Journal of Chromatography A, 1217 (2010) 6778–6784

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Capillary liquid chromatography with diode array and mass spectrometry detection for heterocyclic aromatic amine determination in ready-to-eat food treated with electron-beam irradiation

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ARTICLE INFO

Article history:
Available online 6 July 2010

Keywords:
Heterocyclic aromatic amines
Capillary liquid chromatography
Mass spectrometry
Diode array detection
Electron-beam irradiation
Ready-to-eat food

ABSTRACT

In the present paper, we have developed a capillary liquid chromatography with MS detection for the determination at ng g^{-1} levels of four heterocyclic aromatic amines (MeIQx, norharman, harman and harmine), a group of mutagenic and carcinogenic compounds that can potentially be produced in protein-rich food during processing operations. They have been determined in commercial ready-to-eat (RTE) smoked salmon and soft cheese treated with E-beam irradiation. On the basis of experimental design studies and operating conditions of MS detector, best chromatographic conditions were obtained using a Luna® C_{18} capillary column (150 mm × 0.3 mm I.D.) with a mixture of acetonitrile–ammonium formate 5 mM pH 3.6 buffer (13:87, v/v) as mobile phase. To improve sensitivity, large injection volumes (20 μL) and injection solutions of low elution strength were employed. Sample preparation procedure included a previous treatment with 1 M NaOH, followed by two solid-phase extraction steps; firstly on diatomaceous earth and then on mixed-mode cartridges. Heterocyclic amines were detected neither in irradiated and in non-irradiated samples, indicating that they were not formed by the radiation effect even at doses higher than those indicated in the Food Safety Objective established by regulatory agencies. RTE food samples were spiked at concentration levels in the range 10–30 ng g^{-1}. Recoveries higher than 85% ($n = 3$ for each spiked level) were obtained, showing the effectiveness of the proposed methodology.

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1. Introduction

Heterocyclic aromatic amines (HAs) are potent mutagenic compounds which can be produced in protein-rich foods during processing and cooking. Population is continuously exposed to HAs through diet, as demonstrated by their detection in a wide variety of commercially prepared food and homemade cooked food at levels between 1 and 100 ng g^{-1} [1]. Consequently, the International Agency for Research on Cancer (IARC) recommends a reduced exposure to these compounds [2].

As it is well known, there is an increasing consumer demand for high quality, minimally processed, additive-free and microbiologically safe ready-to-eat (RTE) food, whose processing involves operations that increase contamination risks. Electron-beam (E-beam) irradiation is an effective and recognized way to eliminate pathogens present in food. The recommended dose levels to inhibit insect infestation and to reduce bacterial load are between 1 and 2 kGy for most of foodstuffs, being 10 kGy the maximum authorized dose [3,4]. However, some reports [5] indicate that its application to food can produce secondary effects and promote the appearance of toxic chemical compounds. Thus, irradiation could produce HAs during processing of proteinaceous food [6]. It is, therefore, critical to carefully adjust irradiation doses to achieve an adequate level of microbial safety and to prevent the formation of toxic compounds without significantly affecting consumer acceptance [7]. Moreover, high temperature is occasionally reached during the irradiation treatment and hence promotes the formation of HAs, although there is only one study about this pattern in irradiated cooked ham [8].

Since HAs should be present at trace level (ng g^{-1}) in food samples along with matrix interferences, sensitive analytical techniques have to be employed. Although GC has been used to determinate HAs, most of them are polar and non-volatile compounds and derivatization steps are needed [6,9]. LC techniques mainly with conventional columns and UV detection, which allows the online identification of the analytes by spectral library matching, have been described [10,11].

On the other hand, LC–MS is a powerful technique to determine HAs in complex samples [12]. As low flow rates are usually needed for the electrospray ionization (ESI) LC–MS technique, microbore or semi-microbore columns have been used [13]. In ESI mode, pseudo-molecular ions [M+H]$^+$ of HAs are mainly produced. These amine

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doi:10.1016/j.chroma.2010.06.073

adducts are quite stable and do not undergo significant fragmentation. Different combination of ion sources and analyzers has been used for HA analysis [14,15] and due to their basic nature, acidic pH is normally employed [6].

Nowadays, advantages of capillary liquid chromatography (cLC) are well accepted because cLC handles very low flow rates compatible with MS detection. Its high sensitivity compensates the requirements of low injection volumes [16]. Moreover, in reversed-phase mode, sensitivity can be increased making use of focusing techniques by injection of large solution volumes with very low elution strength (low organic solvent composition). These techniques allow the analyte preconcentration in a small plug on the head of the capillary column, thus minimizing the broadening of chromatographic peak [17,18]. Although gradient cLC has been used in combination with UV detection for HA determination in cooked ham [8], isocratic elution is preferred with MS detection.

Since different variables are involved in reversed-phase LC separations, optimization of the chromatographic conditions should be accomplished by means of multivariate approaches. Methodologies such as response surface analysis (RSA) can help to visualize which are the main experimental variables affecting separation as well as the main interactions between the variables. It models the responses, finds the optimal combination of factors, and it can also predict the most suitable experimental conditions [19–22]. In the case of HAs, mobile phase pH and buffer composition have shown to be critical parameters for retention factor (k) [23] and, experimental design has also been used for optimization purposes [24].

Regarding food sample preparation, usually several extraction steps to minimize matrix effects are required. Several techniques have been applied for the extraction and preconcentration of HAs [6]. General methods are based on the extraction by simple alkalinisation and subsequent extraction on diatomaceous earth. This extract is not suitable for chromatographic analysis and therefore, purification on different sorbents is required [6,25,26]. Recently, this clean-up step has been simplified using Oasis® MCX cartridges for meat samples, allowing the matrix cleaning in only one step [8,27].

