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**Validation of an ion-pair liquid chromatography-electrospray-tandem mass spectrometry method for the determination of heterocyclic aromatic amines in meat-based infant foods**

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# Validation of an ion-pair liquid chromatography-electrospray-tandem mass spectrometry method for the determination of heterocyclic aromatic amines in meat-based infant foods

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## Abstract

A method based on ion-pair liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS) is reported for determining heterocyclic aromatic amines (HAAs) in meat-based infant foods. The HAAs encompassed quinoline (IQ, MeIQ), quinoxaline (MeIQx), pyridine (PhIP) and carboline derivatives (A $\alpha$ C, Harman, Norharman) with d<sub>3</sub>-IQ, <sup>13</sup>C<sub>2</sub>-MeIQx and d<sub>3</sub>-PhIP used as labeled internal standards. The method used extraction into acetone followed by a clean-up on an SCX solid-phase extraction column. LC separation was performed on a TSKgel ODS-80TS column (250 x 2.0 mm, 5 $\mu$ m), the mobile phase being an ammonium formate-formic acid buffer (3.03 mM ammonium formate, pH=2.8) aqueous solution-acetonitrile gradient at a flow rate of 0.2 ml min<sup>-1</sup>. For unequivocal identification of each analyte, three ions were detected and chosen for selected reaction monitoring (SRM). Validation was carried out on lyophilized meat samples. Mean recoveries ranged between 78 $\pm$ 4% and 98 $\pm$ 2% for different analytes. Limits of quantification generally lower than 8 ng/g were demonstrated in meat samples for the analytes investigated. The method exhibited good linearity and repeatability. Robustness testing identified those factors which were statistically significant in influencing chromatographic separation and response, and indicated which parameters have to be strictly controlled for a reliable analysis of HAAs. In particular, mobile phase flow-rate was found to be statistically significant ( $\alpha = 0.05$ ) for the capacity factor (k') of all analytes except for A $\alpha$ C peak, whereas mobile phase pH resulted to be a critical parameter for the k' values of IQ, MeIQ and Norharman. The method proved to be robust *versus* resolution between IQ and MeIQ peaks. Among mass spectrometric parameters, collision energy was found to significantly affect quantitative response of all analytes except that of IQ. The applicability of the method to the analysis of meat-based infant food samples was demonstrated.

1  
2  
3 **Keywords:** Heterocyclic aromatic amines; Ion-pair liquid chromatography-mass spectrometry;  
4 Validation; Robustness  
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## 8 **Introduction**

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11 Heterocyclic aromatic amines (HAAs) are substances of great concern for food safety being  
12 recognized as potent mutagenic compounds to which humans are regularly exposed through diet  
13 and ambient environments [Skog et al. 1998]. These compounds are formed in thermally treated  
14 protein-rich foods at the ng/g level, but they are also detected in several other environments,  
15 including cooking fumes, cigarette smoke, air, rain, and river water [Kataoka, 1997].  
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25 For the unambiguous identification and determination of these mutagenic and carcinogenic  
26 substances in different kinds of food commodities it is therefore necessary to develop and  
27 validate fast and reliable analytical methods. Due to the great complexity of food matrices  
28 and the low concentration levels of HAAs, their identification and determination require a  
29 comprehensive analytical approach involving the use of efficient sample preparation  
30 techniques and the employment of hyphenated analytical techniques. Solid-phase  
31 extraction (SPE) under cationic exchange and reversed-phase mechanisms have been  
32 described as the most widely used methods for their isolation and clean-up [Gross and  
33 Grüter, 1992; Toribio et al., 1999]. High-performance liquid chromatography (HPLC), gas  
34 chromatography (GC), and capillary electrophoresis have been used for separation  
35 purposes [Knize et al., 1992; Pais, and Knize, 2000]. At the present the most widely applied  
36 technique for HAA analysis is liquid chromatography coupled with mass spectrometry (LC-  
37 MS), since LC-MS-based techniques allow to avoid time-consuming derivatization  
38 procedures needed prior to GC-MS determination. In particular, LC coupled with tandem  
39 mass spectrometry (MS-MS) using both triple quadrupole and ion trap mass  
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3 spectrometers have proved highly successful in the determination of HAAs [Klassen et al  
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6 2002; Toribio, et al 2002; Barceló-Barrachina et al., 2004a; 2004b]. The applicability of a LC-  
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9 electrospray-quadrupole time of flight (ESI-QTOF)-MS and LC-ESI-QTOF-MS/MS  
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11 techniques for the identification and determination of HAAs in foods has been also  
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13 investigated using high mass accuracy measurements [Barceló-Barrachina et al., 2004b].  
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15 Very recently a low-time consuming solid-phase extraction procedure was developed  
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17 together with a liquid chromatography-ion trap mass spectrometry method for their  
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20 determination in griddled beef steak [Toribio, et al., 2006].  
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27 In our research work dealing with the investigation of the separation of HAAs (figure 1) by  
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29 reversed-phase ion-pair LC-MS/MS [Bianchi, et al., 2005], we demonstrated that under gradient  
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31 conditions the use of an eluent containing formate buffer at pH 2.8 proved more effective in terms  
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33 of chromatographic behaviour and detectability of these analytes than the use of a mobile phase  
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35 containing the acetate buffer, which is commonly used for the analysis of HAAs.  
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38 The need of a standard methodology and validated analytical methods for HAAs analysis is actually  
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40 of great interest. In particular, interlaboratory exercises of the determination of HAAs in meat  
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42 extract laboratory reference materials have been recently presented highlighting the importance to  
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44 improve the quality of the analytical measurements [Santos et al., 2004.].  
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47 In this context the aim of the present work was the single-laboratory validation of the previously  
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49 developed chromatographic method to be applied to meat-based infant foods to measure their HAA  
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51 content. A procedure entailing solvent extraction and clean-up by SPE was used. Besides to  
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53 linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and recovery, attention  
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55 was focused to the evaluation of method robustness towards both chromatographic and mass  
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57 spectrometric parameters.  
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## 59 **Experimental**

