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Determination of aromatic amines in food products and composite food packaging bags by capillary electrophoresis coupled with transient isotachophoretic stacking

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ABSTRACT

A capillary electrophoretic method was explored to assay aromatic amines in food samples. With an inline-coupled transient isotachophoretic stacking approach, the method has yielded about 200-fold improvement of sensitivity in UV detection of three primary aromatic amines and melamine. By using K^+ as a leading ion and $Tris^+$ as a terminating ion, a plug of 10 cm (equivalent to 0.44 μL) sample solution was allowed to introduce into a 60 cm (50 cm effective) capillary for separation, giving limits of detection down to 2.0×10^{-8} M. Baseline separation was achieved within 10 min, with relative standard deviation of 0.41–0.75% (intra-day) or 1.2–1.5% (inter-day) for migration time and 3.8–4.3% (intra-day) or 5.2–6.7% (inter-day) for peak area. The method was directly applicable to assaying the melamine in powder milk samples, with recovery in between 92.0% and 107.1%. The method could also be applied to the analysis of trace primary aromatic amines migrating from composite food packaging bags after combination of a 10-fold off-line concentration step, with limit of detection down to less than 1 μg/L. By this method, 4,4′-diaminophenylmethane and 2,4-diaminotoluene were thus found in three types of composite food packaging bags.

1. Introduction

Primary aromatic amines (PAA) are well-known toxic and/or carcinogenic pollutants [1] and easily appear in food products since they are widely used in the textile, food, agriculture and pharmaceutical industries. For example, during the process of composite food wrapper, adhesive containing toluene-diisocyanate may be used which may migrate into food and easily becomes more toxic diaminotoluene after hydrolyzation. Besides adhesives, dye on food packages or cooking utensils is another source of aromatic amines and has potential to migrate into food. For example, Mortensen et al. [2] once employed 3% acetic acid as food simulant to contact with cooking utensils at high temperature and found that high levels of 4,4′-diaminophenylmethane (DAPM) and aniline were observed in about half of the samples. Even worse, some amines such as melamine which is a common composition in various adhesives may also appear in food through migration, contamination or even purposive addition by some illegal merchants. Therefore, the high toxicity and exposure of PAA and melamine explain the strict regulation by national and international legislation [3–5].

The early method to detect PAA migrated into foodstuffs was based on spectrophotometry [6] which offers the information of total PAA concentration level but unable to differentiate the possible contribution of inferences. To do so, GC–MS [7,8] and HPLC–MS [2] were developed and have now become the major tools to determine melamine content in foodstuffs [5,9]. Since GC needs to derivatize the PAA of high boiling points [7,8], HPLC–MS is recognized as the more convenient tool to analyze PAA. For example, Mortensen et al. used reversed-phase liquid chromatography–electrospray ionisation–tandem mass spectrometry to directly analyze the migrating composition [2].

Alternatively, capillary electrophoresis (CE) can be superior to the HPLC and GC methods in the analysis of trace samples with limited volumes since CE requires much smaller sample volume but produces much higher efficiency at a shorter runtime and lower cost. However, few papers have been reported on CE of aromatic amines. The reasons are not very clear, but the low content of these compounds (commonly below 0.1 μM) is an obvious obstacle when using UV detection approaches which often have a limit of detection (LOD) above μM. Although highly sensitive detectors have been available such as laser-induced fluorescence (LIF) [10–13], a better way looks to be the use of stacking techniques [13–16] that are convenient in use and cost-effective. Leung and de Mello [13] and Quirino et al. [14] were the first to use field amplification sample

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injection technique in CE of standard aromatic amines. In 2003, Kim et al. [15] employed dynamic pH junction to stack three model anilines. By acidifying the sample matrix to pH 2.0 with phosphoric acid and adjusting the pH value of background electrolyte (BGE) to 4.5, the cationic analytes were successfully focused at the boundary between sample matrix and background electrolyte. In 2008, Zhang et al. [16] adopted sweeping micelle electrokinetic chromatography to improve the limit of detection for some aromatic amines, applicable to the determination of 4-methylaniline, 3,4-dichloroaniline, 4-chloroaniline and 4-aminophenyl spiked in river water with an average recovery ranging from 79.6% to 88.7%.