In this paper, a new method based on cLC-DAD–MS detection has been developed using experimental design methodologies based on response surface analysis. This method has been applied to determine HAs in RTE-smoked salmon and soft cheese treated with E-beam irradiation at doses between 2 and 8 kGy. Selected MeIQx, harman, norharman and harmine are potential toxicological markers and they have been commonly studied and reported in the literature in a wide variety of food products [1]. Special attention has also been paid to sample preparation procedure through the evaluation of several solid phase extraction (SPE) mixed-mode cartridges, aiming to obtain acceptable sample clean-up and recoveries.

This study is a part of an inter-institutional research with the aim to guarantee the chemical and microbiological safety of RTE food processed by ionizing radiation.

2. Experimental

2.1. Reagents and materials

All chemicals and solvents were of analytical grade and purified water from a Milli-Q system was used in all procedures (Millipore, Bedford, MA, USA). Methanol, acetonitrile and dichloromethane of gradient HPLC quality were supplied by Scharlau (Barcelona, Spain). Reagents including ammonium acetate, ammonium formate, sodium hydroxide and hydrochloric acid were purchased from Panreac (Barcelona, Spain). Glacial acetic acid (99.5%) was purchased from Carlo Erba (Milan, Italy) and ammonium hydroxide (33%) was supplied by Riedel de Häen (Seelze, Germany).

Heterocyclic amines studied were 1-methyl-9H-pyrido[3,4-b]indole (harman, H) purchased from Fluka (Buchs, Switzerland), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) provided by Toronto Research Chemicals (Toronto, Canada), 7-methoxy-1-methyl-9H-pyrido[3,4-b]indole (harmine, HMN) purchased from Sigma–Aldrich (Schnelldorf, Germany) and 9H-pyrido[3,4-b]indole (norharman, NH) from Sigma (Steinhem, Germany). 1,3,7-Trimethyl-1H-purine-2,6(3H,7H)-dione (caffeine, CAF) used as internal standard (IS) and (R,R)-2-methylamino-1-phenylpropan-1-ol (pseudoephedrine), α-(1-aminoethyl)-2,5-dimethoxybenzylalcohol (methoxamine), 1S,2R-2-(+)-dimethylamino-1-phenylpropanol (methylephedrine) and 3,5-diamino-6-chloro-*N*-(diaminomethylene)pyrazine-2-carboxamide (amiloride), also tested as internal standard, were also provided by Sigma. According to the manufacturers, the chemical purity of all synthetic reference compounds was over 98%.

Analyte standard stock solutions (200 mg L^{-1}) were prepared in methanol and stored in the dark at 4 °C. Fresh working standard solutions were prepared daily by suitable dilution of the stock solutions to prevent the possible analyte degradation.

High purity inert diatomaceous earth packed in standard disposable SPE cartridges (Spe-ed™ eL/Lute, sample capacity 20 mL, dead volume ≈15 mL) were purchased from Applied Separations (Allentown, PA, USA). Isolute® HCX (mixed-mode C8 and strong cation exchange functionalized silica, 60 mg, 3 mL) cartridges from Biotage (Uppsala, Sweden) were used for sample clean-up. Other cartridges were tested: OASIS® MCX (60 mg, 3 mL) packed with poly-(divinylbenzene-co-N-vinylpyrrolidone) and functionalized with sulfonic acid groups were provided by Waters Corporation (Milford, MA, USA), Strata™ X-C (surface-modified styrene divinylbenzene polymer, 60 mg, 3 mL) by Phenomenex (Torrance, CA, USA) and silica-based Discovery® DSC-SCX (benzene sulfonic acid functional group, 60 mg, 3 mL) by Supelco (St. Louis, MO, USA).

2.2. Apparatus and instrumentation

Sample SPE in cartridges was carried out by means of a VacElut 20-place vacuum manifold supplied by Varian (Harbor City, CA, USA) connected to a membrane vacuum pump from P-Selecta (Barcelona, Spain). This manifold was also employed for solvent evaporation under nitrogen stream. Coupling pieces and stopcocks were also from Varian. A magnetic stirrer purchased from P-Selecta was used for sample homogenization during basic digestion.

Chromatographic analysis by cLC was performed by an Agilent cLC instrument Mod. 1100 Series (Agilent Technologies, Madrid, Spain) which was equipped with a G1376A binary capillary pump, a G1379A degasser and a G1315B diode array detector (500 nL, 10 mm pathlength). Mass spectrometry detection was carried out using an Agilent 6120 Quadrupole LC–MS. The instrument was operated using an ESI source in positive mode and a microelectrospray nebulizer. Nitrogen was used as both drying and nebulizer gas. The drying gas temperature was set at 325 °C and the gas flow rate at 6.0 L min^{-1}. The nebulizer pressure was fixed at 1.17 bar, and the capillary ESI voltage at 3.0 kV. Data acquisition and processing were made using the Agilent Chemstation software package for Microsoft Windows.

An external stainless steel loop with a volume of 20 μL was placed into a Rheodyne® injection valve. Reversed-phase separations were made on a Luna® 3 μm C18 (150 mm × 0.3 mm I.D.) column coupled with a Luna® 3 μm C18 (20 mm × 0.3 mm I.D.) precolumn. Other capillary columns (150 mm × 0.3 mm I.D.) tested were: Synergi™ Fusion 4 μm C18, Synergi™ Max 4 μm C12 and Luna® 3 μm C8. These columns were supplied by Phenomenex

(Torrance, CA, USA) and they were thermostated during the chromatographic run by employing a MISTRAL programmable oven (Spark Holland, Emmem, The Netherlands).

The software package Statgraphics Plus version 5.0, running under Windows XP, was employed for the application of statistical tools.

