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Review

Chromatographic methods for the analysis of heterocyclic amine food mutagens/carcinogens

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ABSTRACT

A series of potent heterocyclic amines that are mutagenic and carcinogenic have been discovered that are formed in some heated foods, most notably, meats derived from muscle. Determining the heterocyclic amine content in foods and food products is required for toxicological research, industry quality control, and possibly in the future, regulatory control. The contents of food needs to be determined using reliable analytical techniques.

Since heterocyclic amines are present in foods at ng/g levels, a variety of liquid–liquid or solid–phase purification techniques are required, followed by gas or high-performance liquid chromatography. Peak detection has been successful using UV, fluorescence, and mass spectrometric detection, and biological activity using the Ames/*Salmonella* test. The low levels present require that chromatographic efficiency, and both detector sensitivity and selectivity be optimized. The cartridge solid-phase extraction and high-performance liquid chromatography method have been used to measure the known food-derived heterocyclic amines for several types of food, and to the authors knowledge, this is the only method undergoing intralaboratory comparison and validation.

Our analysis of the literature shows that chromatographic analysis of the heterocyclic amines by high-performance liquid chromatography or gas chromatography (with derivatization) is satisfactory for heterocyclic amine analysis in foods although the methods are just now being optimized for routine use. The biggest improvements in speed and accuracy will probably come from improved extraction methods as analysis of complex food samples for heterocyclic amines will always be a challenge.

CONTENTS

1. INTRODUCTION

Diet has long been associated with varying cancer rates in human populations, yet the causes of the observed variation in cancer patterns have not been adequately explained [1]. Since 1980, a series of potent heterocyclic amines that are mutagenic and carcinogenic have been discovered. These substances are formed in some heated foods, most notably, muscle meats. The observed higher cancer rates in those populations consuming a western diet which is high in meat has been attributed to high fat or low fiber. Heterocyclic amines are also associated with meat, and unlike fat or fiber, heterocyclic amines have the necessary genotoxic reactivity to lead to the initiating events in the cancer process.

The heterocyclic amines are carcinogens in mice, rats, and monkeys [2,3]. Several of these compounds are among the most potent mutagenic substances ever tested in the Ames/*Salmonella* mutagenicity test [4]. These compounds are also mutagenic in mammalian cells in culture and can cause chromosomal changes in these cells and in mice [5].

These heterocyclic amines are natural products formed from the heating of proteinaceous foods, most notably, muscle meats. Precursors for the formation of these mutagenic and carcinogenic compounds have been shown to be amino acids, such as phenylalanine, threonine and alanine; creatine or creatinine; and sugars [6]. Cooking temperature and time are also important determinants in the formation of these compounds in foods. These variables introduced from the formation process create a large range of possible heterocyclic amine concentrations in foods, requiring the analysis of a large number of samples to determine the heterocyclic amine content in the human diet.

2. ANALYTICAL CONSIDERATIONS

2.1. Goals of analytical method development

The finding of potent mutagenic activity in cooked foods required chromatographic work to isolate and structurally identify the chemicals responsible. Now that many of the chemicals have been identified and synthesized and shown to be animal carcinogens, the next research goals are to quantify the amounts present in foods. Determining the importance of the heterocyclic amines in human health requires accurate exposure (consumption) data with information from comprehensive epidemiology studies. This information can be combined with the rat, mouse and monkey carcinogenic potency assessment for calculation of risk.

Thus far, heterocyclic amines in foods are not regulated by government agencies, although their carcinogenic potency or the amounts detected, in many cases exceed those of many regulated compounds such as chlorinated compounds, pesticides and aflatoxins [7].

The scientific goal in the analysis of these specific carcinogens in foods is to determine the amounts and types of heterocyclic amines present. Besides the need for the determination of the human dose of these heterocyclic amines, the analysis methods could also be used to help devise cooking methods and food preparation strategies to reduce the formation of these compounds in foods. In addition, government or industrial regulation of heterocyclic amines in food may be warranted in the future, and practical methods for food analysis must be available if needed.