In this paper, more attention is paid to CE of real food samples which have complex matrices. They may impact on CE separation seriously, and a tedious procedure of sample pretreatment is often required. It is preferred if the sample preparation can be simplified to some extent by combination with a better sample stacking approach. For this purpose, several easier, stacking injection techniques were tried, of which, transient isotachophoresis (tITP) [17,18] was found to be very favorable in cooperation with CE–UV (tITP–CE–UV) in stacking the PAA and melamine as well. By tITP–CE–UV, the interferences of neutral matrix composition and anion (if at high content) were suppressed in combination with the use of low-pH background electrolyte for CE. The electroosmotic flow is now negligible, only cations with UV absorbance are able to move forwards and can be detected. In theory, isotachpohretic approach can selectively stack the target analytes by choice of a leading cation a bit faster than analytes and a terminating cation a bit slower [19]. After a systematic study and conditions exploration, the newly developed tITP–CE–UV approach was demonstrated to be highly simple: its manipulation is the same as usual CE, needing only to introduce a long plug of sample instead of a short one. With K^+ and $Tris^+$ as the leading and terminating ion, respectively, a length of 17% capillary (10 for a 60 cm capillary in length) was allowed to be loaded with sample solution, able to lower the limit of detection down to 2.0×10^{-8} M. As a consequence, the method was successfully applied to the analysis of both melamine in powder milk samples and PAA migrating from composite food packaging bags.

2. Materials and methods

2.1. Apparatus and conditions

All CE experiments were conducted on a P/ACE MDQ system (Beckman Coulter, Fullerton, CA, USA) with software 32 Karat and direct UV absorbance detection unit. A fused-silica capillary (Yongnian Optical Fiber Factory, Hebei, PR China) of 75 μm i.d. × 60 cm (50 cm to detector) was used throughout. The capillary was pretreated by flushing it with methanol, water, 1 M HNO_3, water, 1 M NaOH, and water for 15 min each. Sample was injected automatically by pressure injection (0.5 psi) and separated at a coolant temperature of 25 °C. The separated bands were detected at 200 nm while the data acquired at 4 Hz.

The length of injected sample zone was calculated by injection time multiplying the flow velocity of the sample solution in capillary that was previously determined at 0.5 psi. The detailed process to measure the velocity is as follows: aniline (10^{-4} M) dissolved in leading electrolyte solution was pumped continually at 0.5 psi from the inlet vial into the BGE-filled capillary with its outlet inserted into a vial also containing BGE. The time when the UV absorbance started to climb was recorded and used to divide 50 cm (the effective capillary length) to get the flow velocity.

2.2. Chemicals and solutions

2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (Bis–Tris) was from Acros Organics (New Jersey, USA). Aniline, melamine, DAPM, 2,4-diaminotoluene (DAT), methanol, Tris, potassium hydroxide, sodium hydroxide, phosphoric acid and hydrochloric acid were all of analytical reagent grade from Beijing Chemical Work (Beijing, China). Milk powders, pet feeds, composite food packaging bags (both unused and used) were collected from the local supermarket, retail store or factory.

Stock solutions of mixed and individual model analytes were prepared, at a final concentration of 10^{-3} M, in 50% ethanol/water and stored at −20 °C in dark to slow down oxidation. Standard sample solutions were prepared by diluting the corresponding stock solutions with a designed matrix prior to use every morning and placed in dark. BGE (also serving as terminating electrolyte) were 80 mM phosphoric acid adjusted to pH 2.65 using saturated Tris solution.

All the water used was purified using a milli-Q purification system from Millipore (Billerica, MA, USA), and all the solutions were filtered through 0.45-μm filters and degassed by ultrasonication for 3 min prior to CE experiments.

2.3. Pretreatment of milk powders and pet feeds

A powder sample (400.0 mg) was mixed in a centrifugal tube with a 4.0-ml aqueous solution of 50% acetonitrile (v/v), ultrasonicated for 10 min and centrifugated at 12,500 rpm for 5 min. An aliquot of 2.0 ml supernatant was dried by rotary evaporator at 50 °C. The resulted residue was dissolved in 2.0 ml leading solution of 80 mM H_3PO_4–KH_2PO_4 at pH 2.85 and filtered through 0.45-μm filters prior to use.