### 60 *Chemicals*

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3 Harman (1-methyl- $\beta$ -carboline) and norharman (9H-pyrido[3,4-*b*] indole) were from Sigma-Aldrich  
4 (Germany). IQ (2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoline), MeIQ (2-amino-3,4-dimethyl-3H-  
5 imidazo[4,5-*f*]quinoline), MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline), PhIP (2-amino-  
6 1-methyl-6-phenylimidazo[4,5-*b*]pyridine), A $\alpha$ C (2-amino-9H-pyrido[2,3-*b*]indole), Isotopically  
7 labelled IQ-D<sub>3</sub>, <sup>13</sup>C<sub>2</sub>-MeIQx e D<sub>3</sub>-PhIP were from Toronto research Chemicals Inc. (Canada).  
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14 Stock standard solutions of HAAs and internal standards at concentrations of 0.5 mg/mL were  
15 prepared in methanol and stored in the dark at 4°C. Working standard solutions were prepared daily  
16 for a better stability by diluting stock solutions with HPLC-grade water. Ammonium formate,  
17 formic acid, HPLC-grade acetonitrile and HPLC-grade water were from Carlo Erba (Milan, Italy).  
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### 23 *Liquid chromatography-mass spectrometry*

24 An Alliance 2690 liquid chromatograph (Waters, Milford, MA, USA) equipped with a 120-vial  
25 capacity sample management system was used. Chromatographic separation was obtained using a  
26 TSKgel ODS-80TS (250 x 2.0 mm, 5 $\mu$ m) (TOSOH BIOSEP, Germany) column under gradient  
27 conditions. The mobile phase consisted of a mixture of ammonium formate-formic acid buffer  
28 (pH=2.8) (v/v) aqueous solution (eluent A) and acetonitrile (eluent B) delivered at a flow-rate of 0.2  
29 mL min<sup>-1</sup>. The gradient was as follows: at 0 min 5% B, in 20 min 54.5% B hold for 3 min, back to  
30 5% B in 3 min and 10 min to re-equilibrate the column.  
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39 A Quattro LC triple quadrupole-mass spectrometer (Micromass, Manchester, UK) with a  
40 pneumatically-assisted electrospray interface was used. Data acquisition was performed in positive  
41 ion mode (ESI(+)). Interface parameters were set as follows: capillary voltage 2.8 kV, cone voltage  
42 50 V (IQ, MeIQ, MeIQx, Norharman, Harman, PhIP) and 40 V (A $\alpha$ C), extractor lens voltage 2 V,  
43 source temperature 110°C, desolvation temperature 240°C, rf lens 0.1 V. The nebulizer and  
44 desolvation gases (nitrogen, 99.999 % high-purity) were delivered at 60 L hr<sup>-1</sup> and 550 L hr<sup>-1</sup>  
45 respectively.  
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53 Experiments for optimization of ESI interface parameters were performed by directly infusing  
54 solutions into the ESI-MS system at a flow-rate of 6  $\mu$ L min<sup>-1</sup>. Full-scan analyses were performed  
55 over the scan range m/z 150-300 using a step size of 0.1 Da and a rate of 0.4 scans/s. Operating in  
56 MS/MS mode, product-ion scan mass spectra of protonated molecules were acquired in the m/z 30-  
57 300 range. Selected reaction monitoring (SRM) analyses were performed as follows: m/z 199 $\rightarrow$ 184  
58 (collision energy, CE 26 eV), m/z 199 $\rightarrow$ 157 and m/z 199  $\rightarrow$ 130 (CE 40 eV) for IQ, m/z 202 $\rightarrow$ 184  
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60