2.3. Procedures

2.3.1. Food sampling and irradiation treatment

Smoked salmon and soft cheese blocks were purchased from Madrid supermarkets. Food samples were sliced (2–3 mm thickness) with an electric machine and slices were vacuum-packaged in laminated film bags of low gas permeability. They were transported in insulated polystyrene boxes and irradiated under an electron-beam radiation source, which operated at 10 MeV, in the IONMED S.A. irradiation sterilization plant (Tarancón, Spain). Radiation doses between 2 and 8 kGy were applied to 3–6 slices included in each bag. Doses of 8 kGy were selected as the highest radiation studied. At this level, although organoleptic changes occurred especially for smoked salmon they could be accepted by consumers, contrary to changes observed at 10 kGy. The dose absorbed by samples was checked by determining the absorption of cellulose triacetate dosimeters attached to packages and simultaneously irradiated with samples [28].

Vacuum bags containing non-irradiated control samples, as well as those containing samples treated with ionizing radiation, were stored at −20 °C and thawed at 4 °C for 24 h before applying the extraction and purification procedures described in the following section.

2.3.2. Extraction and clean-up of RTE food samples

Sample treatment according to the method proposed by Rosales-Conrado et al. [8], was slightly modified for the application to RTE smoked salmon and soft cheese samples and their analysis by cLC-DAD–MS. Briefly, two slices of each sample were finely crushed with a knife. Intermixing was accomplished by the sampling tabling method [29]. Aliquots of the average sample (1 g) were treated with 12 mL 1 M NaOH and the suspension was homogenized by magnetic stirring for 1 h at 500 rpm. The alkaline food sample extracts were percolated by gravity through Spe-ed™ eL/Lute (diatomaceous earth) cartridges. After 5 min, HAs were directly eluted from this cartridge to a silica-based mixed-mode Isolute® HCX cartridge (tandem mode), using 75 mL of dichloromethane (firstly the Isolute sorbent was preconditioned with 2 mL of this organic solvent). The Isolute® HCX cartridges were rinsed sequentially with 2 mL of 0.1 M HCl solution and 2 mL of methanol (MeOH). The retained analytes were eluted using 2 mL of MeOH with NH_3 (25%) (95:5, v/v) and the collected and purified extract evaporated to dryness in a vacuum manifold under a nitrogen stream. Similar procedure was applied to the other SPE cartridges included in this study.

Dry extracts were reconstituted in 1 mL of a mixture of MeOH–ammonium formate 5 mM pH 3.6 (5:95, v/v) [8]. The resulting solutions, with low elution strength (low organic solvent content), have allowed the analyte focusing on the head of the capillary column during the chromatographic analysis carried out under the conditions mentioned below.

When aliquots of the average sample (1 g) were spiked with HAs, they were left to stabilize for 30 min at room temperature before the basic digestion.

2.3.3. cLC-DAD–MS method

Isocratic separation was made on a Luna® C18 (150 mm × 0.3 mm I.D., 3 μm) capillary analytical column coupled with a Luna® 3 μm C18 (20 mm × 0.3 mm I.D.) precolumn, maintaining the temperature at 30 ± 1 °C during the chromatographic run. A mixture of acetonitrile–ammonium formate 5 mM pH 3.6 (13:87, v/v) was used as mobile phase and the flow rate was set at 9.0 μL min^{-1}. Wavelength for the UV-diode array detection was fixed at 275 nm for caffeine (internal standard), 265 nm for MeIQx and at 250 nm for the rest of HAs studied [8]. Regarding MS detection, the full-scan mass spectrum was acquired for each analyte with identification purposes in automatic mode. ESI source operated in the positive ionization mode, using $(M+H(^+$ adducts as molecular ion. These ions were monitored for each parent compound working in selected ion monitoring (SIM). Quantitative analyses were performed using ions at m/z 183.0 for harman, 169.1 for norharman, 214.1 for MeIQx, 213.1 for harmine and 195.1 for caffeine.

To improve sensitivity, large injection sample volumes (20 μL) in buffered aqueous solutions containing 5% of organic solvent for on-column focusing were employed.

This cLC method, combined with the extraction procedure described above, was applied to non-irradiated and irradiated samples at radiation doses in the range 2–8 kGy and spiked samples with HAs at different levels in the concentration range 10–30 ng g^{-1}.

2.3.4. Analytical characteristics

Linearity ranges were determined from HA standards solutions at 10 concentration levels in the range 0.5–50 ng mL^{-1} and 25 ng mL^{-1} of caffeine as IS, prepared on a mixture of MeOH–ammonium formate 5 mM pH 3.6 (5:95, v/v) for focusing purposes. Calibration graphs were also obtained from blank samples spiked after extraction in the range 5–30 ng g^{-1} using 25 ng g^{-1} of caffeine as IS. The results were analyzed by linear regression using peak area ratios.

Limits of detection (LODs) and quantification (LOQs) were evaluated from calibration graphs analyzing HA standard solutions at 0.5 ng mL^{-1} levels or analyzing blank samples spiked with HAs at 0.5 ng g^{-1} levels, calculating the signal to noise ratios (3 and 10 for LOD and LOQ respectively) at a time window around each chromatographic peak.

Precision was calculated from HA standard solutions at 20 ng mL^{-1} level for both retention times and peak areas. Run-to-run precision was evaluated by injecting six standard solutions (n = 6) on the same day, while day-to-day precision was evaluated by injecting six daily standard solutions on three different days (N = 18). Regarding samples, precision was determined using food extracts obtained from samples spiked with HAs at 10 ng g^{-1} level. Run-to-run precision and day-to-day precision were determined as described before for standards.

Relative recoveries were determined by triplicate from blank samples spiked at four different concentration levels between 10 and 30 ng g^{-1} by means of the corresponding sample calibration graphs.

3. Results and discussion

3.1. Optimization of chromatographic conditions

A previous cLC method [8], involving large injection volumes (20 μL) and on-column focusing techniques, provided a suitable sensitivity using an Inertsil® C8 capillary column, DAD and gradient elution. This method showed that composition of the focusing solution, mobile phase and pH have remarkable effects on HA separation. In order to improve this separation, an isocratic cLC method compatible with MS detection was developed and the influence of several chromatographic factors on separation quality evaluated [30].

Table 1
Variables for multifactorial design and experimental responses.