2.4. Migration test for both used and unused composite food packaging bags

The used composite food packaging bags collected from local supermarket were cut off at one edge, emptied the food inside, rinsed with triply distilled water for three times, milli-Q water for three times and drained. Both of the used and unused (with mouth unsealed, unwashed) composite food packaging bags were then subjected to migration tests according to reference [7] as follows: They were all filled with degassed water as aqueous stimulant at a ratio of 0.5 ml/cm^2, stimulant/inner surface area, as has been specified in the European prestandard EN 13130-1. After the open mouths were sealed by heating, they were heated in an oven at 100 °C for 1 h and cooled to room temperature. An aliquot of 5 ml simulant was sucked out from each bag and dried by rotary evaporator at 70 °C, respectively. Each resulted residue was re-dissolved in 0.5 ml leading solution of 80 mM H_3PO_4–KH_2PO_4 at pH 2.85 and filtrated through 0.45-μm filters prior to use.

2.5. Calibration method and data treatment

For quantification, a series of standard solutions (0.05, 0.1, 0.5, 1, 5, 10 μM) were prepared by dilution of stock solution with the leading electrolyte solution. The standards were injected for CE in a sequence from low concentration to high. The peaks recorded were treated by the software 32 Karat, and calibration equations were constructed between concentration (c, μM) and peak area through linear regression and listed in Table 1.

The concentration of each PAA and melamine in migrates (m, μg/L) were then calculated by:

$$m = c \times M \quad (1)$$

while content in milk powders/pet feeds (w, mg kg^{-1}) was calculated by:

$$w = \frac{cVM}{G} \quad (2)$$

Table 1
Quantification features of tITP–CE with leading electrolyte of 80 mM KH_2PO_4-H_3PO_4 (pH 2.85) and sample injected at 0.5 psi for 120 s. The limit of detection (LOD) for common CE was determined with injection at 0.5 psi for 3 s. The running buffer was 80 mM H_3PO_4 adjusted to pH 2.65 with Tris.

Analyte	Equation[a]	Range (μM)	r^2	LOD (μM)	
				tITP–CE	CE
DAPM	$c = -2.515 \times 10^{-2} + 5.482 \times 10^{-5}x$	0.05–5	0.9991	0.02	5
DAT	$c = -5.521 \times 10^{-4} + 7.886 \times 10^{-5}x$	0.1–10	0.9990	0.05	10
Aniline	$c = 1.743 \times 10^{-2} + 6.998 \times 10^{-5}x$	0.1–10	0.9991	0.05	10
Melamine	$c = -9.927 \times 10^{-2} + 6.676 \times 10^{-5}x$	0.1–10	0.9992	0.05	10

[a] c is the concentration of sample in μM and x the peak area.

where M is the molecular weight of the analyte, V the final volume of sample matrix (4 ml) and G the weight of tested milk products (400 mg).

3. Results and discussion

3.1. Method development

3.1.1. Selection of leading and terminating ions for tITP

Since DAPM, DAT and aniline were the most possible existing PAA in food contacting materials and melamine was found in various food products [20,21], they were specially selected in this paper. In order to effectively stack the target analytes in the inlet part of a separation capillary so that a plug of sample can be injected as long as possible, proper leading and terminating ions should be selected which are able to maintain ITP environment sufficiently long. For convenience, the leading ion was added into sample solutions while the terminating ion was added into the BGE. By such as design, a long plug of sample would easily be stacked.