From a practical standpoint, a chromatographic analysis method for heterocyclic amines in foods must be accurate and reproducible. Effort must be made to have methods low enough in cost to be used for many, perhaps hundreds of samples per

year. Acceptable methods need to be low in operator and instrument time, instrument cost, and solvent and reagent cost. The method should also minimize waste generation.

2.2 Chemical characteristics of the heterocyclic amine compounds

The heterocyclic amines found in foods have stable multi-ring aromatic structures and all have an exocyclic amino group. Structures of those commonly detected in foods are shown in Fig. 1. From their structures, liquid chromatography and gas chromatography (with derivatization) appear to be suitable chromatographic analysis methods. All of the heterocyclic amines have characteristic UV spectra and high extinction coefficients, some of the compounds fluoresce and all can be electrochemically oxidized making UV absorbance, fluorescence or electrochemical detection suitable methods.

For gas chromatography (GC), detection by electron-capture or nitrogen–phosphorous thermionic detectors is possible. The aromatic structures of these heterocyclic amines give little fragmentation and therefore show large base peaks, making mass spectrometry (MS) a good detection method following either GC or high-performance liquid chromatographic (HPLC) separations.

2.3. Scope of the analytical problems

The are several factors that make the analysis of heterocyclic amines from foods a difficult problem. Heterocyclic amines are present in foods at low ng/g levels [8]. The low levels require that chromatographic efficiency and both detector sensitivity and selectivity be optimized.

Several of the heterocyclic amines are formed under the same reaction conditions, so the number of compounds of interest requires that the extraction, chromatographic separation and detection be general enough to detect several of the heterocyclic amines per chromatographic experiment.

The complexity and diversity of food sample types needing to be analyzed requires a rugged method not affected by the sample matrix. The extraction methods used are an integral part of the chromatographic analysis because the extractions are suited to the requirements of the chromatography and detection. The sample extraction and preparation methods will be discussed in detail for each chromatographic technique presented in this paper.

3. CHROMATOGRAPHIC ANALYSIS METHODS

3.1. HPLC with detection by bacterial mutagenesis

In the late 1970s the detection of bacterial mutagens in cooked meats led to efforts to isolate the

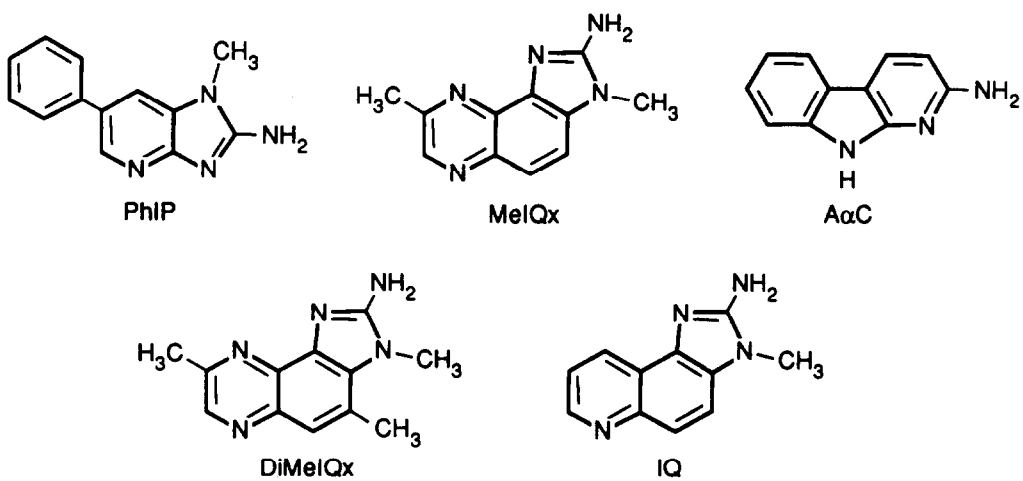

Fig. 1. Structures of five heterocyclic amines commonly found in cooked food. PhIP = 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; MeIQx = 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; AαC = 2-amino-9H-pyrido[2,3-*b*]indole; DiMeIQx = 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; IQ = 2-amino-3-methylimidazo[4,5-*f*]quinoline.

mutagenic chemicals using a variety of extraction methods followed by several HPLC steps. Working on a preparative scale, and with the goal of purifying microgram amounts of the mutagenic chemicals, samples were extracted in acid or organic solvents. Further purification was done using base extractions and open column adsorption or chromatography steps. These workers used the mutagenic activity of collected fractions at each step to guide the purification of the heterocyclic amines for structural determination [9–11]. These studies also gave an estimate of the quantity of the chemical in the original food, but extraction losses could not be accurately determined.

