent adducts were present as compared to at a $V_{Hex1} = 5$ V. It therefore seems that V_{Hex1} accelerates ions in the Quattro Ultima instrument, causing loosely non-covalent bound water and solvent adducts to strip off by in-source collision induced dissociations (CID). In some instruments, V_{cone} can be used for in-source CID. However, changing the V_{cone} settings from 10 to 80 V had little influence on the mother ion intensities, as fragmentation in the Quattro Ultima instrument occurs after the ions are sucked into hexapole 1.

Voltages used to control transmission of ions (e.g. LM1&2, HM1&2, Ion energy 1&2, Hex 2, Aperture) were optimised to achieve unit mass resolution. In the daughter ion scan mode, the collision cell voltage was optimised from 10 to 50 V for each PAA mother ion. Daughter ions with the highest S/N were chosen for quantification purposes, and one extra daughter ion with a signal ≥10% of the quantification ion was chosen for verification purposes (Table 1). Verification ions were however not incorporated into this method, as the measurement of the extra ions would have decreased the sensitivity dramatically. The specificity of all daughter ions was checked with the parent ion scan function.

3.2. Method validation

Method validation was performed in accordance with DANAK (i.e. the Danish accreditation body) guideline RL 1 [29], which is based on international and European guidelines (e.g. ISO, IUPAC and EURACHEM guidelines).

Linearity was evaluated using plots of residuals, and Mandel's fitting test [30] was applied for mathematical verification of linearity. According to Mandel's fitting test ($P = 0.01$), the response of all 20 PAA was linear between the limit of detection and 50 μg amine/L for calibration standards made with Milli-Q water or 3% acetic acid. In addition, no apparent patterns were observed in any of the plots of residuals produced by linear regression, and regression coefficients (r^2) were generally >0.99.

Method accuracy was evaluated both in terms of precision and trueness. Precision can be expressed in terms of the within-laboratory reproducibility (s_R), which DANAK defines as the standard deviation of repetitive determinations performed on identical samples by different analysts over the course of several days. The s_R-values for all 20 PAA were calculated using their repeatability (intra-day) variances (s_r) and between-day variances (s_{day}) in the following manner:

$$s_R = (s_r^2 + s_{day}^2)^{1/2}$$

As precision often varies with analyte concentration and matrix type, s_R (degrees of freedom = 11) was determined at spiking levels of 2, 10, and 25 μg/L in different Milli-Q water and 3% acetic acid simulants (Table 2). s_r was calculated from seven determinations measured by a single technician using a single HPLC column on the same day, and s_{day} was determined by two technicians using two different HPLC columns on three different days. Method precision in terms of intra-day repeatability (s_r) and within-laboratory reproducibility (s_R) varied greatly from analyte to analyte (Fig. 3). s_r at the 2 μg/L level ranged from 3.9 to 19% RSD (mean = 9.2%) in acetic acid, and similar values were determined for PAA in water (data not shown). As expected, precision increased at higher PAA levels, and s_r values for PAA in 3% acetic acid averaged 4.5% at the 10 μg/L level and 3.8% at the 25 μg/L level. The RSD values for s_R were slightly higher than those for s_r, averaging 12% at 2 μg/L, 7% at 10 μg/L, and 7% at 25 μg/L in acetic acid samples (Fig. 3). Similar s_R values were also determined for PAA analysis in water samples (data not shown).

In the absence of reference materials, trueness can be assessed by determining recovery in spiked samples [29]. Trueness was evaluated by spiking different migrates (matrices 1, 2, and 4, see Table 2) with 20 PAA at concentrations of 2, 10 and 25 μg/L and determining their recovery on two different days. Mean recoveries of 20 PAA in two different acetic acid migrates that were analysed on two different occasions are shown in Fig. 3. Mean recoveries in distilled water migrates were comparable to those in acetic acid migrates and ranged from 86 to 107% (data not shown). The RSD

