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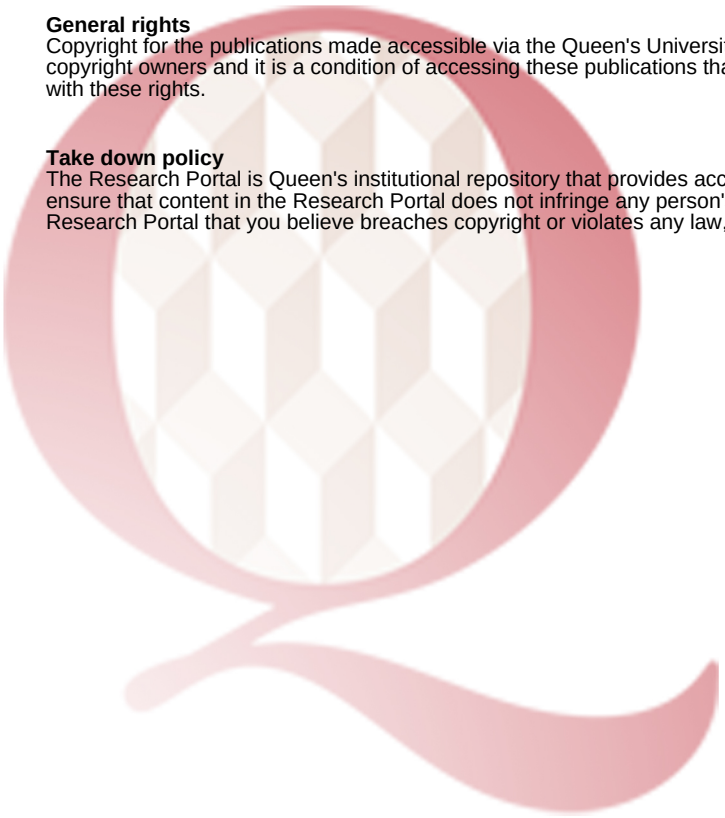

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Optimised extraction of heterocyclic aromatic amines from blood using hollow fibre membrane liquid-phase microextraction and triple quadrupole mass spectrometry

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Abstract

Heterocyclic aromatic amines (HCA) are carcinogenic mutagens formed during cooking of proteinaceous foods, particularly meat. To assist in the ongoing search for biomarkers of HCA exposure in blood, a method is described for the extraction from human plasma of the most abundant HCAs: 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx) (and its isomer 7,8-DiMeIQx), using Hollow Fibre Membrane Liquid-Phase Microextraction. This technique employs 2.5 cm lengths of porous polypropylene fibres impregnated with organic solvent to facilitate simultaneous extraction from an alkaline aqueous sample into a low volume acidic acceptor phase. This low cost protocol is extensively optimised for fibre length, extraction time, sample pH and volume. Detection is by UPLC-MS/MS using positive mode electrospray ionisation with a 3.4 min runtime, with optimum peak shape, sensitivity and baseline separation being achieved at pH 9.5. To our knowledge this is the first description of HCA chromatography under alkaline conditions. Application of fixed ion ratio tolerances for confirmation of analyte identity is discussed. Assay

precision is between 4.5 and 8.8% while lower limits of detection between 2 and 5 pg/mL are below the concentrations postulated for acid-labile HCA-protein adducts in blood.

Keywords:
Heterocyclic aromatic amine, PhIP, MeIQx, Hollow fibre membrane liquid-phase microextraction, Human plasma, UPLC-MS/MS.

1. Introduction

Heterocyclic aromatic amines (HCAs) are formed during the cooking of proteinaceous foods, particularly meat and fish, which provide creatin(in)e and other precursors such as amino acids, sugars or other aldehydes [1]. Their formation in the parts per billion concentration range is highly dependent upon the type of food and degree of cooking; therefore making estimation of dietary exposure to HCAs difficult [2]. The past 30 years have seen extensive investigation into HCAs, in terms of their production, metabolism [3], formation of adducts with DNA [4] and protein [5], their quantification [6] and implications for human health.

2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx) (Fig. 1) are three of the most abundant HCAs present in cooked meat and fish [7,8] although many others have been identified [9]. There is strong evidence from animal and *in vitro* studies of the carcinogenic and mutagenic properties of the HCAs although *in vivo* evidence correlating HCA dietary intake and incidence of cancers can be equivocal [10,11]. To overcome the limitations of estimating HCA intake by food frequency questionnaires, direct measurement of the HCAs, their metabolites or their DNA or protein adducts *in vivo* is necessary. The methodology of analysis of PhIP and its metabolites has been reviewed by Teunissen and colleagues [12] who concluded that LC-MS/MS was clearly the detection method of choice for sensitive qualitative and quantitative analyses of this most abundant of HCAs in biological matrices. Sample pretreatment for HCA analysis usually involves protein precipitation, liquid-liquid extraction (LLE) or solid phase extraction (SPE).

Hollow Fibre Membrane Liquid-Phase Microextraction (HF-LPME) techniques, which employ porous membrane fibres to support an organic solvent during extraction of an aqueous sample, were first introduced by Pedersen-Bjergaard and Rasmussen in 1999 [13] and have received considerable attention for analyses of environmental contaminants and pharmaceuticals and related substances in body fluids, as reviewed by Lee and colleagues [14]. More widespread adoption of HF-LPME is possible, particularly in combination with LC-MS/MS detection techniques [14]. The application of HF-LPME techniques to extraction of HCAs has been limited, yet the traditional

extraction methods for HCAs (usually LLE and/or SPE) are prime candidates for transfer to HF-LPME techniques. HF-LPME was first applied to extraction of PhIP from urine and plasma by a group in Lund University, Sweden [15] who then expanded this to eleven HCAs [16] and metabolites of PhIP in urine [17], proposing urinary PhIP as a possible biomarker of exposure to dietary PhIP [18]. This extraction technique has also been applied to the detection of HCAs in barbecued meats [19].

Despite considerable research into the fate of HCAs *in vivo*, with much now focussing on adducts to DNA, a reliable marker for HCA exposure (either circulating free or adducted to blood proteins such as serum albumin or haemoglobin) remains elusive. There is a need for further validated HCA extraction techniques from blood products to assist in this search.

The three-phase HF-LPME system [14] described in the current study uses a porous polypropylene hollow fibre impregnated with a small volume of organic solvent (the supported liquid membrane phase). An acidic aqueous acceptor phase fills the lumen of the hollow fibre. The third phase is the alkaline aqueous sample (donor phase) containing the weakly basic HCA analytes into which the fibre is immersed. Extraction is by diffusion based on pH differences and is effectively a simultaneous double liquid-liquid extraction from alkaline sample to organic phase to acidic acceptor phase.