(CE 27 eV) for d<sub>3</sub>-IQ, m/z 213→198 (CE 27 eV), m/z 213→170 (CE 40 eV) and m/z 213→117 (CE 50 eV) for MeIQ, m/z 214→131 (CE 40 eV), m/z 214→199 (CE 28 eV) and m/z 214→77 (CE 60 eV) for MeIQx, m/z 215→200 (CE 30 eV) for <sup>13</sup>C<sub>2</sub>-MeIQx, m/z 169→115 (CE 32 eV), m/z 169→89 (CE 50 eV) and m/z 169→142 (CE 30 eV) for Norharman, m/z 183→115 (CE 32 eV), m/z 183→89 (CE 50 eV) and m/z 183→168 (CE 30 eV) for Harman, m/z 225→210 (CE 30 eV), m/z 225→140 and m/z 225→115 (CE 50 eV) for PhIP, m/z 228→210 (CE 32 eV) for PhIP-D<sub>3</sub>, m/z 184→140 (CE 30 eV), m/z 184→167 (CE 25 eV) and m/z 184→113 (CE 50 eV) for AαC. The dwell time and the interchannel delay were set at 0.25 s and 0.01 s respectively.

For data acquisition and processing the Masslynx v4.0 software was used.

### **Samples.**

#### *Solvent extraction and SPE procedure*

Beef-based infant foods were purchased in big trades. Three homogenized and one lyophilized beef-based infant foods coming from different manufacturers were analyzed. Horse-, lamb-, rabbit- and chicken-based infant foods from three different manufactures were also analyzed.

To 1.0 g of homogenized sample weighed into a polypropylene graduated conical tube with cap 12 mL of acetone. After mixing for 5 min on a vortex, the extract was centrifuged at 4800 rpm (1350 g) for 5 min. Extraction was performed twice. The clarified solution was removed and stored in the freeze (-18°C) for one hour to allow protein to precipitate. The mixture was centrifuged at 4800 rpm (1350 g) for 5 min and 24 mL of the final extract were loaded on the SPE cartridges. Strong cation exchange (benzenesulfonic-SCX bonded silica) cartridges (300 mg, 6 mL) (Supelco) were used for concentration and clean-up purposes. Cartridges were first conditioned with 4 mL of methanol and 4 ml acetone, 24-mL sample were then loaded and eluted with 4-ml 2 M ammonium acetate aqueous solution/acetone (50/50, v/v). The volume of the final extract was then reduced to 2 mL in a graduate vial by evaporating acetone under a nitrogen stream. The sample was filtered and directly injected (injection volume 30 µl) onto the LC system.

Sample treatment based on solvent extraction followed by SPE was carried out three times for each sample and three LC replicate analyses were run for each extract.

Recovery studies were carried out by spiking samples of lyophilized meat at two different concentration levels (10 and 50 ng/g) with appropriate volumes of the working mixtures of HAAs.

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3 The mixture was left to stand overnight at 4° C. The sample was equilibrated to room temperature  
4 before following the extraction procedures above described.  
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#### 8 *Validation of the LC-ESI-MS/MS method*

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10 Validation of the developed method was carried out following EURACHEM guidelines [Anon,  
11 1998] on a lyophilized meat matrix extracted and purified using the procedure described.  
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15 Detection limit ( $y_D$ ) and quantitation limit ( $y_Q$ ) were preliminarily calculated as signals based on the  
16 mean blank ( $\bar{x}_b$ ) and the standard deviation ( $s_b$ ) of the blank signals as follows:  
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$$19 \quad y_D = \bar{x}_b + 2t s_b \quad y_Q = \bar{x}_b + 10 s_b$$

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21 where  $t$  is a constant of the  $t$ -Student distribution (one-sided) depending on the confidence level and  
22 the degrees of freedom ( $v=n-1$ ,  $n$ =number of measurements). Ten blank measurements were  
23 performed to calculate  $\bar{x}_b$  and  $s_b$ . A lyophilized beef based sample extract in which the analytes  
24 were verified to be absent was used as the blank matrix.  $y_D$  and  $y_Q$  were converted from the signal  
25 domain to the concentration domain to estimate limit of detection (LOD) and limit of quantitation  
26 (LOQ) respectively, using an appropriate calibration function. In order to satisfy basic requirements  
27 such as homoscedasticity and linearity, the Bartlett test and the Mandel's fitting test were performed  
28 at the 95% confidence level.  
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38 Linearity was established over two orders of magnitude of concentration calculating matrix-  
39 matched calibration curves.  $d_3$ -IQ,  $^{13}C_2$ -MeIQx and  $d_3$ -PhIP labelled internal standard was used. Six  
40 equi-spaced concentration levels were chosen and three replicated injections were performed at  
41 each level. Homoscedasticity test was run and the goodness of fit of the calibration curve was  
42 assessed by applying the lack-of-fit and Mandel's fitting tests [Funk et al., 1995]. A  $t$ -test was  
43 carried out to verify the significance of the intercept (confidence level 95%).  
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50 Accuracy was tested in terms of precision and trueness. Precision was calculated in terms of intra-  
51 day and between-day repeatability as R.S.D.% at two concentration levels (10 and 50 ng/g).  
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53 Trueness was evaluated by calculating the recovery function [Funk et al., 1995], which allows to  
54 assess both constant and proportional systematic errors.  
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57 Robustness of the analytical method was tested using a two-level Plackett-Burman (PB) design.  
58 Eleven factors (eight real factors and three dummy variables) were considered and 12 experiments  
59 were carried out. The PB design was applied in order to identify the critical factors influencing the  
60 following responses: relative peak area (analyte area/IS area) (IQ, MeIQ, MeIQx, Norharman,