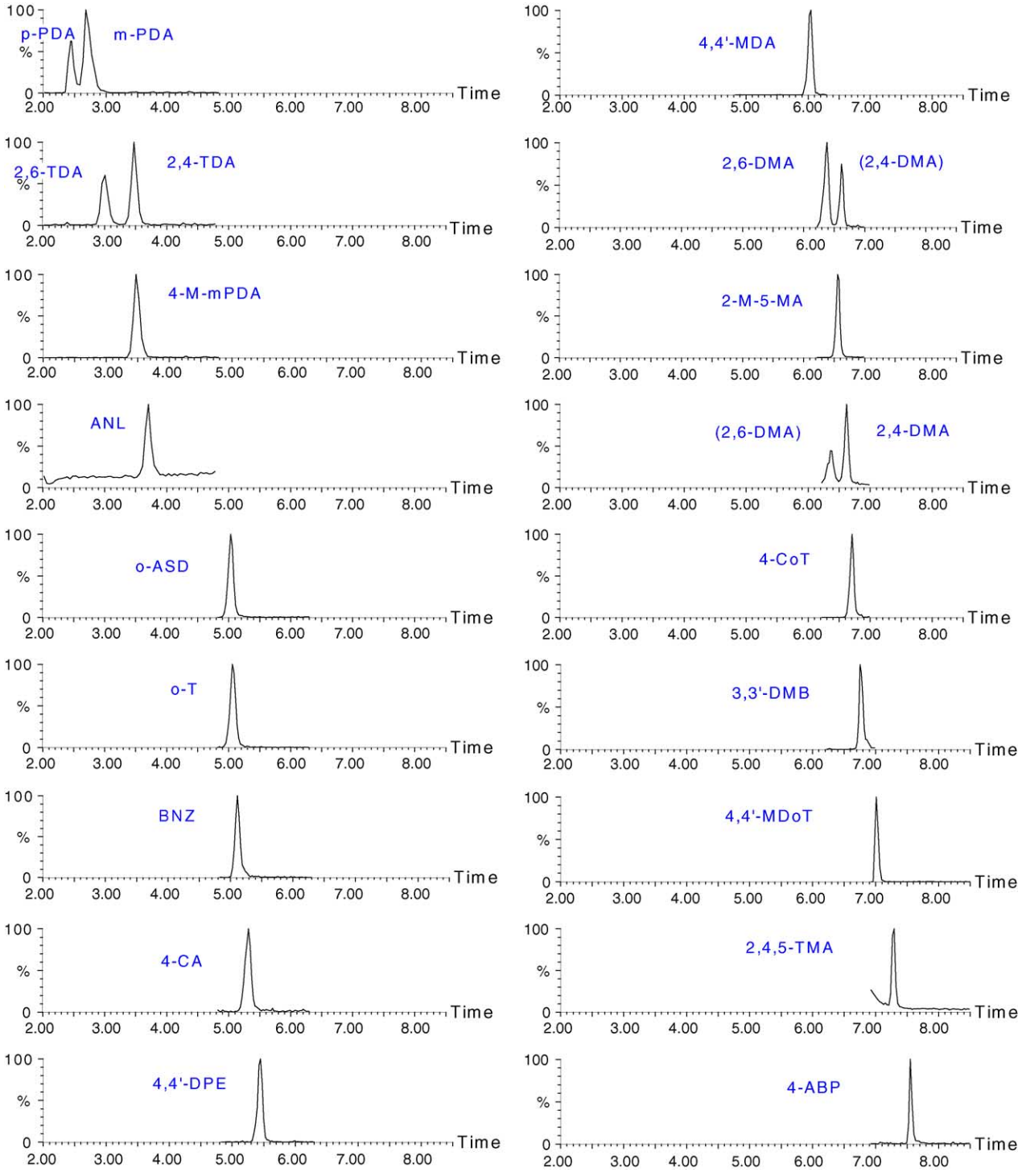


Fig. 1. Chromatograms obtained with a 10 μg/L multi-standard solution in 3% acetic acid. Data acquisition was performed in the MRM mode using the primary MH^+ or $M^{\bullet+}$ precursor ions listed in Table 1.

of the recovery means for *p*-PDA in acetic acid and water were exceptionally high, ranging from 19 to 30%. These high values are a reflection of *p*-PDA's low sensitivity (e.g. LOD = 2.7 μg amine/L) as well as its instability (e.g. stock solutions of *p*-PDA became discoloured with time and could only be stored at 5 °C for 1 month, whereas stock solutions of the other PAA were stable at 5 °C for at least 1 year).