Because the bacterial mutation test is not affected by the hundreds of non-mutagenic chemicals also present following separation by HPLC, complex samples could be characterized by collecting fractions and testing each fraction for mutagenic activity. The mutagenic activity profile of collected fractions from HPLC separations was used to determine that the minimum number of mutagenic compounds formed in cooked beef was six [8]. Mutagenic activity profiles of beef cooked at different temperatures showed that mutagenic compounds with similar retention times and relative amounts were formed despite the generation of tenfold more mutagenic activity with the higher cooking temperatures [12].

Fig. 2 shows the HPLC separation of equal amounts of mutagenic activity from the extract of beef, chicken and fish, all ground and cooked as patties at 220°C for 10 min per side. The samples show a similar pattern of mutagenic activity in peak size and retention time despite the differences in meat source and the large difference in total mutagenic activity. The samples have a ratio of mutagenic acitivity of 1:5:8 for fish, chicken and beef, respectively.

The effect of mutagen-increasing additives before cooking was also investigated using the mutagenic activity of collected HPLC fractions. It was determined that creatine was a precursor for almost all of the HPLC separable mutagenic compounds in fried-ground beef and chicken [13]. The effect of the addition of individual amino acids, and creatine to ground pork before frying was noted by Övervik *et al.* [14] using the HPLC–bacterial mutagenesis analysis method.

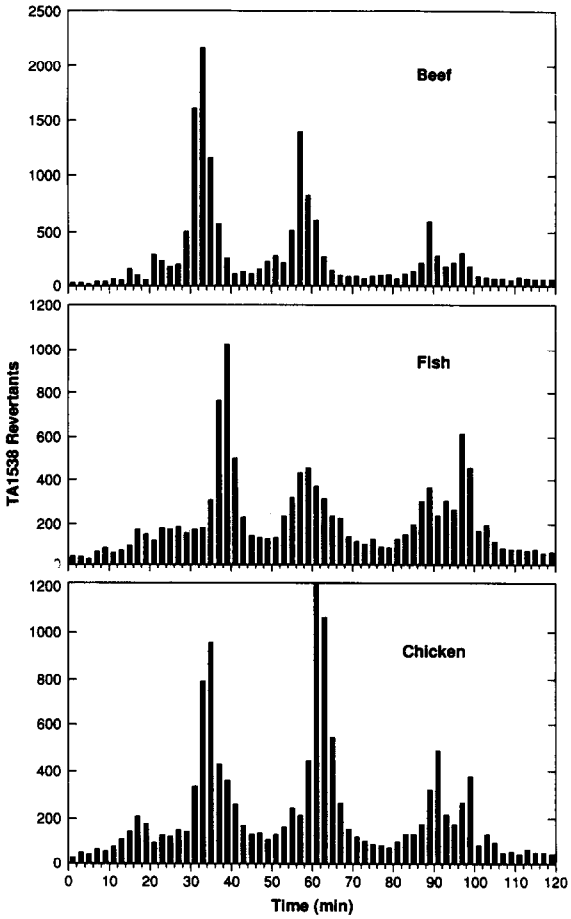

Fig. 2. Mutagenic activity profiles of collected fractions from the HPLC separation of ground-fried beef, fish and chicken. Elution from a PRP-1 styrene–divinylbenzene column was with 15% acetonitrile, 0.1% diethylamine in water for 70 min, with a gradient to 100% acetonitrile at 140 min. Equal amounts of mutagenic activity were injected for each sample.

The HPLC–bacterial mutagenesis method is useful for characterizing samples containing unknown mutagenic chemicals for which no standard compounds are available. Quantitation is crude, but it is useful for sample-to-sample comparisons. Chromatographic coelution experiments using the analysis of collected fractions can also be done despite the complexity of the samples as determined by UV absorbance.