Harman, PhIP, A $\alpha$ C); resolution (Rs) (IQ, MeIQ); capacity factor (k') (IQ, MeIQ, MeIQx, Norharman, Harman, PhIP, A $\alpha$ C); asymmetry factor (T) (IQ, MeIQ, MeIQx, Norharman, Harman, PhIP, A $\alpha$ C). Eight critical factors which were believed to affect the chromatographic results were chosen, three of which referred to the mass spectrometer: the mobile phase flow-rate (ml/min), the percentage of organic modifier (acetonitrile) at the beginning and at the end of the gradient (%), the pH and the concentration of ammonium formate in the mobile phase, the column temperature ( $^{\circ}$ C), the cone voltage (V), the capillary voltage (kV) and the collision energy applied in the collision cell of the mass spectrometer (eV). Experimental conditions were set selecting symmetrical values around the nominal level which reflected the variations that could be encountered in laboratory during the mobile phase preparation or due to instrumental fluctuations (9-20% of the nominal value) (Tables 1 and 2). The three dummy variables included in the design were used to evaluate the variability of the procedure. The effects were calculated as follows:

$$E_x = \frac{\sum Y(+)}{N/2} - \frac{\sum Y(-)}{N/2}$$

where  $E_x$  is the effect of factor X on response Y; Y(+) and Y(-) are the responses where X is at the level (+) and (-) respectively, N=12 is the total number of experiments.

The standard error  $(SE)_e$  was evaluated using the dummy variables as follows:

$$(SE)_e = \sqrt{\frac{\sum E_{dummy}^2}{n_{dummy}}}$$

Significance of an effect was evaluated at both  $\alpha = 0.05$  and  $\alpha = 0.01$  significance levels. An effect was considered significant at a given  $\alpha$  level if  $|E_x| > E_{critical} = t_{critical}(SE)_e$ , where  $t$  is a constant of the  $t$ -Student distribution (one-sided) depending on the confidence level and the degrees of freedom ( $v=n-1$ ,  $n$ =number of measurements). The matrix of experiments is illustrated in Table 3. The experiments were carried out in random order and for each experiment a single injection was performed. Two experiments at the nominal level (one before and one after the experimental design execution) were run in order to assess the absence of instrumental drift. Method robustness was evaluated at 100  $\mu$ g/L level on standard solutions. A blank injection was performed prior to each analysis.

All statistical analyses and tests were performed by using the statistical package SPSS v. 9.0 for Windows (SPSS, Bologna, Italy).

## Results and Discussion

### *LC-ESI(+)-MS and- MS/MS performances*

The separation of the investigated analytes was performed in less than 20 min with good resolution [Toribio, et al., 2006] (figure 2).

Under ESI(+)-MS conditions, mass spectra of all compounds showed predominant  $[M+H]^+$  ions without fragmentation. The description of the product ion MS/MS spectra as obtained by low-energy collision-induced dissociation of protonated HAA molecules were previously depicted [Toribio, et al., 2006]. HAA extraction was performed from meat samples by using acetone as already proposed by Felton et al. [1981; 1984] who successfully applied this solvent for HAA separation from food by liquid–liquid extraction.

In the development of the SPE procedure for purification and concentration of HAAs in meat products, mixtures of acetone/aqueous solution of ammonium acetate at different concentrations (0.5, 2 and 3 M) (50/50) were evaluated as eluting solvents. The method was assessed on the basis of the qualitative and quantitative results obtained on the meat-based food used in this work. The best results were obtained by using 2M ammonium acetate aqueous solution/acetone (50/50, v/v). Under these conditions, recovery values ranging from  $78\pm 4\%$  to  $98\pm 2\%$  (RSD<12%, n=12) were obtained for the analytes.

SPE extracts did not show the presence of any interference in correspondence to the transitions monitored for the amines. However, suppression effects were observed in correspondence to the peaks of all the analytes, suggesting the presence of not detected co-eluting matrix compounds in the extracts. On the basis of these findings, further investigation on matrix suppression effect was performed during method validation.

### *Calibration and method performances*

To check method performance, quality parameters such as robustness, detection limit, quantification limit, linearity range, precision and trueness were studied in matrix.