Design points	pH	ACN (%)	$R_{s,min}$	t_{end} (min)
1	3.8	16	1.60	11.5
2	3.6	18	1.31	9.70
3	3.8	12	2.64	19.8
4	4.1	18	1.12	8.80
5	3.6	14	2.15	14.0
6	3.8	18	1.24	9.10
7	3.6	12	3.10	20.3
8	4.1	16	1.41	11.1
9	3.8	14	1.92	13.7
10	4.1	14	1.62	14.7
11	3.6	16	1.75	11.2
12	4.1	12	2.11	21.3

Different reversed-phase packings such as Luna® C8, Luna® C18, Synergi™ Max-RP (C12) and Synergi Fusion (C18 and polar embedded group) were tested using UV-DAD detection. These columns were chosen due to their high efficiencies and bonded phase surface coverage [31,32]. Considering the different particle sizes, flow rate was set at 15 μL min^{-1} for both Synergi Fusion™ and Synergi™ Max-RP, 12 and 9 μL min^{-1} for Luna® C8 and Luna® C18 columns respectively.

Chromatographic conditions were optimized using experimental design methodologies such as central composite design. Factors and ranges selected for the optimization of mobile phase composition were ACN (11.6–28.4%), ammonium acetate concentration (13.2–46.8 mM) and buffer pH (3.6–4.4). Response variables were expressed in terms of resolution, calculated at baseline, between the worst resolved peak pair NH–H ($R_{s,min}$), and retention time of the last eluting peak (t_{end}). From the obtained equations, it could be concluded that $R_{s,min}$ was significantly affected by both ACN percentage and pH while t_{end} only by ACN. In addition, the ammonium acetate concentration had no significant effect on separation.

Chromatographic separation efficiency was optimized for $R_{s,min} \geq 2$ and minimum t_{end} value. Luna® C8 capillary column provided poor resolution. Luna® C18 and Synergi™ Max-RP columns provided similar $R_{s,min}$ to Synergi™ Fusion but higher efficiencies. However, Synergi™ Max-RP column provided poor peak symmetry. Therefore, Luna® C18 column was selected for further optimization with the quadrupole MS detector.

Taking into account the instrumental limitations of the MS capillary nebulizer, the ammonium acetate concentration in the mobile phase was fixed to 5 mM. Chromatographic separation was optimized using multifactorial design. Factors and ranges selected were ACN (12–18%) and pH (3.6–4.1). For the injection, buffered focusing solutions with 5% MeOH were used.

Table 1 includes the values of the experimental responses ($R_{s,min}$ and t_{end}), which were fitted into the following normalized polynomial equations:

$$R_{s,min} = 1.74 - 0.51\text{pH} - 1.35\text{ACN} - 0.06\text{pH}^2 + 0.39\text{pH ACN} + 0.40\text{ACN}^2 \quad (1)$$

$$t_{end} = 12.18 + 0.18\text{pH} - 11.02\text{ACN} + 0.76\text{pH}^2 - 0.94\text{pH ACN} + 4.83\,\text{ACN}^2 \quad (2)$$

Determination coefficients were 0.988 and 0.992 for $R_{s,min}$ and t_{end} respectively, showing the reliability of the equations. In the studied domain, both ACN percentage and pH affected significantly to $R_{s,min}$ (p values 0.0000 and 0.0002 respectively) (Eq. (1)) while ACN (%) affected only t_{end} (p value 0.0000 and 0.0005 for the single and quadratic term respectively) (Eq. (2)). As can be expected, the interactions between factors (ACN-pH) were significant only for $R_{s,min}$ response (p = 0.0033). In Fig. 1 it can be observed that the ACN percentage decrease in the mobile phase increases $R_{s,min}$ and t_{end}. However, when pH decreases, $R_{s,min}$ increases and t_{end} does not change significantly.

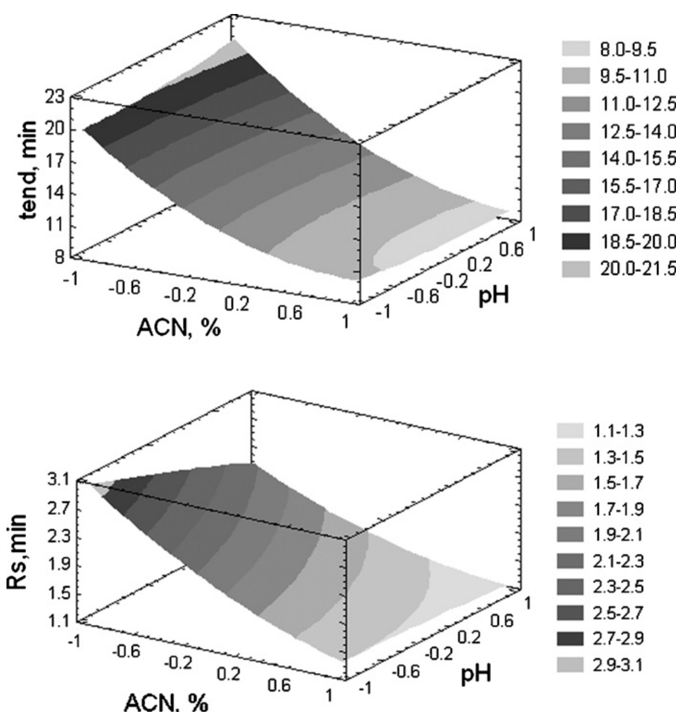

Fig. 1. Response surfaces of t_{end} and $R_{s,min}$ obtained for ACN percentage and pH at 5 mM buffer concentration for the Luna® C18 capillary column.