To select a better leading cation, H^+, Na^+ and K^+ were tried and compared, which were available from hydrochloric or phosphoric acid titrated to pH 2.85 with sodium or potassium hydroxide. Fig. 1A shows that K^+ is the best, offering the fastest separation (<10.0 min) with baseline resolution and high peak efficiencies (110.5–43.1 × 10^4 theoretical plates); H^+ is in the second place, yielding the highest resolution but slowest separation (about 12 min), broadest and lowest peaks; and Na^+ is the worst which impacts on the separation seriously although it produces the highest stacking efficiency. With a mobility of 362.5 × 10^{-9} m^2 V^{-1} s^{-1}, H^+ is too fast to offer a sufficient stacking time. Although its stacking performance could be improved somewhat by increasing the concentration of hydrochloric acid, the cost is to further delay the separation [19]. Na^+ with a mobility of 51.9 × 10^{-9} m^2 V^{-1} s^{-1} which is close to the apparent mobility of the first analyte (DAPM) could maintain the longest ITP stacking, but just due to this reason it left very limited time for separation, resulting in poor resolution. Only K^+ has a moderate mobility of 76.2 × 10^{-9} m^2 V^{-1} s^{-1} and was hence selected. Similar to H^+, K^+ caused a slight increase of peak height as its concentration changed from 80 to 160 mM in a form of KH_2PO_4–H_3PO_4 at pH 2.85, due to a longer duration of ITP. Considering that a longer duration of ITP always reduces separation time and hence resolution, high concentration of leading cation is not suggested except for 80 mM KH_2PO_4.

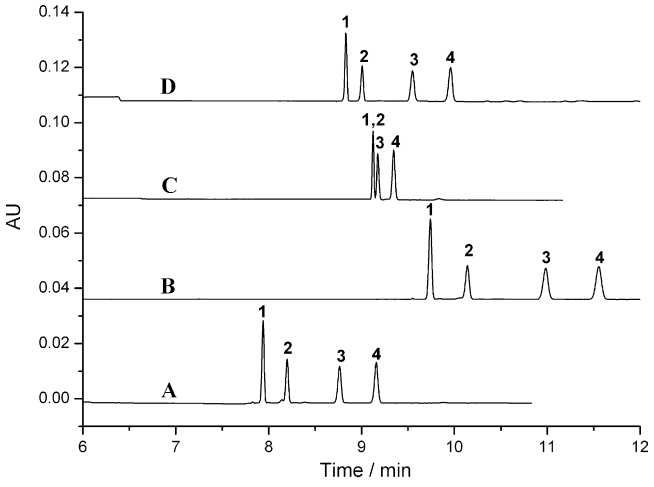

Fig. 1. Impact of leading and terminating ions on stacking and separating efficiencies.
Separation voltage: +20 kV; Injection of aromatic amines (2 μM): 0.5 psi for 120 s; running buffer: 80 mM H_3PO_4 adjusted to pH 2.65 using Tris in (A–C) and Bis–Tris in (D). Leading electrolyte and sample matrix: (A and D) 80 mM KH_2PO_4–H_3PO_4 (pH 2.85), (B) 70 mM HCl and (C) 80 mM NaH_2PO_4–H_3PO_4 (pH 2.85). Peaks identities: (1) DAPM, (2) DAT, (3) aniline and (4) melamine.

Besides the leading ion, the terminating ion in BGE was also examined. After a screening of different co-ions, Tris and Bis–Tris were found to be the most acceptable which could easily be introduced into BGE as a pH adjuster. Fig. 1A and D shows that both Tris and Bis–Tris are able to produce similar stacking efficiency with high resolution, though, Bis–Tris delays a bit separation. For faster separation Tris is suggested. By Tris, the pH values of terminating electrolyte or BGE had only a slight impact on the stacking between 2.00 and 3.00, giving the best at pH 2.65.

Since electroosmotic flow is negligible at the selected pH [22], the neutral impurities will be unable to move forward. On the other hand, the anionic impurities move backward until out of the capillary inlet during electrophoresis. As a result, the system stacks only the cations with mobility in between K^+ and $Tris^+$, helpful the treatment of complex samples.

3.1.2. Injection modes and loadable length

Both pressure and electrokinetic injections were showed to be adoptable. However, the latter introduces much more potassium ions into the capillary than the former, resulting in a serious increase of electrical current and in turn not allowing a free selection of injection time as compared with the pressure injection. In addition, the electrokinetic approach caused injection bias and worse reproducibility than the pressure injection. It is hence not suggested in conducting quantitative analysis, instead, pressure injection is proposed.