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5 Since the LC-MS/MS method developed is intended for routine use, its robustness, i.e. the capacity  
6 to remain unaffected by small variations in nominal experimental conditions, was tested at the  
7 beginning of the validation procedure. For this purpose, a screening design such as the Plackett-  
8 Burman was used. Factors were selected among those that are most likely to be controlled when a  
9 method is transferred between laboratories and that could influence the responses of the method.  
10 The results of the robustness test in terms of the effects of the different factors on the considered  
11 responses are shown in Table 4. The factors having a statistically significant effect on a response at  
12 significance level of 1 and 5% were indicated. Relative peak area resulted to be significantly  
13 affected both by chromatographic and MS/MS parameters. In particular, under the conditions used  
14 in this work, the analyte-internal standard area ratio resulted to be affected by mobile phase flow-  
15 rate, ion pair reagent concentration, column temperature (only for MeIQx), cone voltage (only for  
16 A $\alpha$ C), collision energy (for all analytes except for MeIQx and IQ). These findings suggest that  
17 these parameters should be strictly controlled for a reliable quantification of HAAs. None of the  
18 factors resulted to have significant effect on resolution between IQ and MeIQ peaks. The results  
19 obtained for the other factors evidenced that mobile phase flow-rate was found to be statistically  
20 significant ( $\alpha = 0.05$ ) for the capacity factor of all analytes except for A $\alpha$ C peak. In addition,  
21 mobile phase pH resulted to be a critical parameter for the  $k'$  values of IQ, MeIQ and norharman.  
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37 Under the optimized chromatographic and MS/MS conditions, operating in SRM mode, LOD and  
38 LOQ of HAAs in lyophilized meat samples were at levels lower than 5 and 8 ng/g respectively,  
39 except for PhIP (Table 5).  
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44 Using external calibration, good linearity of the method was established over two orders of  
45 magnitude ( $r^2 = 0.998-0.999$ ,  $n=18$ ) on both solvent and matrix. Linearity was demonstrated by  
46 verifying homoscedasticity over the range tested (Bartlett test) and by applying the Mandel fitting  
47 test (Table 5). The latter showed that a quadratic regression did not provide a better fitting than the  
48 linear one.  
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55 Excellent precision in terms of intra-day repeatability was calculated, RSD% values ranging from 1  
56 to 4% ( $n=5$ ) for all the analytes. Between-day precision calculated over three days provided RSD  
57 values lower than 10% for IQ, MeIQx and PhIP, whereas greater variability was observed for the  
58 analysis of MeIQ and A $\alpha$ C, being calculated RSD of ca. 25% for these compounds.  
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3 Finally, the calculation of the recovery functions to test trueness of the developed method was  
4 performed by spiking and analyzing lyophilized meat samples. The intercepts of the recovery  
5 functions calculated from these data were compared with 0 by means of a *t*-test. For all analytes the  
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Finally, the calculation of the recovery functions to test trueness of the developed method was performed by spiking and analyzing lyophilized meat samples. The intercepts of the recovery functions calculated from these data were compared with 0 by means of a *t*-test. For all analytes the *t* values calculated for the intercepts were lower than the tabulated value at 95% confidence level, thus demonstrating that constant systematic errors are not present. Instead, the presence of proportional systematic errors was evidenced, since slope values of the recovery functions were demonstrated to be significantly different from 1 ( $p < 0.05$ ). These findings suggest that to attain accuracy in the quantitative analysis of HAAs in lyophilized meat samples the use of the internal labelled standard method is recommended.

#### *Determination of HAAs in meat based infant foods using the LC-ESI-MS/MS method*

The applicability of the LC-ESI-MS/MS method was then evaluated for the identification and determination of heterocyclic aromatic amines in meat-based infant foods.

With the aim to adding confidence to the identity of the analyte, three fragmentation transitions per compound present in the samples were monitored, thus complying with MS confirmation criteria as established by 1999/333/EG [20]. After confirmation, the quantitative assay of HAAs identified in the samples was performed using suitable calibration curves (Table 6). Among the samples investigated, norharman and harman were detected in all samples considered at concentrations up to  $8.9 \pm 0.3$  and  $19 \pm 3$  ng/g respectively, whereas MeIQx was detected only in one beef-based infant sample at a concentration value under the quantification limit. Figure 3 illustrates the LC-ESI(+)-MS/MS extract transition chromatograms of the HAAs investigated in one infant food sample.

## **Conclusions**

This study validated the ion pair HPLC under gradient conditions and ESI(+)-MS/MS detection method for the simultaneous determination of a number of heterocyclic aromatic amines in beef-based infant foods. Even if lower detection limits are demanded in this kind of application, valuable information was achieved from the robustness study, which indicated those factors with statistical significance which influence chromatographic separation and response.