The chromatographic separation efficiency was optimized by maximizing $R_{s,min}$ and minimizing t_{end} values which maximized the desirability function over the selected region. The maximum desirability function yielded a t_{end} = 13.6 min and $R_{s,min}$ = 2.2 at pH 3.6 and 14.5% ACN. The estimated response surfaces (Fig. 1) predicted that $R_{s,min}$ > 2 and t_{end} in the range 12.5–15.0 min could be obtained at pH 3.6–3.7 and 13–15% ACN. As a compromise, pH 3.6 and 13% ACN were selected as optimum values. Under these conditions t_{end} = 15.0 min and $R_{s,min}$ = 2.3 were expected and experimentally assessed. Finally, ammonium formate 5 mM was also tested and selected to avoid problems into the capillary MS nebulizer.

3.2. Analytical characteristics of HA standard solutions

An internal standard was used for quantitation purposes. Several compounds such as pseudoephedrine, methoxamine, methylephedrine, amiloride and caffeine were tested. Among all, caffeine was selected because it was separated to baseline between MeIQx and NH peaks.

After applying the developed cLC-DAD–MS method, linearity range was determined from HA standards solutions and analyzed by linear regression obtaining good correlation coefficients in all cases (r > 0.995). The detection and quantitation limits determined are included in Table 2. As can be expected, the best LODs and LOQs were obtained when MS detection was used. These LODs were better than those reported in other published methods: 0.2 ng for both NH and H and 0.2 or 0.8 ng for MeIQx using HPLC-DAD [10,11], 2–6 pg for NH, H, MeIQx and HMN using cLC-DAD [8], and 6 and 8 pg for H and NH respectively using HPLC–single quad MS [14]. This improvement is mainly attributed to the large injected volume (20 μL) and the focusing solution employed.

Table 2
Analytical characteristics for HA standard solutions.

Analyte	LOD[a] ($\mu g L^{-1}$)		LOQ[a] ($\mu g L^{-1}$)		Run-to-run precision (RSD, %)			Day-to-day precision (RSD, %)		
	UV	MS	UV	MS	t_R (min)	Peak area		t_R (min)	Peak area	
						UV	MS		UV	MS
MeIQx	0.043 (0.86)	0.023 (0.46)	0.143 (2.86)	0.077 (1.54)	0.4	2.1	7.1	0.9	4.6	9.1
NH	0.039 (0.78)	0.004 (0.08)	0.130 (2.60)	0.013 (0.26)	0.9	4.2	4.5	1.1	4.6	7.8
H	0.061 (1.22)	0.010 (0.20)	0.203 (4.06)	0.033 (0.66)	0.7	3.8	7.1	1.2	5.0	9.5
HMN	0.095 (1.90)	0.005 (0.10)	0.316 (6.32)	0.017 (0.34)	0.6	2.1	7.3	1.2	4.8	9.2

[a] LOD and LOQ expressed as mass (pg) are given in brackets (injection volume 20 μL).

Relative standard deviation (RSD) for retention times (t_R) and peak areas are also summarized in Table 2. As can be seen, precision was lower than 1.2% for retention times, and than 5% (UV detection) and 9.5% (MS detection) for peak areas. In addition, the variance analysis of one factor showed that there are no significant differences between the results obtained by UV and MS detection with a confidence level of 95%.

3.3. Analysis of smoked salmon and soft cheese samples

3.3.1. Optimization of sample preparation

The optimized cLC method and an extraction procedure using Oasis® MCX cartridges for cooked ham clean-up [8] were applied to non-irradiated and irradiated smoked salmon and soft cheese. HAs were not detected at the LOD method both by UV and MS, and the extract obtained was not clean enough for MS detection. The clean-up procedure was modified in order to obtain higher preconcentration factors by evaporating the eluted extracts until dryness and further reconstituting of the sample extract to 1 mL. According to the requirements of the cLC for focusing purposes, reconstitution was done in buffered aqueous solution with low organic content (5% MeOH). HAs were detected neither by UV nor by MS and some interferences were present in the UV and MS chromatograms for example at MeIQx retention time in the soft cheese extracts.

Consequently, to optimize the clean-up, two silica-based mixed-mode sorbents consisting of an ion exchange moiety (sulfonic acid) and an alkyl chain (usually C_8) on the silica surface [33] (Discovery® DSC-SCX and Isolute® HCX) as well as a polymeric-based mixed-mode sorbent (Strata™ X-C) were evaluated. Non-irradiated and irradiated smoked salmon and soft cheese were analyzed and no peaks at retention times of the HAs were detected both by UV and by MS. The HA adduct ions were observed neither in SCAN nor in SIM mode. Thus, extracts of non-irradiated smoked salmon and soft cheese samples were used as blank samples and calibration graphs from blank samples spiked after extraction in the range 5–30 ng g^{-1} using 25 ng g^{-1} caffeine as IS were obtained. Then, the suitability of the cartridges was evaluated from spiked samples at 20 ng g^{-1}.

The Strata™ X-C cartridge yielded high recoveries for MeIQx (>80%), probably due to the polar nature of this compound and to the polar retention mechanism of the sorbent [31], although less polar amines were completely lost. The Discovery® DSC-SCX provided good recoveries (>78%) for all HAs studied but dirty extracts were obtained mainly for soft cheese, thus this cartridge was discarded. The Isolute® HCX sorbent provided good recoveries and the cleanest extracts for both smoked salmon and cheese, thus this one was selected for clean-up purposes.

3.3.2. Determination of HAs in smoked salmon and soft cheese

The optimized cLC-DAD–MS method combined with the sample preparation procedure developed was applied to the analysis of non-irradiated and irradiated smoked salmon and soft cheese. Samples were spiked with HAs by triplicate at four different concentrations in the range 10–30 ng g^{-1}, levels which can be frequently expected in cooked food. Representative chromatograms from non-irradiated smoked salmon and soft cheese samples non-spiked and spiked at a concentration level of 15 ng g^{-1} are shown in Fig. 2. No matrix interferences at the retention time of target compounds were observed. These results obtained from UV detection, were also obtained from MS detection in SCAN and SIM mode.