Hollow fibres require preparation by the operator prior to use. We have noted that the literature employing hollow fibres for extractions from low volume biological samples does not always provide clear descriptions of the procedures involved. Therefore, in the current study we have focussed on some of the detailed practicalities of preparing and handling hollow fibres in addition to the optimisation and validation of the extraction protocol and LC-MS/MS detection of HCAs in human plasma.

2. Materials and methods

2.1. Reagents and samples

Reference standards 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3,8-dimethyl-imidazo [4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo [4,5-f]quinoxaline (4,8-DiMeIQx), 2-amino-3,7,8-trimethylimidazo [4,5-f]quinoxaline (7,8-

DiMeIQx) and deuterium labelled internal standards (I.S.) D3-PhIP, D3-MeIQx and D3-4,8-DiMeIQx were obtained from Toronto Research Chemicals (North York, ON, Canada). Mixed standard solutions prepared in LCMS grade methanol were stored at 4°C. Unless stated, all other chemicals were obtained from Sigma-Aldrich (Dorset, UK).

Blood samples, obtained from the research project *Food*CAP funded by the World Cancer Research Fund (Grant ID 2010/255), were from healthy volunteers who had fasted for 10-12 h to ensure removal of free circulating HCAs from serum. This study was conducted according to the guidelines laid down in the declaration of Helsinki and all procedures involving human subjects were approved by the School of Medicine, Dentistry and Biomedical Sciences Research Ethics Committee of Queen's University Belfast. Each participant provided written, informed consent prior to blood donation.

2.2. Apparatus

Accurel® PP 300/1200 polypropylene hollow fibre membranes (300 µm wall thickness, 1200 µm inner diameter and 0.2 µm pore size, F-No-5129, manufactured by Membrana GmbH, Wuppertal, Germany) were kindly donated by the Danish Meat Research Institute (Roskilde, Denmark). Sample extractions were carried out in LC-GC certified clear glass 2 ml HPLC microvials (12 x 32 mm) with screw neck caps containing bonded pre-slit PTFE/silicone septa obtained from Waters Corporation (Manchester, UK), and also used 18 gauge by 2.5 cm (0.8 mm needle outer diameter) syringe needles (Sigma Aldrich) and 8 x 3 mm PTFE stirrer magnets (Scientific Laboratory Supplies, Nottingham, UK).

An Acquity I-class UPLC® binary pump and sample management system (Waters Corporation, Manchester, UK) coupled to a Xevo TQS tandem mass spectrometer (Waters Corporation), both controlled by MassLynx™ software, were used for sample extract analysis. The mass spectrometer operated under positive electrospray ionisation mode (ESI). Data acquisition was in Multiple Reaction Monitoring mode (MRM) with a total run time of 3.4 min. Data analysis was performed using Waters TargetLynx™ software. MS source settings were as follows: capillary voltage 0.5 kV, source temperature 150°C, desolvation temperature 650°C, cone nitrogen gas flow 150 L/h, desolvation nitrogen gas flow 1000 L/h. Separation of HCAs was carried out on an Acquity BEH C18 1.7 µm UPLC analytical column (50 x 2.1 mm) equipped with an in-

line filter unit (0.2 µm, 2.1 mm) (Waters Corporation), maintained at 40°C. A binary gradient mobile phase was applied at a flow rate of 0.8 mL/min, phase A being 5 mM ammonium formate pH 9.5 (aq) and phase B being acetonitrile. The rapid gradient profile was: (1) 0-0.2 min, held at 93% A, (2) 0.2-2 min, falling linearly to 75% A, (3) 2.01-2.2 min, held at 70% A, (4) 2.25-2.75 min, held at 50% A, (5) 2.8-3.4 min, held at 93% A. The UPLC purge wash was acetonitrile:water (10:90, v/v) and the wash solution was acetonitrile:water (50:50, v/v). Injection volume was 5 µl. Table 1 provides details of HCA fragmentation transitions and other MS/MS conditions.

2.3. Preparation of hollow fibres

The optimised conditions for extraction of HCAs from plasma using HF-LPME are described below. Description of the method optimisation study follows in section 2.6. Porous Accurel® PP 300/1200 polypropylene hollow fibre membranes were cut into 2.5 cm lengths and one end was heat-sealed using a hot soldering iron – the fibre tip was touched lightly onto the hot iron surface for 1-2 sec and then immediately squeezed repeatedly using fine tipped metal tweezers to form a seal of approximately 3-4 mm length. The fibre was cleaned by soaking in acetone for 10 min before drying at 37°C for approximately 15 min (in a glass or paper container, not plastic, to avoid damage by residual acetone). Sealed fibres were stored in a capped glass tube prior to use. If condensation of acetone is evident during storage, fibres should be re-dried at 37°C. Immediately prior to use, fibres were prepared for extraction by filling the lumen of the hollow fibre with acidic acceptor phase and filling the pores of the fibre membrane with organic solvent as follows. An 18 gauge hypodermic needle fitted to a 1 mL disposable plastic syringe containing acidic acceptor solution (0.1 M sulphuric acid) was inserted carefully into the unsealed end of the fibre ensuring the membrane was not punctured and a strong seal was achieved (use of a fibre with different internal diameter would require a different gauge needle). Acceptor solution was injected firmly into the lumen of the fibre until droplets were clearly visible on the outer surface of the porous fibre and no leakage was evident from the sealed end. The fibre was removed from the needle and a clean hypodermic needle protruding through the pre-slit septum of a microvial screw cap was carefully inserted. Holding the needle Luer-Lok connector, the fibre was then dipped into 1-octanol for 30 sec, allowing the organic solvent to fill the membrane pores. Excess 1-octanol on the fibre surface was removed by manually shaking the fibre in deionised 18Ω water for 30 sec. The prepared fibre could then be conveniently

immersed in a sample vial, allowing the screw cap to be sealed and the height of the fibre adjusted through the pre-slit septum without removing or touching the fibre. Each 2 mL sample vial contained an 8 x 3 mm stirrer magnet and the needle height was adjusted to avoid the stirrer damaging the sealed end of the fibre during extraction (Fig. 2).