Limits of detection (LODs) were calculated using the analyte concentration corresponding to the mean signal of sample blanks (y_b) and the s_R values of samples fortified

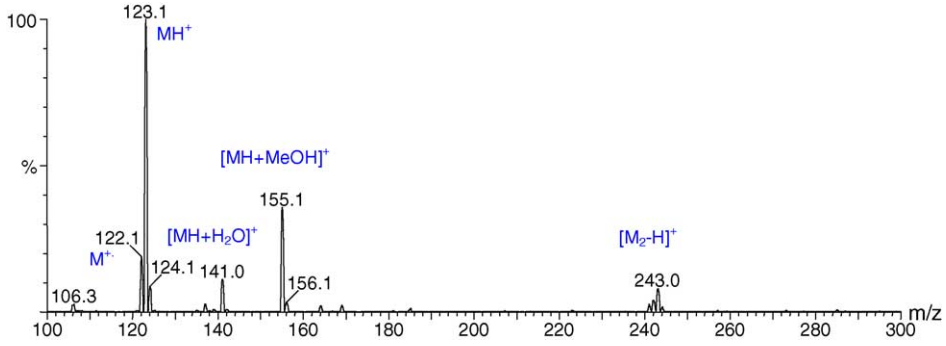


Fig. 2. Full scan spectrum of a 5 μg/mL 2,4-TDA standard solution in distilled water that was injected into 20:80 MeOH/water with 2.3 mM PFPA. The spectrum is an average of two scans minus the background, and Savitsky Golay smoothing was applied ($n = 2$).