This method is very labor intensive and has a slow sample throughput since samples must be collected and tested for mutagenic activity following

each separation. Quantitation is limited since many sample-handling steps are needed. The range of mutagenic activity per microgram of the heterocyclic amines reported in the literature spans several orders of magnitude and illustrates the problem in estimating of the mass of the mutagenic chemicals from measurement of mutagenic activity.

3.2. Cartridge solid-phase extraction–HPLC

Analyzing heterocyclic aromatic amines at nanogram levels with HPLC and ultraviolet or fluorescence detection requires chromatograms free from interfering peaks. Co-extracted matrix components influence analyte detection limits more than does the absolute detector sensitivity. The sample work-up therefore, is the most critical part of heterocyclic amine analysis. Work at Nestec [15–17] focused on developing practical solid-phase extraction (SPE) based procedures allowing high sample throughput and high analytical sensitivity in food analysis. SPE refers to procedures using disposable cartridges typically containing 100 to 500 mg of a solid, often silica-based, sorbent. Advantages of SPE are considerable simplification and speeding up complicated sample preparation prior to chromatographic analysis.

Food samples such as fried meat or fish require an initial extraction. Diatomaceous earth (Kieselgur, Extrelut), a sand-like porous material is a very practical carrier for extracting all known heterocyclic amines from solid or liquid foods. The inert diatomaceous earth carrier allows efficient and rapid organic solvent extraction without any risk of emulsion formation, a very common problem when extracting food. Fig. 3 shows the extraction profile of radiolabeled 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), indicating that more than 70% of MeIQx and 50% of PhIP are recovered with 40 ml dichloromethane. The crude extract contains basic and neutral substances, however, sensitive analysis of low heterocyclic amine levels in such an extract is not possible due to co-extracted interfering substances. Therefore, additional sample preparation procedures using solid-phase-extraction steps were developed.

Sequential application of several SPE media with different selectivities reduces matrix background as

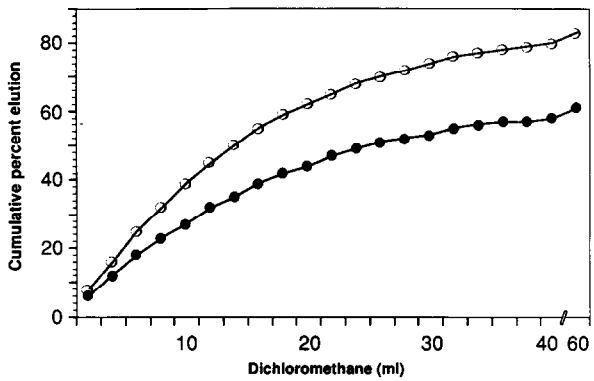

Fig. 3. Extraction of MeIQx and PhIP from fried meat with Kieselgur carrier. Fried meat samples were spiked with [^{14}C]MeIQx or [^{3}H]PhIP and loaded on Extrelut-20 colums as described [15]. During extraction with dichloromethane 20 fractions of 2 ml and one 20-ml fraction were collected. The graph shows the cumulative recovery of both [^{14}C]MeIQx (○) and [^{3}H]PhIP (●) as a function of the extraction volume.

a means to lower analyte detection limits but with the knowledge that multiple purification steps increase the time required for the sample preparation and can introduce sources of errors. Coupling SPE cartridges to "tandems" eliminates time-consuming sample handling steps such as evaporations and resuspensions. The key finding leading to the development of this tandem extraction method was that cation-exchange resins retain heterocyclic amines in dichloromethane which is used for the initial sample extraction with diatomaceous earth.