2.4. Sample extraction

Plasma samples (0.2 mL) were placed in 2 mL glass HPLC microvials and fortified with 50 pg/mL internal standard (I.S.; 10 µl of 1 ng/mL mixed deuterium labelled HCAs D3-PhIP, D3-MeIQx and D3-4,8-DiMeIQx) for validation and routine analyses. For optimisation of the method the I.S. were added to samples after extraction by addition of 10 µl to the HPLC microvial insert containing the recovered acidic acceptor phase to enable calculation of HCA extraction efficiency. For optimisation and validation of the method, mixed standard HCAs were added to samples prior to extraction at 30 pg/mL (60 µl of 100 pg/mL mixed PhIP, MeIQx, 7,8-DiMeIQx and 4,8-DiMeIQx). Samples were made alkaline by addition of 1.3 mL of 0.5 M NaOH and a 8 x 3 mm stirrer magnet was placed in each microvial. A prepared hollow fibre was immersed in each sample as described above (Fig. 2) and vials were placed in a Perspex microvial rack on a single position magnetic stirrer at room temperature for 5 h, stirring at 550 rpm. Use of a microvial rack avoided the need for a multiple-position magnetic stirrer. After extraction the fibre was removed from the sample with the needle still attached. The fibre sealed end was cut off with sharp scissors and an air filled 1 mL disposable syringe used to expel the acceptor phase (typically 20 µl from a 2.5 cm fibre) into a pre-weighed 200 µl glass insert inside a 2 mL HPLC microvial. The recovered liquid was weighed and an equal volume of 0.1 M NaOH was added to neutralise the acidic accepter phase. The neutralised extract was then made up to a final volume of 100 µl by addition of 30 mM ammonium formate (aq.) pH 9.5 to ensure compatibility with the LC-MS/MS mobile phase.

2.5. Calibration

LC-MS/MS solvent calibration standards were prepared by addition of I.S. and increasing volumes of 100 pg/mL mixed standard HCAs in microvials. Solvent was evaporated to dryness under nitrogen and standards were reconstituted in 20 µl

acetonitrile and 80 µl of 30 mM ammonium formate pH 9.5 before transferring to 200 µl glass microvial inserts.

2.6. Method optimisation

The optimum conditions for extraction using HF-LPME are highly dependent upon the target compound and the fibre being used. Each new extraction method requires specific optimisation to take account of the structure of the analytes and the gauge of fibre employed. The critical variables assessed in this optimisation study were the length of hollow fibre, the molarity of NaOH used to dilute the plasma sample, the final volume of diluted sample and the extraction time. The four HCA analytes being extracted from plasma included three compounds based on an imidazo-quinoxaline skeleton (MeIQx, 4,7-DiMeIQx, 7,8-DiMeIQx) and one based on a phenyl-imidazo-pyridine skeleton (PhIP). Plasma from a single volunteer was used during method optimisation. Plasma was fortified with HCAs prior to extraction under various conditions (duplicate samples for each condition) and I.S. added after extraction.

2.7. Method validation

Validation of the optimised extraction method was carried out using plasma from a single volunteer, seven aliquots being fortified with 30 pg/mL HCA mixed standards and 50 pg/mL I.S. prior to extraction on each of 3 days. Extracted HCAs were quantified against solvent calibration curves. Plasma fortified with I.S. only was included as a negative control. Samples fortified with progressively lower HCA concentrations were extracted in order to estimate limits of detection and quantification.

3. Results and discussion

Development of this HF-LPME technique demonstrated the convenience of leaving the hollow fibre attached to a hypodermic needle during extraction, allowing easy handling and recovery of acceptor phase when extracting a limited sample volume. The fibre need not be touched after immersion in the sample as the needle Luer-Lok acts as a convenient handle for the remainder of the procedure. Other authors have immobilised the fibre on a wire during extraction [17], however this requires the fibre to be transferred after extraction from the wire to a new needle for recovery of the acceptor

phase, increasing the number of handling steps and the risk of cross-contamination. For larger liquid sample volumes, a longer fibre may be used and both ends sealed before complete immersion of the fibre in the sample without any support. This approach has been used at the University of Seville for extraction of pharmaceuticals from urine and environmental water samples [20,21]. The use of HPLC microvials with pre-slit septa screw caps as sample containers proved to be a simple way to secure the fibres and needles during extraction and a convenient way to adjust fibre depth in the sample. Acceptor phase could even be recovered from the fibre without removing the needle from the pre-slit septum. A little practice in preparation of the fibres, particularly the sealing of the ends with a soldering iron, yielded consistent results, with less than 5% of fibres leaking from the sealed end when filling with acceptor phase (these fibres were discarded).

3.1. Method optimisation

3.1.1. LC-MS/MS optimisation

Several sub-2 µm, octadecylsilyl (C18-based) UPLC columns manufactured by Waters were assessed for the separation of the four HCAs (HSS T3, HSS, BEH C18, CSH C18 and AccQTag Ultra) in addition to a Phenomenex Kinetex pentafluorophenyl (PFP, 2.6 µm) column. All were assessed under acidic mobile phase A conditions ranging from pH 3.5 to 6.4, while BEH C18 and Kinetex PFP were also assessed at alkaline pH 8.0 to 9.5. Notably, the BEH column yielded the best peak shape, sensitivity (peak intensity) and baseline separation of the DiMeIQx isomers at pH 9.5 - the natural pH of 5 mM ammonium formate, avoiding the need for pH adjustment of mobile phase A (Fig. 3). Separation of HCAs has traditionally been achieved under acidic LC conditions [1,12], on the principle that mobile phase pH should be lower than the analyte pKa (<pH 5 for the HCAs) in order to fully protonate the HCA amine groups prior to positive mode electrospray ionisation. However, baseline chromatographic separation of the DiMeIQx isomers is sensitive to pH and is incomplete under the commonly used pH 4.7 or lower [22,16]. Holland and colleagues [23] unusually employed a mobile phase ranging from pH 6.8 to 7.85 to separate HCAs, including 4,8-DiMeIQx, in hydrolysed urine but the degree of chromatographic separation from 7,8-DiMeIQx was not described. In the current study, use of pH 9.5 and the UPLC gradient described above (5mM aqueous ammonium formate and acetonitrile) facilitated baseline separation of MeIQx (t_R 1.07

min), 7,8-DiMeIQx (1.31 min), 4,8-DiMeIQx (1.38 min) and PhIP (2.21 min) with a total gradient runtime of 3.4 min and typical peak widths of 3.4-3.8 sec (Fig. 4). Ammonium formate was employed as an ion pairing agent, in keeping with Bianchi and colleagues [24] who demonstrated better HCA peak shapes with formate than with acetate. It may be that investigators, on observing improved HCA peak shape as mobile phase pH was lowered below pH 4.7, have not previously studied the benefits of LC conditions closer to or higher than neutral. Nevertheless, as stated by Bianchi and colleagues [24], "depending on the specific purpose and design of the experiment, fine adjustments for pH and mobile phase concentration are always recommended to achieve optimal separation of HCAs". To our knowledge this is the first report of chromatographic separation of HCAs under alkaline LC-MS/MS conditions. The benefits of alkaline mobile phase conditions with positive mode ionisation are clearly compound dependent, as shown by Gerssen and colleagues [25] who observed improved recovery from shellfish of the marine toxin azaspiracid-1 using pH 11 LC conditions compared with pH 2.6 under positive ESI, attributing this to altered matrix suppression effects, whilst other toxins performed better under acidic conditions. Kipper and colleagues [26] also found that optimum signal intensities and peak separation of several antibiotics under positive ESI were achieved at pH 9.