Irradiated salmon and cheese samples were modified at different levels of irradiation, as can be seen in the UV chromatograms of Figs. 3I and 4I respectively. In salmon samples a peak close to 7 min was observed at 2 and 4 kGy but not at 8 kGy, and other peak close to 19 min was also observed at all irradiation levels. Regarding cheese samples, a peak close to 16 min was mainly detected at

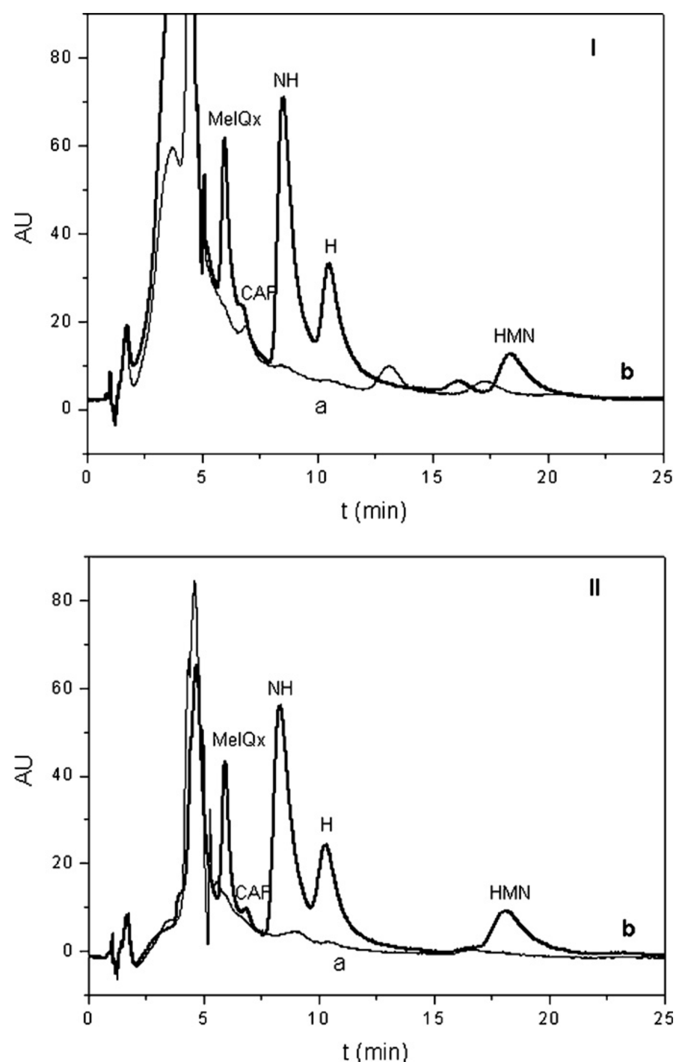

Fig. 2. Representative chromatograms of non-irradiated smoked salmon (I) and soft cheese samples (II), non-spiked (a) and spiked (b) with HAs at a concentration level of 15 ng g^{-1} (detection wavelength 250 nm).

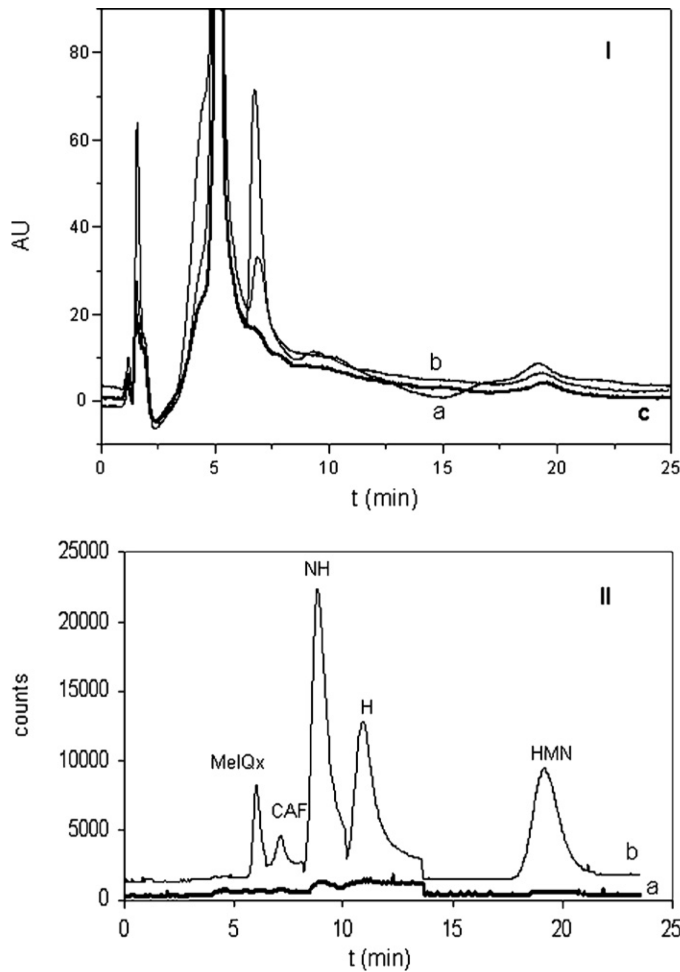

Fig. 3. Chromatograms obtained by UV detection (250 nm) (I) and MS detection in SIM mode (II) for smoked salmon irradiated at levels of 2 kGy (Ia), 4 kGy (Ib) and 8 kGy (Ic) and for smoked salmon irradiated at 8 kGy (IIa) and spiked with HAs at 15 ng g^{-1} (IIb).