In tITP–CE, the two consecutive steps of tITP and capillary zone electrophoresis (CZE) compete for the effective length of a given capillary: An increase of sample plug length to have a longer tITP procedure for better stacking efficiency can result in worse resolution of samples due to the reduction of separation length for CZE, and vice versa. As expected in testing with a capillary having 50 cm effective length and a tITP system of K^+–$Tris^+$, the peak height of aromatic amines increased with the increase of injection time from 30.0 s (2.5 cm) to 150.0 s (12.5 cm), accordingly the resolution decreased from 19.3 to 2.43 for DAPM and DAT (Fig. 2), for example. If the sample plug was increased further to 15.0 cm in length, the pair of DAPM and DAT could not more be resolved. This means that unlimited increase of the sample loading is not possible by tITP–CE or compromise between the sample loading and separation should be considered. Since the capillary length is in most cases fixed or cannot be lengthened over the limitation of high voltage power supply, the sample loading should be confined within a certain length. From Fig. 2, it can be found that the longest injection time allowed is 150 s (ca. 12.5 cm) to keep all the resolution above 2. To be safe, we suggest injecting for 120 s or 10 cm which is 20% of separation length. Under this limitation, the sample loading volume can still be increased for 120/t folds where t is the injection time in common CE, typically 1–3 s at 0.5 psi. The effectiveness of tITP–CE–UV has been validated by CE of mixed PAA and melamine

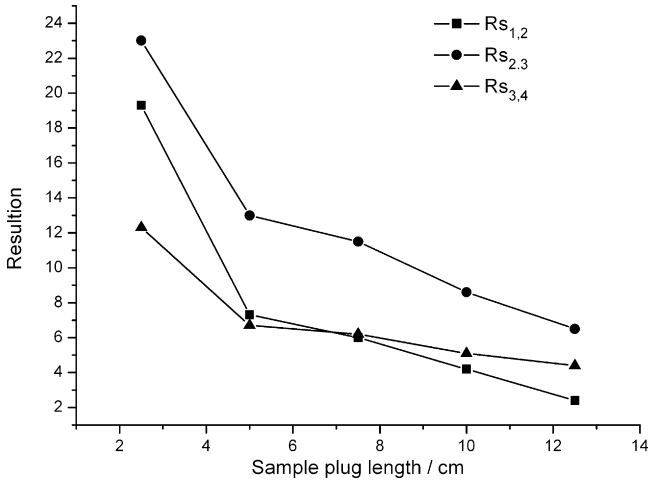

Fig. 2. Plot of sample zone length against resolution measured by tITP–CE with 80 mM KH_2PO_4–H_3PO_4 (pH 2.85) as a leading electrolyte and 80 mM H_3PO_4–Tris (pH 2.65) as running buffer. Other conditions were the same as in Fig. 1. $Rs_{1,2}$ is the resolution between DAPM and DAT, $Rs_{2,3}$ that of DAT and aniline, and $Rs_{3,4}$ of aniline and melamine.

prepared in different matrices. Fig. 3 shows that the method can increase the detection sensitivity for about 200 folds by a direct comparison between Fig. 3A and B.

3.2. Quantification feature of tITP

Standard working equations for the four target aromatic amines were constructed between concentration (c) and peak area (x) listed in Table 1. The linear range of the working equations covered about two orders of magnitude, with a linear correlation coefficient (r^2) of higher than 0.999.

The reproducibility of the developed method was examined in both cases of inter-day and intra-day through six replicate runs of mixed standard analytes at 2.0 μM. Table 2 lists the relative standard deviations (RSDs) measured. It shows that the reproducibility is ideal: the variation of migration time is less than 0.75% and 1.3%, for intra- and inter-day runs, respectively, and that of peak area is within 4.3% and <6.7% for intra- and inter-day, respectively. They are all less than 7.0%. By keeping the injection time at 120 s, the LOD measured at S/N = 3 was down to 20 nM for DAPM, about