The choice of fragmentation transitions for identification of the isomers of DiMeIQx is important. Both 4,8-DiMeIQx and 7,8-DiMeIQx share m/z 228>213 as their most intense transition. In the absence of demonstrable chromatographic separation, studies which use this peak for quantification of DiMeIQx (for example [23,27]) risk misidentification of the isomers. It is advisable to use the m/z 213 fragment as the qualitative (confirmatory) ion and to use the less intense, but essentially unique, fragments m/z 212 and m/z 131 for reliable quantification of 4,8-DiMeIQx and 7,8-DiMeIQx respectively (Table 1 and Fig. 4).

3.1.2. Fibre length optimisation

The bar charts in Fig. 5 illustrate the proportion of HCAs extracted under various conditions from plasma fortified with 30 pg/ml mixed HCAs. Data are normalised to percentages of the maximum HCA concentration extracted under the assessed conditions. HF-LPME extraction conditions were as described above in sections 2.3 and 2.4, with each of the following four variables being independently optimised.

Fig. 5a demonstrates the influence of the length of hollow fibre used in a fixed sample donor volume of 1.5 ml, with 0.5 M NaOH diluent and 5 h extraction time. A 1 cm length of fibre clearly provided insufficient acidic acceptor phase for successful extraction (8-14 µl recovered) due to loss of lumen volume following sealing of the fibre and insertion of the supporting needle. A 2.5 cm length was convenient for the 2 ml HPLC vials. Using a pair of 2.5 cm fibres in a single sample vial yielded twice the volume of acceptor phase following extraction (45 µl) but only a marginal increase in PhIP and MeIQx recovery and required twice the time and consumable materials to prepare. Longer fibres had a detrimental effect on MeIQx recovery and had to be folded once or twice to be fully submerged in the sample, risking damage to the integrity of the supported organic layer during extraction. Therefore, a single 2.5 cm fibre was used for all further extractions.

3.1.3. Donor NaOH molarity optimisation

Fig. 5b demonstrates that HCA extraction efficiency by HF-LPME is influenced by the molarity of NaOH used to create the alkaline sample conditions for extraction (0.2 ml plasma sample was diluted with 1.3 ml NaOH). At least 0.5 M NaOH was required to achieve maximum analyte recovery (1 M in the case of MeIQx) with extraction efficiency dropping when more concentrated NaOH was used. This is in contrast to Busquets and colleagues [17,18] who suggested that PhIP or its metabolites could be extracted from urine using HF-LPME with greater signal to noise ratio when mildly acidic conditions were employed (pH 5.5) compared with a donor pH greater than 10. However, this observation was not consistent across Busquets' studies. It is also in contradiction of the conventional use of alkaline sample conditions for the extraction of a weakly basic compound such as PhIP (p*K*a=5.6) into an organic solvent followed by back-extraction into an acidic acceptor phase [15]. Furthermore, the current study shows that simply raising sample pH above 10 may not be optimal for HCA extraction. Observed pH in duplicate diluted plasma samples prior to extraction were as follows: 0.01 M NaOH (pH 11.0), 0.05 M (pH 12.5), 0.2 M (pH 13.2), 0.5 M NaOH (pH 13.5), 1 M (pH 13.6) and 2 M (pH 13.7). It can be seen from Fig. 5b that even at sample pH 12.5 (0.05 M NaOH), extraction of HCAs may still be less than 50% of maximum. A NaOH molarity of 0.5 M (pH 13.5) was employed as a compromise for the optimal extraction of the quinoxaline and pyridine HCAs. The use of 0.5 M NaOH is in agreement with the studies of HCA

extraction from urine by HF-LPME emanating from Lund University, Sweden [15,16,17,18]. However, low parts per billion concentrations of PhIP and 4,8-DiMeIQx were also successfully extracted from cooked meats by HF-LPME using 0.05 M NaOH [19], although extraction efficiency was not described.

3.1.4. Donor volume optimisation

Fig. 5c illustrates the influence of the final volume of a 0.2 ml plasma sample diluted with 0.5 M NaOH prior to extraction using a 2.5 cm hollow fibre. Donor volume did not influence the volume of acidic acceptor phase recovered (typically 22 µl from a 2.5 cm fibre) but the dilution effect on the sample resulted in poorer extraction into the fixed acceptor phase volume. A final donor volume of 1.5 ml was chosen as optimum for the current system.

3.1.5. Extraction time optimisation

Fig. 5d illustrates how optimum extraction time using HF-LPME is dependent upon the chemical characteristics of the analytes. PhIP (a phenyl-imidazo-pyridine) reached maximum recovery after 5 h, while the three imidazo-quinoxaline compounds extracted more slowly, with recovery still rising after 7 h. A compromise protocol of 5 h was adopted to cover all analytes and to facilitate completion of a sample batch extraction within a single working day.

This 5 h extraction time is in keeping with Ramos Payan and colleagues [20] who extracted fluoroquinolone antibiotics from bovine urine and environmental water samples in a 5.5 h timescale using HF-LPME. The same research group similarly extracted sulphonamide antibiotics from human urine in 6 h, but extraction of non-steroidal anti-inflammatory drugs was achieved within 20 min [21], illustrating again the need to optimise HF-LPME techniques for each analyte of interest. A review of environmental and bioanalytical applications of HF-LPME [14] also highlighted the variable extraction times and recoveries achievable by this technique, depending upon analyte chemistry and sample type. Extraction times were often 1 h or less, but recoveries were consequently incomplete.

382 *3.2. Method validation*

383 Solvent calibration curves were linear in the range equivalent to 5-80 pg/mL plasma
384 (10-160 pg/mL reconstitution solvent, or 50-800 fg on-column), coefficients of
385 determination R^2 typically being greater than 0.998 for all HCAs. The mean recovery
386 and precision of the HF-LPME extraction method are shown in Table 2. Data are based
387 on 21 replicates of human plasma fortified with 30 pg/mL HCAs prior to extraction (7
388 replicates extracted on each of 3 days). Mean observed HCA concentrations (recovery)
389 were greater than 92% of fortified concentrations for all four HCAs. Precision (RSD)
390 was below 9% in all cases, both within and between runs, demonstrating satisfactory
391 performance at physiologically relevant HCA-adduct concentrations in human blood [28,
392 29]. Estimated lower limits of detection and quantification based on signal-to-noise
393 ratios greater than 3 and 10 respectively (using Peak-to-Peak calculations on
394 unsmoothed raw data) in plasma fortified with HCAs prior to extraction are shown in
395 Table 2. Limits are based on the lowest intensity peak for each analyte. Limits for PhIP
396 are higher than for the quinoxaline HCAs due primarily to the low intensity of the
397 secondary, confirmatory transition peak for PhIP (m/z 225.2>183.2). HF-LPME assay
398 sensitivity and performance are similar to those reported by Lezamiz and colleagues
399 [15] who studied only PhIP in plasma.
400
401 Confidence in the identification of analytes is important when measuring low
402 concentrations of compounds in complex biological matrices, particularly for analyte
403 groups like the HCAs which share common structures and fragmentation patterns. The
404 presence of a second transition peak to confirm analyte identity should be a
405 prerequisite, although this is not always the case in the published literature of HCAs.
406 Furthermore, in the present study the ratio of confirmatory to primary quantitation peaks
407 (ion ratio) was monitored in every sample to ensure agreement with the same ratio in
408 calibration standards. Guidance on the tolerances to apply to compliance of ion ratios
409 with their standards was taken from the document laying out the required analytical
410 performance of methods in the veterinary pharmaceuticals field: Commission Decision
411 2002/657 [30]. While this European Commission document sets out to ensure animal-
412 derived food products are free of harmful residues and is not directly applicable to the
413 study of natural carcinogens such as the HCAs, it is the opinion of the authors that
414 similar performance criteria should be applied to analysis of suspected carcinogens
415 whenever possible. Consequently, we applied the tolerances as defined in Decision