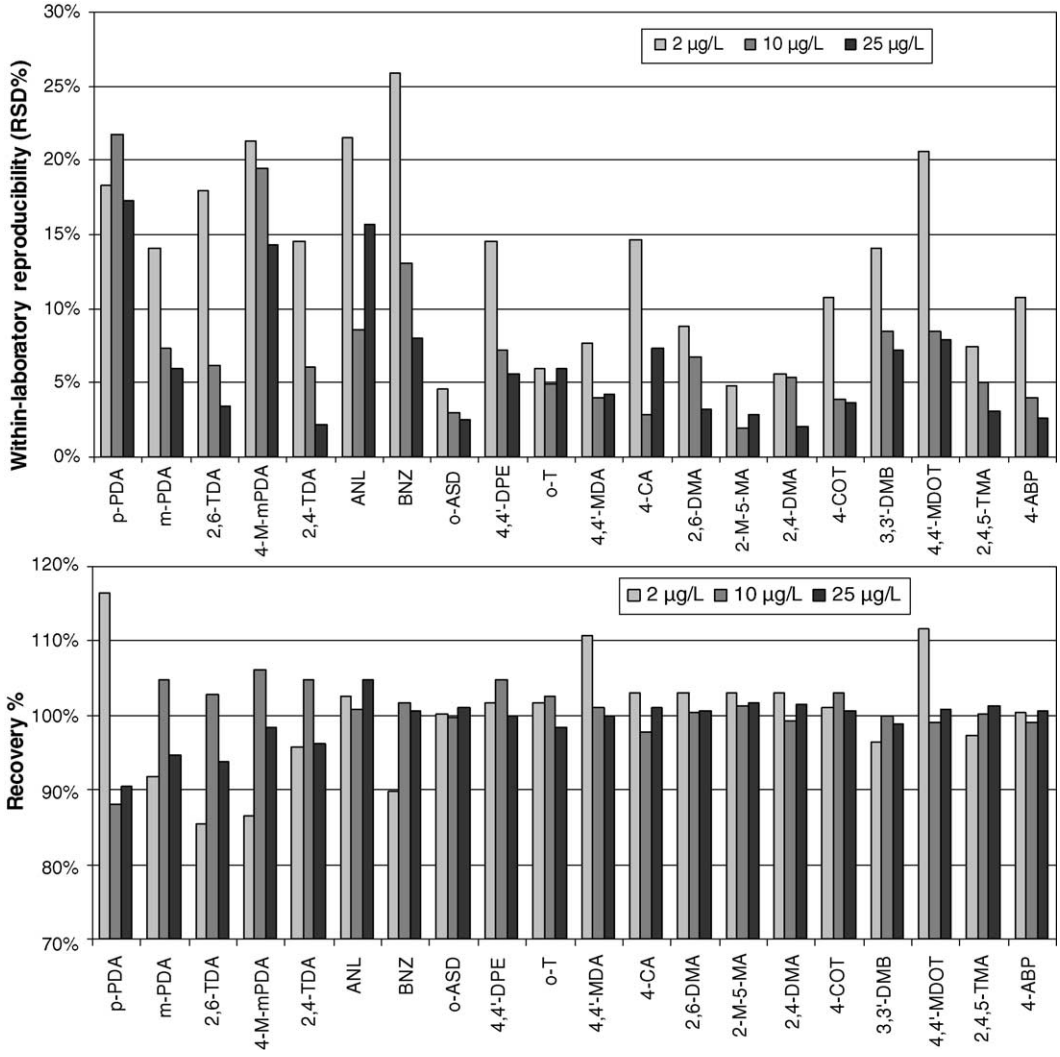


Fig. 3. Method accuracy: Within-laboratory reproducibility (top) and mean recoveries (bottom) of 20 PAA in 3% acetic acid matrices. RSD values for recovery means ranged from 1 to 16%, except for *p*-PDA and 4-M-mPDA at 2 μg/L (RSD = 30% and 35%, respectively) as well as *p*-PDA at 10 and 25 μg/L (RSD = 26% and 19%, respectively).

Table 3
Limits of detection (LODs) for 20 PAA in water and 3% acetic acid matrices are expressed using two different units, where the number of significant figures varies according to the measurement uncertainty of each PAA

PAA	Water matrices		3% acetic acid matrices	
	μg amine/L	μg aniline eq./kg	μg amine/L	μg aniline eq./kg
p-PDA	1	0.4	3	0.7
m-PDA	0.6	0.2	0.9	0.2
2,6-TDA	0.3	0.1	1	0.3
4-M-*m*PDA	0.7	0.1	1.5	0.3
2,4-TDA	0.5	0.1	1	0.2
ANL	0.5	0.1	0.8	0.3
BNZ	0.9	0.1	2	0.3
o-ASD	0.31	0.07	0.28	0.06
4,4′-DPE	0.5	0.1	0.9	0.1
o-T	0.38	0.10	0.37	0.10
4,4′-MDA	0.90	0.13	0.69	0.10
4-CA	0.8	0.2	1	0.2
2,6-DMA	0.31	0.07	0.60	0.14
2-M-5-MA	0.27	0.05	0.31	0.06
2,4-DMA	0.94	0.22	0.44	0.10
4-C*o*T	0.9	0.2	0.7	0.1
3,3′-DMB	0.7	0.1	1.0	0.1
4,4′-MD*o*T	0.8	0.1	1.4	0.2
2,4,5-TMA	0.86	0.18	0.73	0.15
4-ABP	0.4	0.1	0.7	0.1

L was converted into kg taking into account that (1) 2 dm^2 laminate sample was in contact with 100 mL food simulant and that (2) the conventional surface area to volume ratio is 6 dm^2 to 1 kg food/food simulant.

with 20 PAA at 2 μg/L:

$$\mathrm{LOD} = y_b + 3s_R$$

Detection limits for 20 PAA are listed in Table 3 and range from 0.27 to 3 μg amine/L. Migration results are usually expressed in μg aniline eq./kg in order to enable a straightforward comparison with the specification for PAA migration in the Plastics Directive [9]. As an example, the LODs in Table 3 were converted to μg aniline eq./kg using the same exposure conditions as in the migration tests for the laminate samples, i.e. a food simulant volume of 0.1 L and an exposed contact material area of 2 dm^2. Furthermore, when no specific knowledge exists about the surface to volume ratio of the final packaging, the conventional surface to volume ratio according to the Plastics Directive is 6 dm^2 to 1 kg of foodstuff. As this was the case for the laminates tested in the present study, the conversion between the concentration of *each* specific PAA in the migration solution and aniline equivalents per kg food/food simulant was calculated using the following formula:

$$\frac{\mu\mathrm{g\ aniline\ eq.}}{\mathrm{kg}} = \frac{\mu\mathrm{g\ amine}}{\mathrm{L}} \times \frac{\mathrm{Mw_{aniline}}}{\mathrm{Mw_{amine}}} \times \frac{6\,\mathrm{dm^2}}{1\,\mathrm{kg}} \times \frac{\text{volume of food simulant in test (L)}}{\text{area of contact material exposed (dm}^2\text{)}}$$

In general, the LODs for PAA in water-based matrices were slightly lower than those determined for acetic acid. LODs averaged 0.6 μg amine/L in water in comparison to 0.9 μg amine/L in acetic acid. In addition, the LOD for *p*-PDA was substantially higher than the limits for the other 19 PAA and was therefore not included in the above-mentioned averages. The higher detection limits for acetic acid matrices are in part due to higher sample blank values, as there are apparently more interferences present in migrates produced from exposing laminates to 3% acetic acid. Also, *p*-PDA generates two mother ions, $M^{\bullet+}$ and MH^+, of which only the latter is quantified.