The evaluation of matrix effects by means of the recovery function allowed us to exclude the presence of constant systematic errors, not otherwise detectable by using a labelled internal standard. The validation data demonstrate that this method is convenient for routine analysis of

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3 heterocyclic aromatic amines in meat products, since a good repeatability was shown and detection  
4 limits and quantification limits were in the low ng/g level.  
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10  
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13 natural trace contaminants".  
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Table 1  
Factors investigated in the Plackett-Burman design of robustness test

Factors	Units	Level (-)	Nominal level (0)	Level (+)
Mobile phase flow-rate	ml/min	0.18	0.2	0.22
Ion pair reagent concentration	mM	2.73	3.03	3.33
pH of the buffer	-	2.52	2.8	3.08
Column temperature	°C	30	35	40
Percentage organic modifier (%B) in the mobile phase at the start of the gradient	%	4.5	5	5.5
Percentage organic modifier (%B) in the mobile phase at the end of the gradient	%	52	54.5	57
Collision energy	eV	- *	0 *	+ *
Cone voltage	V	- *	0*	+*

\*Different conditions were used for each analyte (see Table 3)



Table 2. Cone voltage and collision energy values used for each analyte in the PB design.

Analyte	Level (-)		Nominal level (0)		Level (+)	
	CE (eV)	cone voltage (V)	CE (eV)	cone voltage (V)	CE (eV)	cone voltage (V)
IQ	24	27	26	50	29	55
MeIQ	45	45	27	50	30	55
MeIQx	24	27	40	50	44	55
Norharman	45	36	32	50	35	55
Harman	36	24	50	50	55	55
PhIP	45	45	30	50	33	55
A $\alpha$ C	29	27	30	45	33	44
D <sub>3</sub> -IQ	45	45	27	50	30	55
<sup>13</sup> C <sub>2</sub> -MeIQx	45	29	30	50	33	55
D <sub>3</sub> -PhIP	45	50	32	55	35	61

Table 3

Matrix of the Plackett- Burman design (- = low factor level, + = high factor level)

Experiment No.	Factors <sup>a</sup>										
	A	B	C	D <sup>b</sup>	E	F	G	H	I <sup>c</sup>	J	K
1	+	+	-	+	+	+	-	-	-	+	-
2	+	+	-	-	-	+	-	+	+	-	+
3	-	+	+	-	+	+	+	-	-	-	+
4	-	+	+	+	-	-	-	+	-	+	+
5	-	+	-	+	+	-	+	+	+	-	-
6	+	+	+	-	-	-	+	-	+	+	-
7	+	-	+	+	+	-	-	-	+	-	+
8	+	-	+	+	-	+	+	+	-	-	-
9	-	-	+	-	+	+	-	+	+	+	-
10	+	-	-	-	+	-	+	+	-	+	+
11	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	+	-	+	+	-	+	+	+

<sup>a</sup> (A) mobile phase flow rate (mL/min); (B) salt concentration (mM); (C) dummy 1; (D) collision energy (eV); (E) dummy 2; (F) acetonitrile at the start of the gradient (%); (G) dummy 3; (H) acetonitrile at the end of the gradient (%); (I) cone voltage (V); (J) column temperature (°C); (K) pH of the buffer.

<sup>b,c</sup> The values used for each analyte are reported in Table 2

Table 4  
Results of the robustness test in terms of the effects of the different factors on the considered responses

Analyte	Factors							
	A <sup>1</sup>	B <sup>1</sup>	C <sup>1</sup>	D <sup>1</sup>	E <sup>1</sup>	F <sup>1</sup>	G <sup>1</sup>	H <sup>1</sup>
IQ	k'***							k'***
MeIQ	k'***	As**	A/A <sub>IS</sub> **	k'***			k'* As**	k'***
MeIQx	k'*** A/A <sub>IS</sub> **	A/A <sub>IS</sub> **					A/A <sub>IS</sub> **	
Norharman	k'*** As**		A/A <sub>IS</sub> **					k'*** As**
Harman	k'***		A/A <sub>IS</sub> **					
PhIP	k'*** As**		A/A <sub>IS</sub> **					
AαC			A/A <sub>IS</sub> **			A/A <sub>IS</sub> *		

<sup>1</sup> (A) mobile phase flow-rate; (B) ion-pair reagent concentration; (C) collision energy; (D) organic modifier percentage at the start of the gradient; (E) organic modifier percentage at the end of the gradient; (F) cone voltage; (G) column temperature; (H) pH of the buffer

\* Significance level 5%.

\*\* Significance level 1%.

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Table 5

LOD and LOQ values and matrix-matched calibration curves established in lyophilized meat extracts using LC-ESI-MS/MS method.

Calibration function:  $y=b_1x$ .