2002/657 to the ion ratios in all samples: if ion ratios did not agree with the same ratios in calibration standards to within 20 to 30% (dependent on the magnitude of the ratio), the identity of the analyte peak could not be confirmed. Ion ratios greater than 0.5 are permitted a tolerance of ±20% relative to calibration standards, 25% tolerance is applied for ratios between 0.2 and 0.5, 30% for ratios of 0.1-0.2, and 50% for ratios below 0.1. For example, the ratio of the peak areas of MeIQx daughter ions 131.1/199.1 (Table 1) was typically around 0.4, permitting this ratio in a sample to be within 25% of the mean ratio of the same peaks in calibration standards.

3.3. Application to blood samples

Preliminary studies demonstrated that the HF-LPME plasma extraction method may also be applied to serum and whole blood, although whole blood matrix effects caused an additional 30-40% signal suppression and quantitative accuracy was adversely affected, with I.S.-corrected recoveries being around 85% for the quinoxaline HCAs and around 72% for PhIP (data not shown), compared with 93-99% and 92% respectively in plasma (Table 2).

Human plasma samples were obtained from the World Cancer Research Fund project *Food*CAP. Samples were from volunteers whose dietary HCA intake was estimated using WISP nutritional analysis software on the basis of 7 day food diaries and the US National Cancer Institute's CHARRED database (http://dceg.cancer.gov/tools/design/charred). Volunteers fasted for 10-12 h before providing blood samples to ensure removal of free circulating HCAs from their plasma. Unsurprisingly, no HCAs were detected in these samples using the optimised HF-LPME extraction method, even from volunteers with a nominally high HCA intake of greater than 1 µg/day.

Adducts of HCAs bound covalently to blood proteins such as haemoglobin and serum albumin have, since the 1990s, been proposed as potential biomarkers of exposure to HCAs (as reviewed by [2,31]). However, the suitability of protein adducts as reliable indicators of potential carcinogenic damage by HCAs is still unproven [31]. Furthermore, Magagnotti and colleagues [29] detected PhIP released by mild acid hydrolysis from purified haemoglobin and serum albumin, demonstrating differences between meat consumers and vegetarians in the range 7-67 pg/mL for PhIP-albumin

adducts. However, this work has not been replicated since, despite ongoing studies in the field.

The current HF-LPME method was applied to plasma samples from high dietary HCA intake volunteers in an attempt to measure protein-bound HCAs following their release by the acid hydrolysis protocol of Magagnotti and colleagues [29]. Plasma was diluted to a final concentration of 0.1 M hydrochloric acid and incubated at 80°C for 1 h before adjusting the pH with NaOH and extracting by HF-LPME as described above. No acid-labile HCAs were detected under these conditions, demonstrating the need for further work on the release of labile HCA adducts from blood proteins.

4. Conclusions

The described HF-LPME method presents a convenient and low cost technique for extraction of carcinogenic heterocyclic aromatic amines from plasma. LC-MS/MS assay sensitivity is in the range postulated for acid-labile HCA-protein adducts in blood [29], with satisfactory assay precision and recovery. The hollow fibre membrane liquid-phase microextraction technique benefits from very low organic solvent usage compared with conventional liquid-liquid extraction (LLE) and low cost compared with solid phase extraction methods (SPE). To our knowledge this is the first description of a validated HF-LME protocol for extraction of a group of HCAs from plasma and also of their chromatographic separation under alkaline conditions.

Extensive optimisation of the HF-LPME protocol demonstrates the need for such microextraction techniques to be carefully optimised for each analyte of interest. A final compromise protocol is described, balancing the variable recoveries between different analytes and enabling a batch of approximately 20 samples to be completed in a normal working day. It should be noted that samples can be left unattended during the lengthy HF-LPME extraction step, freeing the operator for other duties, unlike manual LLE and SPE techniques. This technique represents an additional, low cost extraction tool in the ongoing search for biomarkers of exposure to carcinogenic heterocyclic aromatic amines in plasma.

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FIGURE CAPTIONS

Fig. 1. Structures of heterocyclic aromatic amines.

Fig. 2. Format of hollow fibre membrane liquid-phase microextraction vials (2.5 cm sealed fibre, 18 gauge needle, pre-slit septum cap and 1.5 mL diluted sample with 8 mm magnetic stirrer in 2ml microvial).

Fig. 3. ESI-UPLC-MS/MS MRM chromatograms of primary quantitation peaks of 4,8-DiMeIQx and 7,8-DiMeIQx (eluting first from a 5cm BEH C18 UPLC column) illustrating the beneficial effects on peak shape, sensitivity and baseline separation of increasing mobile phase pH. Analyte m/z transitions and peak heights are listed.

Fig. 4. ESI-UPLC-MS/MS MRM chromatograms of primary quantitation peaks of heterocyclic aromatic amines and their deuterated internal standards extracted by HF-LPME from human plasma fortified at 30 pg/mL (I.S. at 50 pg/mL). Baseline separation of DiMeIQx isomers is achieved at pH 9.5. Analyte names, m/z transitions and peak heights are listed.

Fig. 5. Optimisation of the extraction of four heterocyclic aromatic amines from plasma by hollow fibre membrane liquid-phase microextraction. Data are means of duplicate extractions of plasma fortified at 30 pg/mL. Four HF-LPME variables were optimised - A: length of hollow fibre, B: molarity of NaOH diluent, C: donor (final sample) volume, and D: extraction time.

Optimised extraction of heterocyclic aromatic amines from blood using hollow fibre membrane liquid-phase microextraction and triple quadrupole mass spectrometry

Kevin M. Cooper*, Natcha Jankhaikhot, Geraldine Cuskelly

Highlights

- A Hollow Fibre Liquid Microextraction technique for heterocyclic amines is described.
- HF-LPME extraction of carcinogenic HCAs from plasma is extensively optimised.
- Optimum UPLC-MS/MS chromatography and positive mode ES ionisation achieved at pH 9.5.