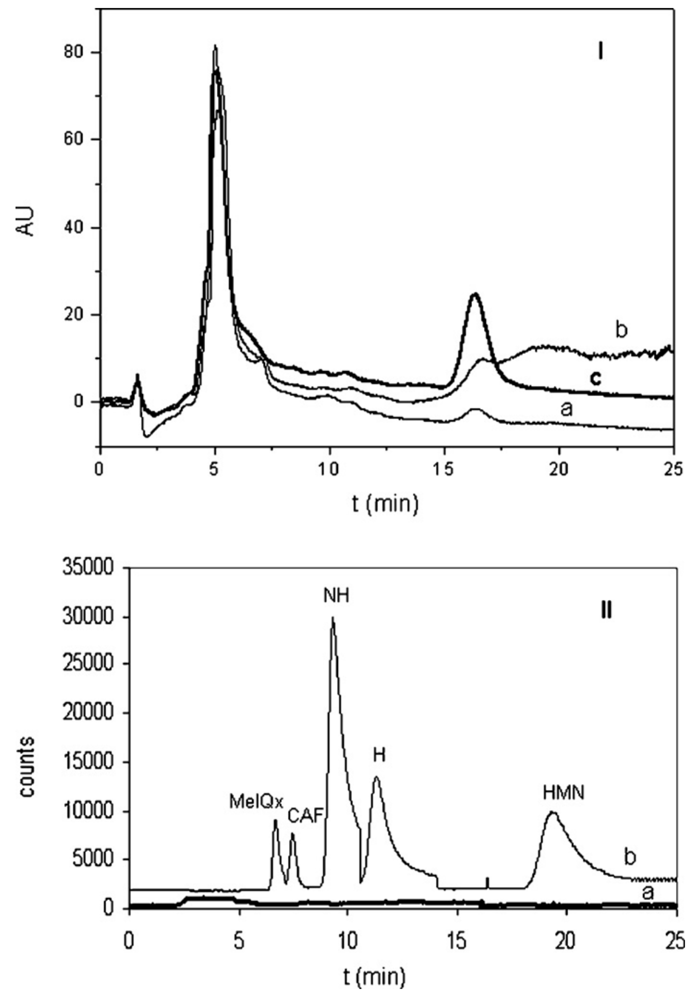

Fig. 4. Chromatograms obtained by UV detection (250 nm) (I) and MS detection in SIM mode (II) for soft cheese irradiated at levels of 2 kGy (Ia), 4 kGy (Ib) and 8 kGy (Ic) and for soft cheese irradiated at 8 kGy (IIa) and spiked with HAs at 15 ng g^{-1} (IIb).

8 kGy. The UV spectra registered for these matrix peaks were different to those corresponding HAs. In addition, the MS chromatograms of irradiated food samples were obtained. Figs. 3II and 4II show the chromatograms obtained in SIM mode for smoked salmon and soft cheese respectively, corresponding to non-spiked and spiked irradiated samples at 8 kGy. As can be observed, no peaks at the HA retention times were detected indicating that no matrix interferences are present. On the other hand, no signals at the HA

Table 3
Analytical characteristics for HAs in non-irradiated food samples.

Analyte	Smoked salmon						Soft cheese					
	LOD (ng g^{-1})		LOQ (ng g^{-1})		Recoveries (n = 3 for each spiked level)		LOD (ng g^{-1})		LOQ (ng g^{-1})		Recoveries (n = 3 for each spiked level)	
	UV	MS	UV	MS	Added (ng g^{-1})	Mean ± RSD (%)	UV	MS	UV	MS	Added (ng g^{-1})	Mean ± RSD (%)
MeIQx	0.059	0.030	0.197	0.100	10	93 ± 9	0.066	0.070	0.220	0.233	10	89 ± 8
					15	89 ± 1					15	90 ± 5
					20	97 ± 4					20	93 ± 7
					30	86 ± 7					30	90 ± 9
NH	0.050	0.008	0.167	0.027	10	93 ± 8	0.057	0.005	0.190	0.017	10	85 ± 8
					15	91 ± 3					15	91 ± 4
					20	90 ± 9					20	97 ± 6
					30	89 ± 7					30	95 ± 8
H	0.078	0.017	0.260	0.057	10	88 ± 6	0.090	0.016	0.300	0.053	10	87 ± 7
					15	90 ± 7					15	87 ± 5
					20	90 ± 9					20	88 ± 7
					30	90 ± 7					30	96 ± 7
HMN	0.147	0.011	0.490	0.037	10	88 ± 4	0.10	0.013	0.333	0.043	10	86 ± 6
					15	85 ± 5					15	89 ± 5
					20	94 ± 7					20	90 ± 6
					30	89 ± 6					30	85 ± 8

Table 4
Run-to-run and day-to-day precision in RTE food samples spiked at 10 ng g^{-1}.

Analyte	Smoked salmon				Soft cheese			
	Run-to-run precision (RSD, %)		Day-to-day precision (RSD, %)		Run-to-run precision (RSD, %)		Day-to-day precision (RSD, %)	
	UV	MS	UV	MS	UV	MS	UV	MS
MeIQx	2.8	10	7.2	16	1.3	8.4	8.0	13
NH	2.2	7.2	8.5	17	2.9	7.7	6.8	11
H	6.4	5.3	8.9	11	6.0	7.8	9.5	12
HMN	5.0	10	6.9	16	5.6	11	9.2	12

corresponding ions were observed in the non-spiked both irradiated and non-irradiated samples indicating that they are not formed by the ionizing radiation effect even at high doses.

Analytical characteristics for food-spiked samples were determined with DAD and MS detection for comparison. The results were analyzed by linear regression using peak area ratios and good correlations were obtained ($r > 0.993$). The LODs and LOQs from UV and MS detection are summarized in Table 3. As can be observed, MS provides low LODs and LOQs allowing discriminating false HA identification at their retention times. Table 3 also includes the spiked recoveries calculated as the mean of three determinations for each spiked level. As can be seen, they were in the range 85–97% with RSD values lower than 9% in all cases. Day-to-day precision, expressed as RSD (Table 4), were lower than 8.9% (UV) and 17% (MS) for salmon samples, and lower than 9.5% (UV) and 13% (MS) for cheese samples.

In general, reasonable recoveries and good precision results were obtained, not only at the same day but also at different days, demonstrating the suitable extraction efficiency of the analytical method developed. In addition, relatively constant recoveries were obtained for the studied HAs all over the ranges evaluated.