Using a SPE-HPLC-UV method for the specific determination of PAA in aqueous food simulants, Brauer and Funke reported LODs ranging from 0.10 to 0.27 μg amine/L [20]. Furthermore, a SPE-GC–MS method was able to detect eight different PAA in water samples after derivatisation at levels above 0.1–0.4 μg amine/L [21]. Both of these methods as well as the present method enable the detection of PAA at levels well below the detection limit that is specified in Directive 2002/72/EC (i.e. 20 μg aniline eq./kg food simulant), thus paving the way for a reduction in the PAA migration limits established by EU legislation. In addition, these new analytical methods allow for the specific determination of the individual PAA-species, which makes expressing detection limits in terms of aniline equivalents irrelevant. Using aniline equivalents to set a "non-detectable" migration limit for legislative purposes is clearly problematic, as the legal amount of, e.g. 4,4′-MDA is double that of aniline (20 μg aniline eq./kg = 43 μg 4,4′-MDA/kg) because of differences in their molar weights.

In regard to method selectivity, relevant interferences were not detected in the migrate matrices that were used to determine method accuracy. Specificity of the analysis relies on MS/MS selection of the daughter ion with the highest S/N for quantification purposes and using extra daughter ions for verification purposes.

3.3. Sample analysis

In order to test the applicability of the validated method, two different types of samples were analysed. As the primary aim of the sample analysis was to test the newly developed method with real matrices, the sampling and migration test conditions for some of the samples were less elaborate than the usual enforcement standards.

3.3.1. Plastics laminates containing PUR adhesives

The method was applied to quantify PAA levels in migrates from multi-layered plastic laminates. 4,4′-MDA was detected in migrates from 4 of the 10 laminates tested, and 2,6-TDA was detected in migrates from a single laminate (Table 4). Detection of these compounds is not surprising, as residual monomers of 4,4′-methylene diphenyl diisocyanate (4,4′-MDI) and 2,4-toluene diisocyanate (2,4-TDI) are often found in PUR adhesives used to produce laminates, and 2,6-toluene diisocyanate (2,6-TDI) is a common impurity in technical grade 2,4-TDI [31,32]. A recent study likewise

Table 4
PAA migration expressed in aniline equivalents from 10 laminate samples after exposure to 3% acetic acid for 24 h at 40 °C

Laminate no.	Contact layer		PAA concentration ± SD of triplicates (μg aniline eq./kg)	
	Polymer	Thickness (μm)	2,6-TDA	4,4′-MDA
1	PE octene	40	–	1.4 ± 0.3
2	PE octene	60	–	2.3 ± 0.2
3	PE coex	60	–	2.5 ± 0.3
4	PE coex	40	–	2.0 ± 0.3
5	OPA	12	–	–
6	CPP	60	–	–
7	EVAPE	60	–	–
8	LLDPE	60	–	–
9	PE	50	0.5 ± 0.1	–
10	PE	55	–	–

Dash (–) denotes migration below the LOD: 2,6-TDA = 0.3 aniline eq./kg and 4,4′-MDA = 0.1 μg aniline eq./kg.

reported migration of 4,4′-MDA, 2,6-TDA as well as 2,4-TDA from multi-layered FCM [21]. However, the PAA levels detected by Brede et al. were substantially lower than the levels determined in this study. Differences in the reported amounts of PAA migration are likely due to differences in food contact layer, e.g. the type and quantity of PUR-adhesive applied, and in curing conditions. The laminates tested in this study were intentionally allowed to cure for only 3 days, which is a shorter period than that commonly used by the industry.

PAA migration levels determined after only 24 h of exposure did not significantly differ from those found after 10 days (data not shown). However, the thickness of the contact layer seems to influence PAA migration levels. The migration of 4,4′-MDA in laminates 1–4 seems to be proportional with contact layer thickness. Laminate no. 2 had a contact layer thickness that was 1.5 times thicker than the contact layer of laminate no. 1, and 4,4′-MDA levels in migrates from laminate no. 2 were approximately 1.7 times the levels detected in migrates from laminate no. 1.