Figure 1 Cooper

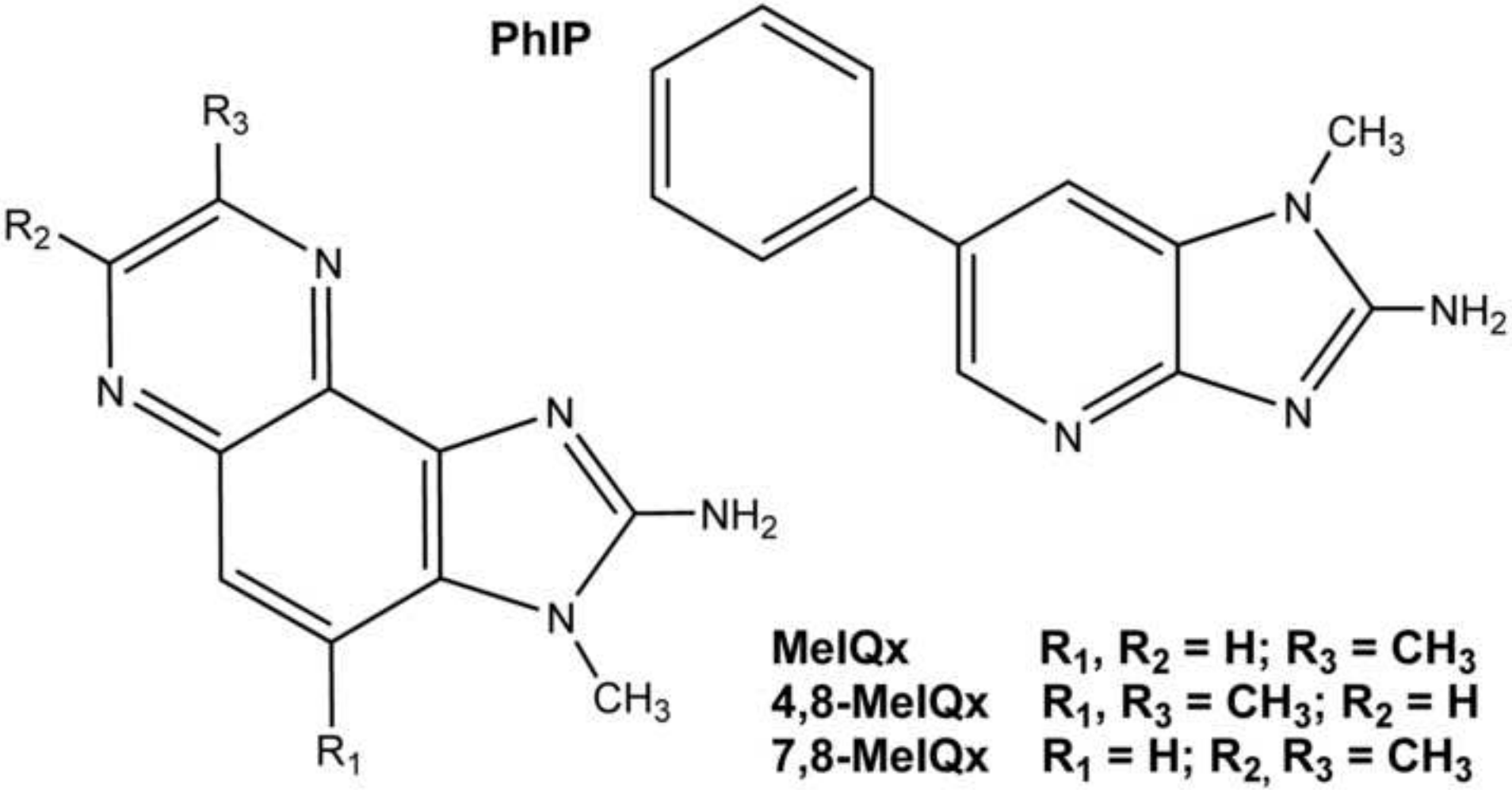