Analyte	LOD	LOQ	Concentration range (ng/g)	Homoscedasticity test <sup>b</sup>	Mandel's test <sup>b</sup>	$b_1(\pm s_{b1})^b$
	Matrix: lyophilized meat (ng/g) <sup>a</sup>					
IQ	5.4	7.8	10-1000	0.11	0.23	0.93(±0.03)
MeIQ	1.8	3.8	10-1000	0.06	0.51	1.98(±0.08)
MeIQx	4.1	8.1	10-1000	0.08	0.06	0.367(±0.013)
Norharman	0.5	1.0	1-500	0.12	0.05	2.37(±0.12)
Harman	0.5	1.0	1-500	0.34	0.21	4.3(±0.2)
PhIP	12	26	40-2200	0.25	0.47	0.073(±0.002)
AαC	3.7	7.4	40-1350	0.45	0.19	0.63(±0.02)

<sup>a</sup> Referred to 1g sample extracted with 2 mL solvent. See Experimental.

<sup>b</sup> Confidence level 95%.

Table 6

Determination of HAAs in infant meat based samples <sup>a</sup>

Analyte	Beef <sup>a</sup>	Chicken <sup>a</sup>	Horse <sup>a</sup>	Lamb <sup>a</sup>	Rabbit <sup>a</sup>
	(ng/g)				
IQ	- <sup>b</sup>	-	-	-	-
MeIQ	-	-	-	-	-
MeIQx	-	-	-	-	-
Norharman	8.9±0.3	6.3±0.5	7.4±0.3	8.7±0.3	5.4±0.2
Harman	19±3	6.4±0.8	11.1±0.7	10.7±0.8	9.2±0.6
PhIP	-	-	-	-	-
AαC	-	-	-	-	-

<sup>a</sup> Three different brands. Number of samples for each brand: 3. Results not corrected by the recovery.<sup>b</sup> - = not detected.

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5 **Figure 1.** Chemical structures of heterocyclic aromatic amines investigated.  
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8 **Figure 2.** LC-ESI(+)-MS/MS extracted ion chromatograms of a standard solution of HAAs (100 µg/l).  
9 For chromatographic and MS/MS conditions see Experimental.  
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13 **Figure 3.** LC-ESI(+)-MS/MS product ion mass spectra of a standard solution of HAAs (100 µg/l). For  
14 chromatographic and MS/MS conditions see Experimental.  
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18 **Figure 4.** LC-ESI(+)-MS/MS extracted ion chromatograms of HAAs identified in an homogenized  
19 beef-based infant food sample extract.  
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Figure 1