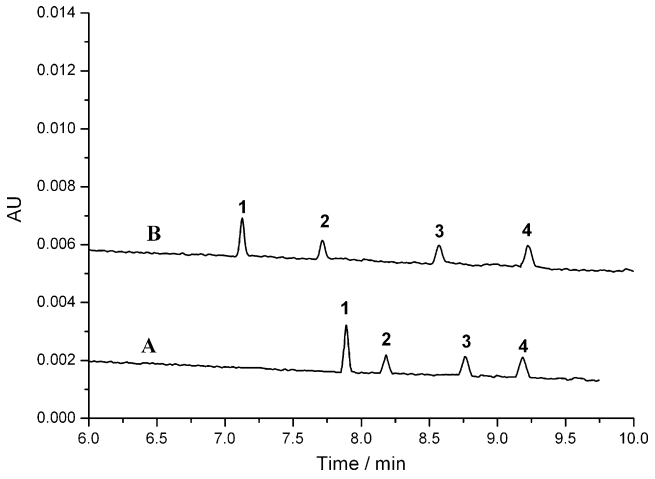

Fig. 3. Comparison of (A) tITP with (B) normal pressure injection measured from (A) 0.10 μM and (B) 20 μM aromatic amines prepared in 80 mM KH_2PO_4–H_3PO_4 (pH 2.85) and running buffer, respectively. Injection was conducted at 0.5 psi for (A) 120 s and (B) 3 s. Other conditions were the same as in Fig. 1.

Table 2
Intra-day and inter-day relative standard deviation (RSD%, $n = 6$) of migration times and peak areas measured using the conditions as shown in Fig. 1.

Analyte	Intra-day RSD (%)		Inter-day RSD (%)	
	Migration time	Peak area	Migration time	Peak area
DAPM	0.41	3.9	1.2	5.2
DAT	0.47	4.0	1.3	6.7
Aniline	0.52	4.3	1.3	6.2
Melamine	0.75	3.8	1.5	5.9

Table 3
Assaying of powder foods averaged from six measurements.

Food sample	Concentration[a] (mg kg^{-1} ± SD)			
	Melamine	Aniline	DAT	DAPM
Milk powder 1	0.242 ± 0.023	–	–	–
Milk powder 2	63.6 ± 4.9	–	–	–
Soy milk powder 1	–	–	–	–
Pet feed 1	–	–	–	–
Pet feed 2	–	–	–	–

[a] The dash (–) represents the concentration is below LOD.

200–250-fold improvement in detection sensitivity compared with the common CE (Table 1, the last column).

3.3. Assay of milk powders and pet feeds

For validation, the developed method was first used to assay commercial milk powders and pet feeds available in local supermarkets (Table 3). After extracted from milk powders or pet feeds, deproteined by centrifugation and reinstituted in leading electrolyte solution, the real samples were then subjected to tITP–CE–UV, further treatment of the samples by solid phase extraction was not required, which is necessary for GC and HPLC [5]. Fig. 4 shows that the electropherogram is clean and there is no aromatic amines detected other than melamine (peak 4 in Fig. 4A) for milk powder 1. To further validate this tITP method, known amounts of all the four aromatic amines were added directly into milk powder 1 at three levels just before extraction. Their recoveries measured and averaged over six measurements were between 92.0% and 107.1%, with RSDs between 3.52% and 6.23% (Table 4).