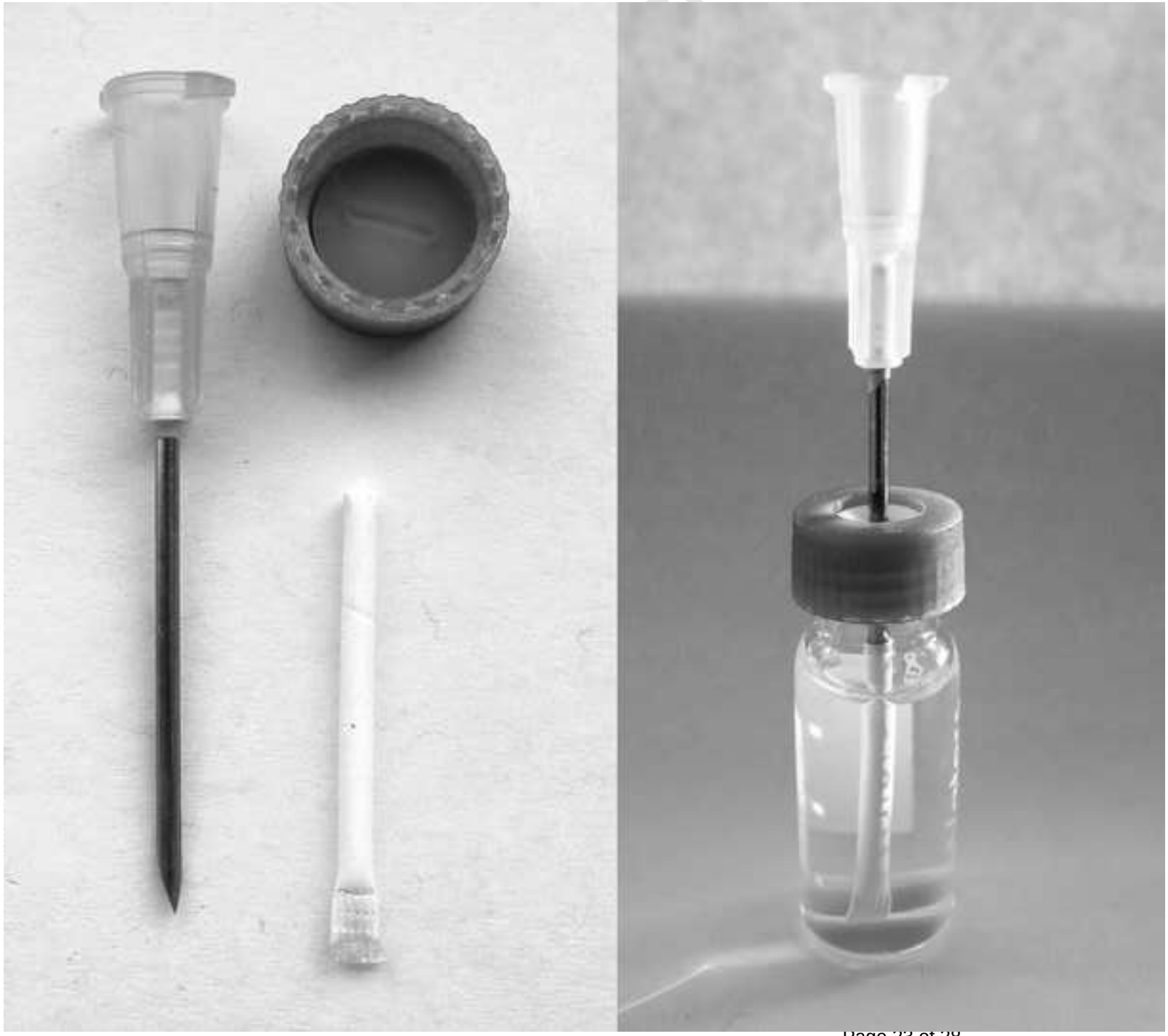
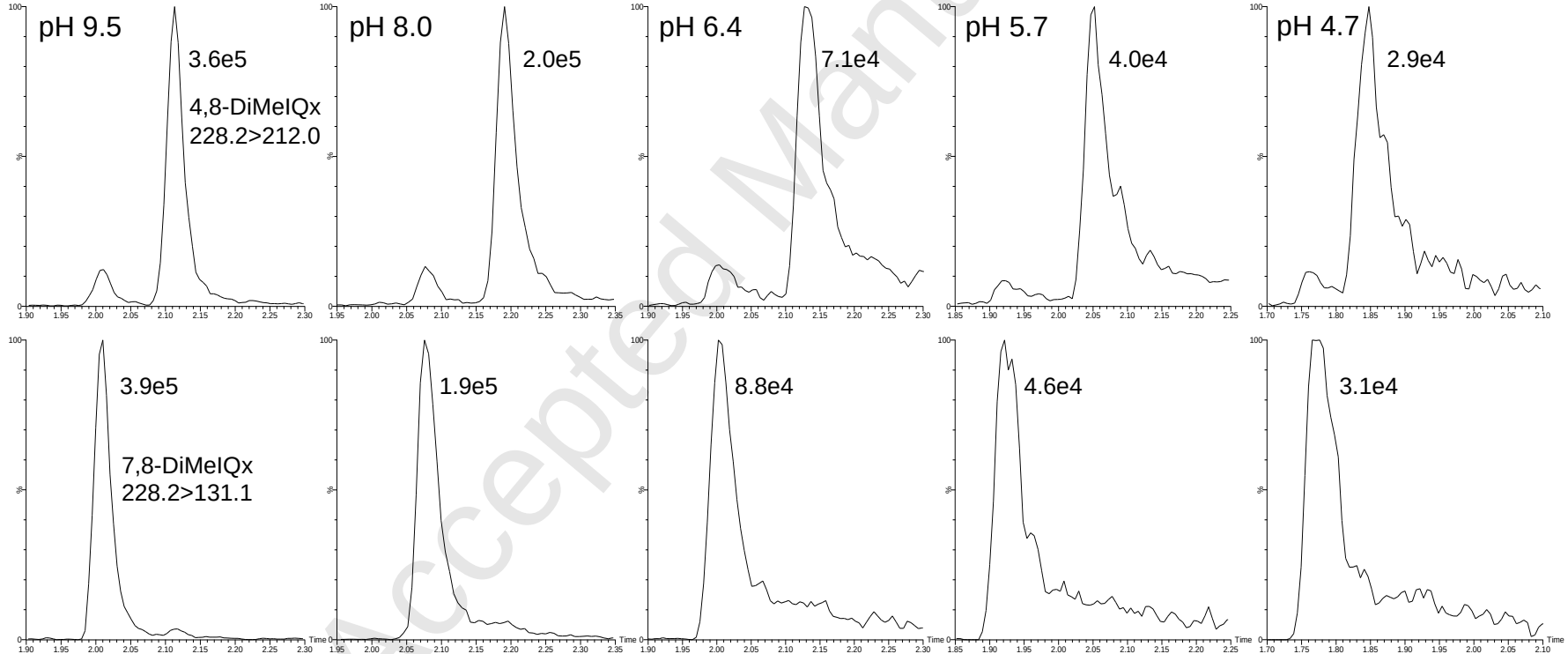