RSD values for the triplicate determinations of 4,4′-MDA migration from laminate samples were at least twice the s_R values determined for 4,4′-MDA in spiked food simulants, which indicates that the migration test in itself doubled the uncertainty of the overall result. However, there was no added uncertainty for the triplicate determinations of 2,6-TDA migration from laminates. In this case, the uncertainty resulting from the migration tests was likely masked by the relatively high RSD value for the reproducibility of the LC–MS/MS analysis for 2,6-TDA (Fig. 3).

3.3.2. *Cooking utensils made from black nylon*

When articles are intended for repeated use, it is the result of the third migration test that has to comply with the migration limit [9]. Six of the eleven tested samples released large amounts of PAA after the third 2 h migration test, using a fresh portion of food simulant in each test. The predominant PAA migrants from these black nylon cooking utensils were 4,4′-MDA and aniline (Table 5), as previously found by the Norwegian, Slovenian and German authorities [33–35]. The present study only focused on PAA migration from black-coloured nylon cooking utensils, because recent findings suggest that the source of PAA is the application of black colorant in the polyamide raw material [33]. The identities of the PAA were verified by comparing the ratio of the signals of supplementary daughter ions (Table 1) to those detected in a calibration standard. Even after repeated exposure simulating 1–2 years of household use, a final 10 min test of the ladles showed that they continued to release PAA above the legal limit (Table 5). Presently, a further survey of PAA migration from black nylon FCM on the Danish market is being conducted.

Table 5
PAA-migration in aniline equivalents from cooking utensils immersed in the food simulant 3% acetic acid at high temperatures

Type of cooking utensil	Result of the third 2 h test at 100 °C with a new portion of food simulant[a] (μg aniline eq./kg)	Result of a 10 min test at 100 °C with a new portion of food simulant after about 250 h exposure time at 95–100 °C[a] (μg aniline eq./kg)
Soup spoon	2200 (4,4′-MDA)	n.a.
Ladle	3500 (4,4′-MDA)	66 (4,4′-MDA)
Ladle	5800 (4,4′-MDA)	38 (4,4′-MDA)
Skimmer	1500 (4,4′-MDA)	n.a.
Dipper	1700 (4,4′-MDA)	n.a.
Whisk	50 (aniline)	n.a.

n.a. = not analysed.
[a] Principal analyte detected is shown in parentheses.

4. Conclusion

A fast, simple and sensitive LC–MS/MS method without any preliminary derivatisation or pre-concentration steps was developed for the simultaneous quantification of 20 PAA in aqueous food simulants. Method validation using spiked migrates from laminate samples demonstrated that the method is capable of detecting PAA with excellent accuracy at levels much lower than the "non-detectable" limits set by EU legislation. In addition, the method has been shown suitable for quantifying PAA migration from FCM such as plastic laminates containing PUR adhesives and black nylon cooking utensils. Analytical methods for FCM are arguably at an earlier stage than fields such as food analysis; e.g. not all relevant PAA are included in current methods, only PAA in aqueous matrices can be measured, and only free PAA are measured instead of total PAA (i.e. free + bound PAA in azocolours, polyurethanes, and isocyanates). Future work will involve incorporating the MRM traces used for verification as well as traces for additional PAA into the present method, and developing a LC–MS/MS method for the determination of PAA in the official food simulant for fatty foods, namely ethanol and olive oil.