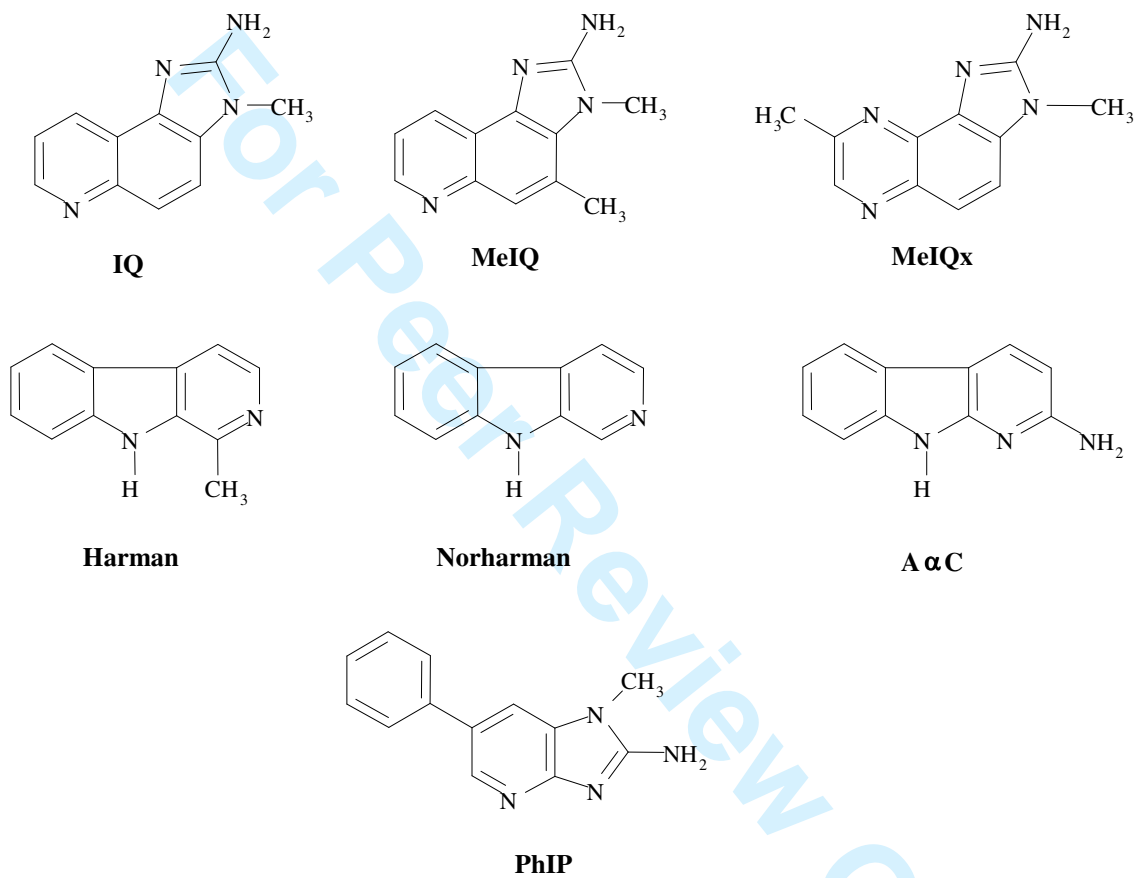




Figure 2

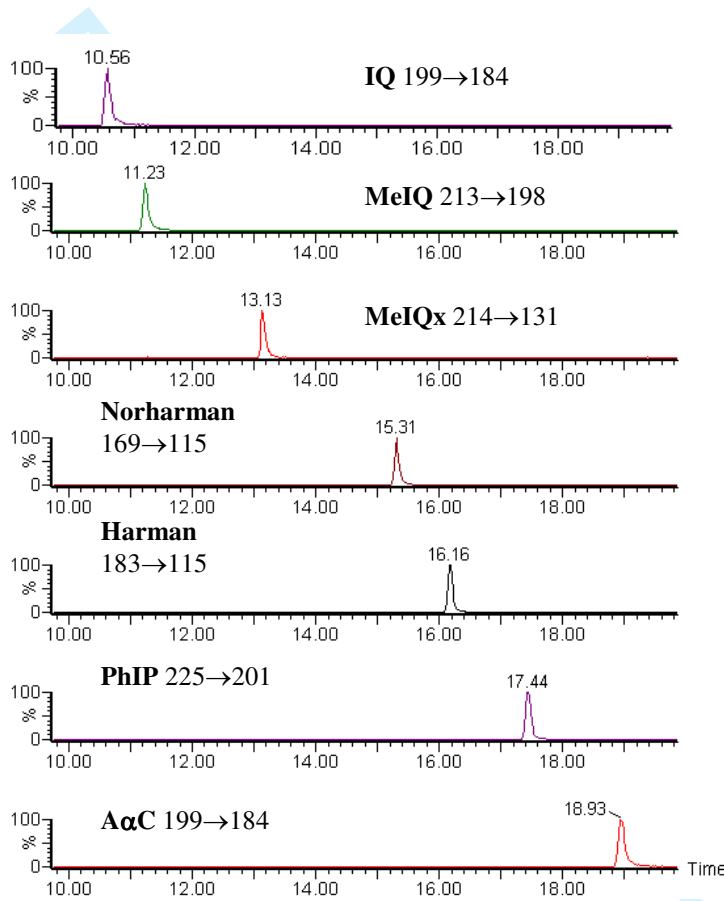
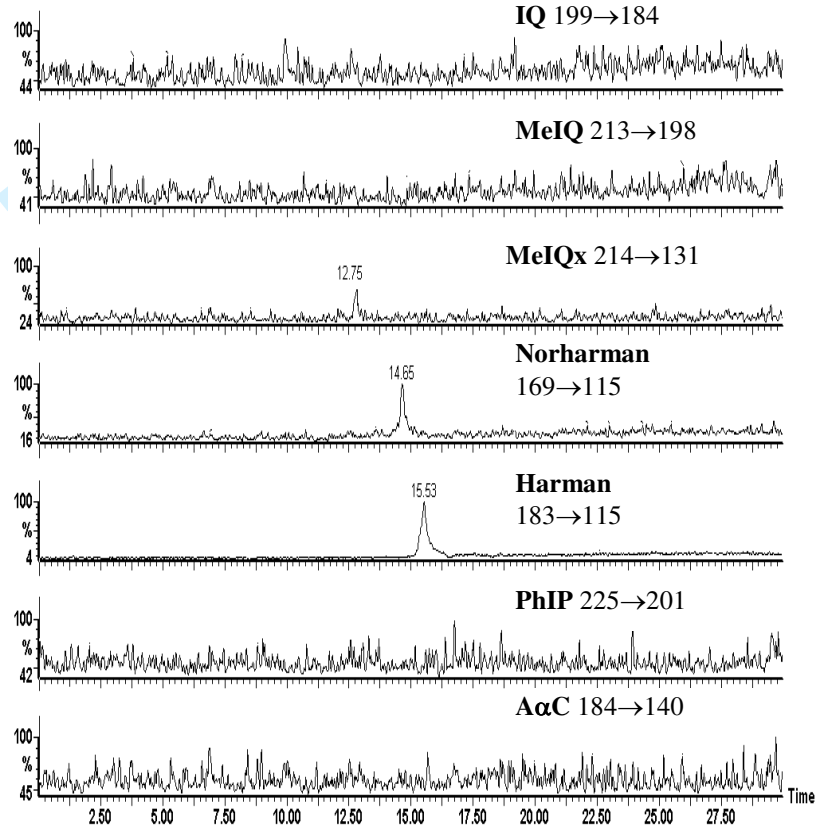


Figure 3



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