Figure 2 Cooper colour version

Figure 2 Cooper b&w version





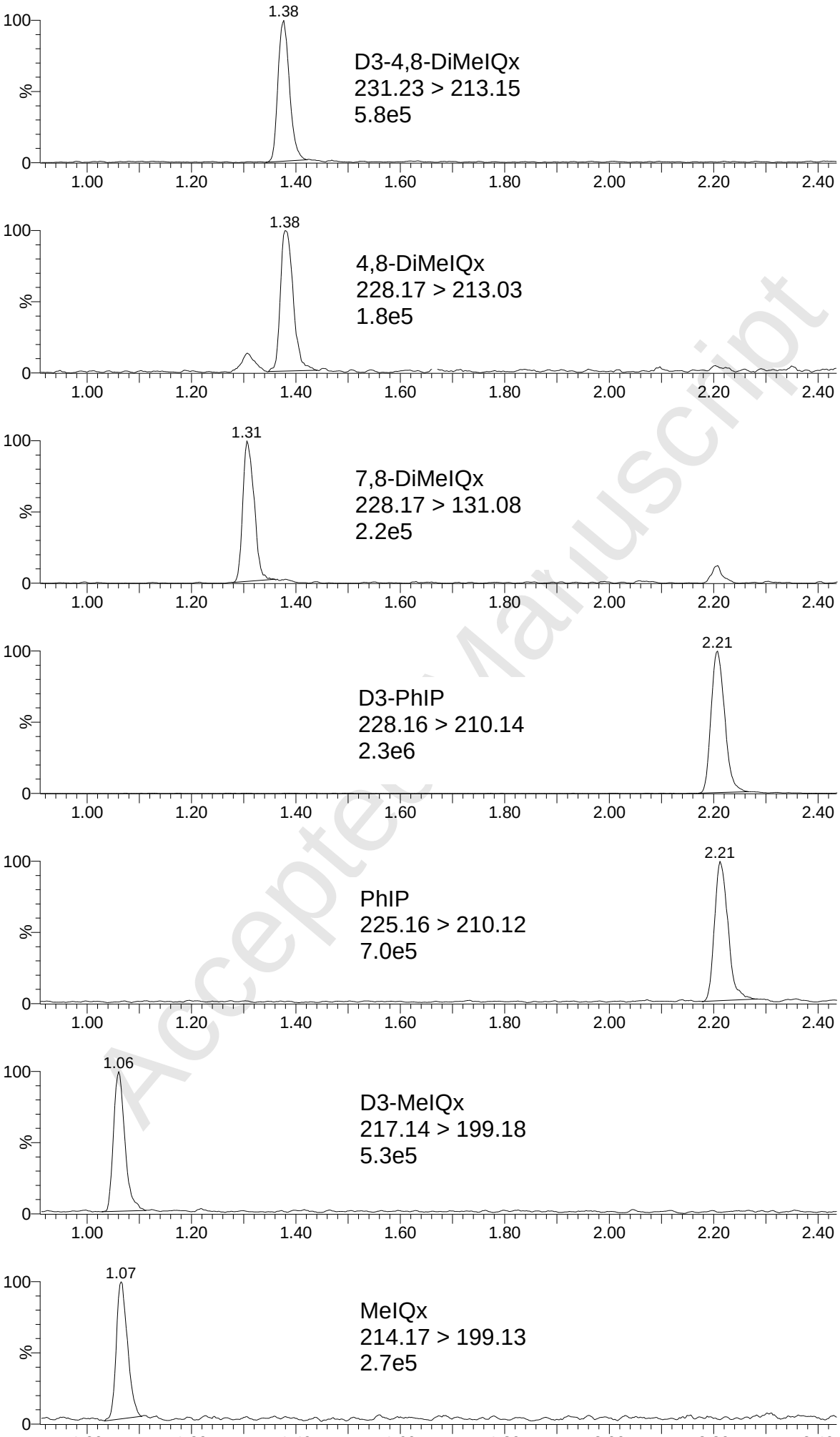


Figure 5 Cooper

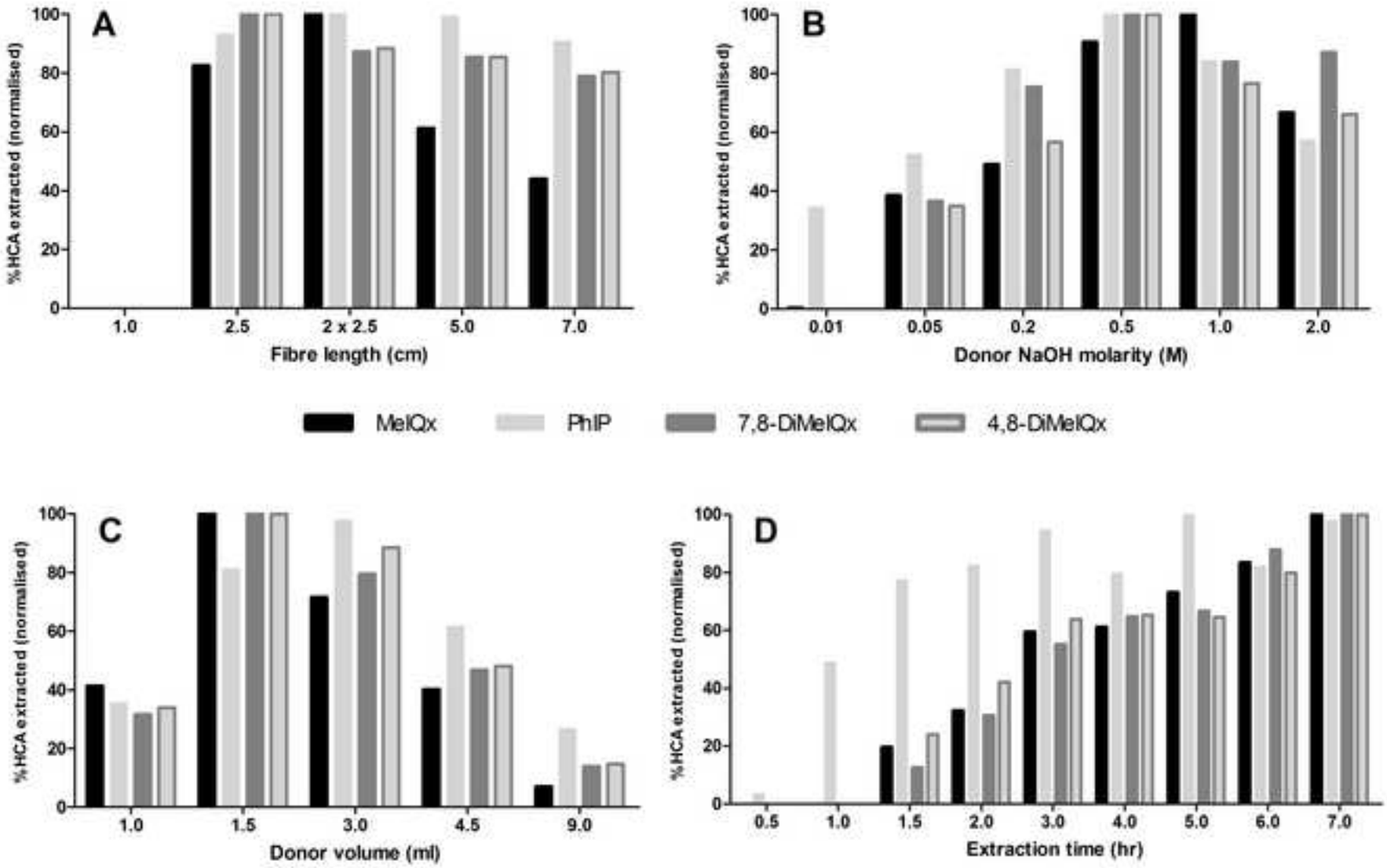


Table 1

Heterocyclic aromatic amine UPLC-MS/MS fragmentation conditions.

Analyte	t_R (min)	Primary transition (m/z)	Confirmatory transition (m/z)	Collision energy (V)
PhIP	2.21	225.2>210.1	225.2>183.2	26 / 28
D3-PhIP	2.21	228.2>210.1	-	28
MeIQx	1.07	214.2>199.1	214.2>131.1	24 / 36
D3-MeIQx	1.07	217.1>199.2	-	26
7,8-DiMeIQx	1.31	228.2>131.1	228.2>213.1	36 / 24
4,8-DiMeIQx	1.38	228.2>212.0	228.2>213.1	35 / 26
D3-4,8-DiMeIQx	1.38	231.2>213.1	-	24

Table 2

Validation of HCA extraction by HF-LPME from plasma fortified with 30 pg/mL HCAs (*n*=21; 7 replicates on 3 days).

	PhIP	MeIQx	7,8-DiMeiQx	4,8-DiMeIQx
Mean concentration (pg/mL)	27.6	29.8	27.8	28.4
Mean recovery (%)[a]	92.0	99.4	92.5	94.6
Mean within day RSD (%)	5.4	4.5	6.4	7.1
Between day RSD (%)	7.5	4.6	7.7	8.8
Limit of detection (pg/mL)	5	3	2	2
Limit of quantification (pg/mL)	15	10	7	7

[a] I.S.-corrected recovery