4. Conclusions

The developed cLC-DAD–MS method was found to be suitable for the HAs analysis at ng g^{-1} levels in RTE food samples, and it improves other published methods in terms of analysis time, resolution of the critical pair H–NH and detection limits. It is worth to mention that this cLC method uses low flow rates (μL min^{-1} level) and it spends low amount of organic solvents, producing low waste and it is an environmentally friendly technique, cost-saving and more suitable for coupling with MS detection.

In addition, E-beam irradiation could be applied for sanitation of both smoked salmon and soft cheese even at doses higher than those recommended by the Food Safety Objective (FSO) established by EU and USDA statements, with the advantage that toxic HAs are not produced during the irradiation treatment.

Acknowledgments

The present work has received the financial support from the project TEMINYSA/CM “Emerging technologies and minimal processing: application to chemical and microbiological safety of ready-to-eat (RTE) foods” (S-0505/AGR-0314), funded by the Dirección General de Universidades e Investigación, Consejería de Educación, Comunidad de Madrid (Spain). R. Gonzalo-Lumbreras also wishes to thank to the Comunidad de Madrid for the support through a postdoctoral grant.

References

[1] G.A. Keating, K.T. Bogen, J. Chromatogr. B 802 (2004) 127.
[2] International Agency for Research on Cancer, IARC Monographs of the Evaluation of the Carcinogenic Risk of Chemicals to Humans, vol. 56, Lyon, 1993.
[3] H. Matloubi, F. Aflaki, M.J. Hadjiezadegan, J. Food Compos. Anal. 17 (2004) 133.
[4] World Health Organization, Wholesomeness of irradiated food: report of a join FAO/IAEA/WHO expert committee, WHO Technical Report Series, vol. 659, Geneva, 1981.
[5] Z. Seregély, J. Farkas, E. Tuboly, I. Dalmani, Chemometr. Intell. Lab. 82 (2006) 115.
[6] M. Sanz-Alaejos, J.H. Ayala, V. González, A.M. Afonso, J. Chromatogr. B 862 (2008) 15.
[7] M.C. Cabeza, I. Cambero, L. de la Hoz, J.A. Ordoñez, Innov. Food Sci. Emerg. 8 (2007) 299.
[8] N. Rosales-Conrado, M.E. León-González, L.V. Pérez-Arribas, L.M. Polo-Díez, Anal. Bioanal. Chem. 391 (2008) 1433.
[9] H. Kataoka, J. Chromatogr. A 774 (1997) 121.
[10] C.M. Lan, T.H. Kao, B.H. Chen, J. Chromatogr. B 802 (2004) 27.
[11] F.L. Warzecha, B. Janoszka, U. Blaszczyk, M. Strózyk, D. Bodzek, C. Dobosz, J. Chromatogr. B 802 (2004) 95.
[12] H. Moriwaki, Curr. Anal. Chem. 3 (2007) 69.
[13] E. Barceló-Barrachina, E. Moyano, L. Puignou, M.T. Galceran, J. Chromatogr. B 802 (2004) 45.
[14] E. Barceló-Barrachina, E. Moyano, L. Puignou, M.T. Galceran, J. Chromatogr. A 1023 (2004) 67.
[15] P.A. Guy, E. Gremaud, J. Richoz, R.J. Turesky, J. Chromatogr. A 883 (2000) 89.
[16] J.P.C. Vissers, H.A. Claessens, C.A. Cramers, J. Chromatogr. A 779 (1997) 1.
[17] N. Rosales-Conrado, M.E. León-González, L.V. Pérez-Arribas, L.M. Polo-Díez, J. Chromatogr. A 1076 (2005) 202.
[18] A. Prüß, C. Kempter, J. Gysler, T.J. Jira, J. Chromatogr. A 1303 (2004) 167.
[19] S. Pous-Torres, J.R. Torres-Lapasió, J.J. Baeza-Baeza, M.C. García-Álvarez-Coque, J. Chromatogr. A 1193 (2008) 117.
[20] E.J. Klein, S.L. Rivera, J. Liq. Chromatogr. R. T. 23 (2000) 2097.
[21] Y. Vander-Heyden, LC GC Eur. 19 (2006) 469.
[22] Y.P. Zhang, Y.J. Ahang, W.J. Gong, A.I. Gopalan, K.P. Lee, J. Chromatogr. A 1098 (2005) 183.
[23] F. Calbiani, M. Careri, L. Elviri, A. Mangia, I. Zagnoni, Food Addit. Contam. 24 (2007) 883.
[24] F. Bianchi, M. Careri, C. Corradini, L. Elviri, A. Mangia, I. Zagnoni, J. Chromatogr. B 825 (2005) 193.
[25] G.A. Gross, A. Grüter, J. Chromatogr. 592 (1992) 271.
[26] F. Toribio, R. Busquets, L. Puignou, M.T. Galceran, Food Chem. Toxicol. 45 (2007) 667.
[27] C. Messner, M. Murkovic, J. Chromatogr. B 802 (2004) 19.
[28] ASTM, American Society for Testing and Materials, E1650-97e1 Standard practice for use of cellulose acetate dosimetry systems, vol. 12.02, 2000.
[29] G.H. Jeffer, J. Basset, J. Mendham, R.C. Denney, Quantitative Chemical Analysis, 5th ed., Longman Scientific & Technical, England, 1989.
[30] T. Sivakumar, R. Manavalan, C. Muralidharan, K. Valliappan, J. Pharmaceut. Biomed. 43 (2007) 1842.
[31] Phenomenex Catalog for Chromatographic Columns and Supplies, Torrance, 2009–2010.
[32] L.R. Snyder, J.W. Dolan, P.W. Carr, J. Chromatogr. A 1060 (2004) 77.
[33] S. Hug, J. Chromatogr. A 1673 (2005) 335.