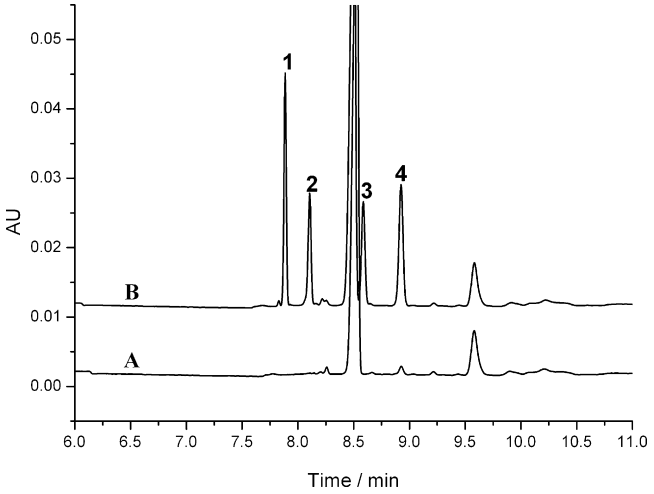

Fig. 4. Electropherograms measured by tITP–CE with a leading electrolyte of 80 mM KH_2PO_4–H_3PO_4 (pH 2.85) and running buffer of 80 mM H_3PO_4–Tris (pH 2.65). The sample was extracted from the milk powder 1 (referred to Table 3) spiked (A) without or (B) with 5.56 mg kg^{-1} DAPM, 3.42 mg kg^{-1} DAT, 2.60 mg kg^{-1} aniline and 3.08 mg kg^{-1} melamine. Other conditions and peak identity were the same as in Fig. 1.

Table 4
Recovery test using milk powder 1 as a matrix ($n = 6$).

Analyte	Added (mg kg^{-1})	Found (mg kg^{-1})	Recovery[a] (%)	RSD (%)
DAPM	5.56	5.33	95.9	4.53
	0.922	0.900	97.6	3.52
	0.397	0.410	103.3	6.23
DAT	3.42	3.28	95.9	5.10
	0.611	0.598	97.9	3.52
	0.186	0.196	105.4	5.33
Aniline	2.60	2.40	92.0	4.10
	0.466	0.438	94.0	3.99
	0.186	0.196	105.4	5.33
Melamine	3.08	3.20	95.9	6.10
	0.631	0.862	98.3	4.33
	0.252	0.512	107.1	5.17

[a] Recovery was calculated by equation of 100 × (Found − Background)/Added, where the Bacground value of zero (under LOD) was used except for melamine that gave a value of 0.242 mg kg^{-1} as shown in Table 3.

Table 5
Migration testing of three unused and five used composite food packaging bags with water as a food simulant.

Bags number	Concentration in simulant ($n = 6$)[a] (μg L^{-1} ± SD)			
	DAPM	DAT	Aniline	Melamine
Unused 1	1.22 ± 0.11	–	–	–
Unused 2	–	–	–	–
Unused 3	–	–	–	–
Used 1	–	–	–	–
Used 2	–	–	–	–
Used 3	1.19 ± 0.11	2.20 ± 0.19	–	–
Used 4	1.92 ± 0.16	–	–	–
Used 5	–	–	–	–

[a] The dash (–) represents the concentration is below LOD.

From Table 3, melamine was found in two kinds of milk powders and no aromatic amines were found in the five tested samples. According to the FDA of USA, a melamine content less than 2.5 mg kg^{-1} in milk products does not raise concerns for human health. By our method, the limit of quantification (LOQ, signal-to-nose is 10) was 0.126 mg kg^{-1}, about 19 folds better than the FDA regulation.

3.4. Assay of both unused and used composite food packaging bags

In combination with an off-line 10-fold concentration step, the tITP–CE–UV was demonstrated to be also applicable to the analysis of migrates from composite food packaging bags. Its LOD was 0.40, 0.62, 0.47 and 0.63 μg/L for DAPM, DAT, aniline and melamine, respectively, and LOQ 0.80, 1.24, 0.94, 1.26 μg/L, respectively. These LOQs are lower than the EU regulation suggested level (20 μg/L, total amines [4,7]). With such a low LOQ, we have found only one unused and two used food packaging bags containing low quantity of DAPM and one used bag containing DAT (Table 5).

4. Conclusions

After optimization, the method of tITP–CE–UV explored in this article was able to produce up to 250-fold stacking efficiency for PAA/melamine and to reduce the separation time to less than 10.0 min runtime which is comparable with HPLC [2] but better than GC [7]. The method was validated to be applicable to the analysis of both powdered foods (milk powders and pet feeds) and migrates from composite food packaging bags (both unused and used), with a linear working range over two orders of magnitude and recoveries of 92.0–107.1%. In assay of migrates from composite food packaging bags, by combing an off-line concentration step of 10 folds, the LODs of PAA and melamine in aqueous food simulant were all lower than 1 μg/L, capable of satisfying the legislation of EU. If these quantification features are considered together with the fast running speed, minute consumption of samples, facile operation and more or less simplification of sample preparation procedure, the present method should be preferred.

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