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References

[1] H.M. Pinheiro, E. Touraud, O. Thomas, Dyes Pigments 61 (2004) 121.
[2] M. Wirts, T. Salthammer, Environ. Sci. Technol. 36 (2002) 1827.
[3] J.E. Bailey Jr., Anal. Chem. 57 (1985) 189.
[4] J.E. Bailey Jr., C.J. Bailey, Talanta 32 (1985).
[5] S.W. Oh, M.N. Kang, C.W. Cho, M.W. Lee, Dyes Pigments 33 (1997) 119.
[6] R. Benigni, L. Passerini, Mutat. Res. 511 (2002) 191.
[7] IARC Monographs on the Evaluations of Carcinogenicity to Humans, vols. 1–82, International Agency for Research on Cancer, Lyon, 2003.
[8] EU-Commission, Synoptic document—draft of provisional list of monomers and additives used in the manufacture of plastics and coatings intended to come into contact with foodstuffs updated to 25 January 2003, Scientific Committee for Food, Brussels, Belgium, 2003.
[9] Commission, Directive 2002/72/EC of 6 August 2002 relating to plastic materials and articles intended to come into contact with foodstuffs, Official Journal of the European Communities, L 220, 2002, p. 18.
[10] Directive, 2002/61/EC of 19 July 2002 amending for the nineteenth time Council Directive 76/769/EEC relating to restrictions on the marketing and use of certain dangerous substances and preparations (azocolourants), Official Journal of the European Communities, L 243, 2002, p. 15.
[11] G. Lawson, C.T. Barkby, C. Lawson, Fresenius J. Anal. Chem. 354 (1996) 483.
[12] X.T. Trier, J.H. Petersen, Migration of Primary Aromatic amines from Flexible Laminated Plastics, FødevareRapport 2002:13, The Danish Veterinary and Food Administration, Søborg, Denmark, 2002, http://www.foedevarestyrelsen.dk/FDir/Publications/2002013/Rapport.pdf.
[13] K. Bouma, E. Wijma, Migration of Primary Aromatic Amines from Multilayer Films for Food Packaging, Report no. ND1FC004/01, Inspectorate for Health Protection and Veterinary Public Health, Regional Service North, Groningen, The Netherlands, 2002, http://www.vwa.nl/download/rapporten/Voedselveiligheid/020521_aromatic_amines.pdf.
[14] J.S. Gándara, P. López Mahía, P. Paseiro Losada, J. Simal Lozano, S. Paz Abuín, Food Addit. Contam. 10 (1993) 555.
[15] C. Lambert, M. Larroque, J.C. Lebrun, J.F. Gerard, Food Addit. Contam. 14 (1997) 199.
[16] C. Brede, I. Skjevrak, P. Fjeldal, Colour substances in food packaging materials, Norwegian Food Control Authority, Norway, 2003, http://snt.mattilsynet.no/dokumentasjon/rapporter/2003/snt_arbeidsrapporter/200303.pdf.
[17] K. Marcali, Anal. Chem. 29 (1957) 556.
[18] R.J. Rando, Y.Y. Hammad, Am. Ind. Hyg. Assoc. J. 46 (1985) 206.
[19] G. Norwitz, P.N. Keliher, Talanta 33 (1986) 311.
[20] B. Brauer, T. Funke, Dtsch. Lebensm. -Rdsch. 98 (2002) 405.
[21] C. Brede, I. Skjevrak, H. Herikstad, J. Chromatogr. A 983 (2003) 35.
[22] T.C. Schmidt, M. Less, R. Haas, E. von Löw, K. Steinbach, G. Stork, J. Chromatogr. A 810 (1998) 161.
[23] T. Zimmerman, W.J. Ensinger, T.C. Schmidt, Anal. Chem. 76 (2004) 1028.
[24] P.M. Gates, E.T. Furlong, T.F. Dorsey, M.R. Burkhart, Trends Anal. Chem. 15 (1996) 319.
[25] Materials, and articles in contact with foodstuffs – Plastics substances subject to limitation – Part 1: Guide to test methods for the specific migration of substances from plastics to foods and food simulants and the determination of substances in plastics and the selection of conditions of exposure to food simulants, EN 13130-1:2004(E), European Committee for Standardization (CEN), Brussels, Belgium, 2004.
[26] B.A. Mansoori, D.A. Volmer, R.K. Boyd, Rapid Commun. Mass Spectrom. 11 (1997) 1120.
[27] A.M. Kamel, P.R. Brown, B. Munson, Anal. Chem. 71 (1999) 5481.
[28] G. Wang, R.B. Cole, in: R.B. Cole (Ed.), Electrospray Ionization Mass Spectrometry: Fundamentals, Intrumentation, and Applications, John Wiley & Sons, New York, 1997, p. 148.
[29] DANAK, RL, 1 Guideline, 2003, http://www.danak.org.
[30] W. Funk, V. Dammann, G. Donnevert, Quality Assurance in Analytical Chemistry, VHC Verlagsgesellschaft, Weinheim, Germany, 1995.
[31] K. Ellendt, B. Gutsche, G. Steiner, Dtsch. Lebensm. -Rdsch. 99 (2003) 131.
[32] L. Castle, Food Pack. Bull. 10 (2001) 2.
[33] C. Brede, I. Skjevrak, Food Addit. Contam. 21 (2004) 1115.
[34] A. Zoric, Department of Sanitary Chemistry, Institute of Public Health of the Republic of Slovenia, Personal communication.
[35] EU-Commission, Rapid alert notification 2004.489, Health and Consumer Protection Directorate-General, D3, 2004.