Cartridge extraction of food products using coupled diatomaceous earth and propylsulfonic acid silica (PRS) cartridges efficiently concentrates basic compounds but PRS also retains other dichloromethane-soluble polar compounds which may interfere with the amines during HPLC. Using the ion-exchange properties of PRS cartridges, a simple procedure to remove most of the unwanted co-extracted interfering peaks was developed. Drying the PRS cartridge with the adsorbed basic extract followed by reequilibration with 0.1 *M* hydrochloric acid initiates ion-exchange binding between heterocyclic amines and the resin. Two simple elution steps subsequently separate heterocyclic amines into two groups, the carboline derivatives and the 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-type compounds. Concentration of these eluates is con-

veniently achieved on a C_{18} silica cartridge. The heterocyclic amines can be eluted and conveniently concentrated by passing over a C_{18} silica cartridge, rather than by rotary evaporation, and finally eluted into autosampler vials with a methanol-ammonia solution mixture [15].

Despite being a multi-step procedure, the diatomaceous earth–PRS method extraction and clean-up does not included any off-line transfers requiring rotary evaporator concentration and resuspension and is well suited for application in routine quality control. Quantitative determinations are done by HPLC with multichannel UV and fluorescence detection. The levels detected were usually in the low ng per gram range with the most abundant compounds found in food samples being MeIQx, PhIP and 2-amino-9H-pyrido[2,3-*b*]indole (AαC) [16].

3.2.1. HPLC

Baseline separation of all heterocyclic amines was a prerequisite for the multi-compound analysis method. The TSK gel ODS80TM (TosoHaas, Montgomeryville, PA, USA) column showed superior peak symmetry and separation efficiency as compared to other HPLC columns tested. Binary mobile phase gradients with acidic buffer (between pH 3 and 4) and acetonitrile gave good peak shapes, but above pH 3.2, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) co-eluted, and below pH 3.5, 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-2) and PhIP were not baseline separated. They could be resolved though, using a ternary gradient including pH switching from pH 3.2 to pH 3.6 during the run as shown in Fig. 4 [16].

3.2.2. Quantification model

The extraction efficiency of heterocyclic amines following a purification procedure as outlined above is less than one hundred percent. Using a single internal standard added to each sample at the beginning of the extraction is a possible way to estimate analyte losses [15]. This is not ideal because heterocyclic amines include several classes of compounds which are extracted with different efficiency. Even within a group of compounds significant variations may exist as shown for MeIQx and PhIP in Fig. 3. Moreover, the extraction efficiency of heterocyclic

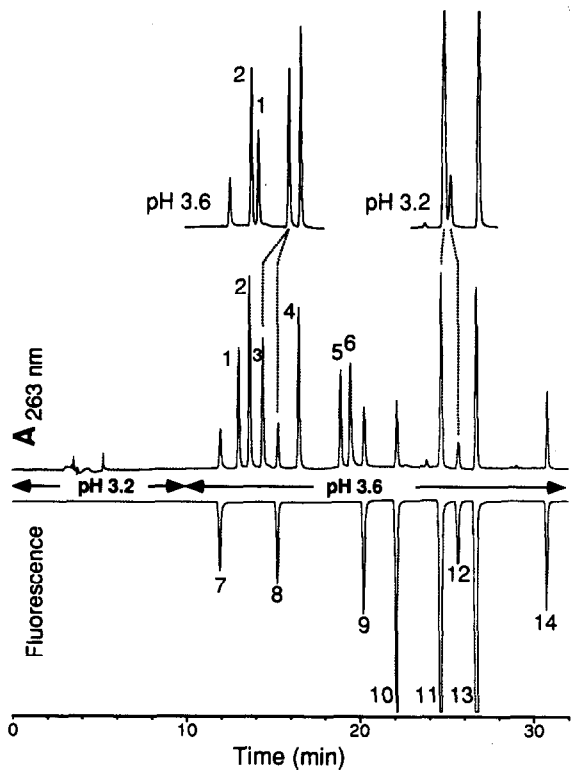

Fig. 4. Separation of heterocyclic amine reference standards. A ternary mobile phase system was used to separate 14 heterocyclic amines on a Toyo Soda TSK gel ODS80TM column (250 mm × 4.6 mm I.D.). A = Triethylamine phosphate (TEAP) 0.01 *M* pH 3.2; B = TEAP 0.01 *M* pH 3.6; C = acetonitrile. Linear gradient: 5–15% C in A from 0–10 min.; 15–25% C in B from 10–20 min; 25–55% C in B from 20–30 min. Peaks are identified as follows (in parentheses excitation/emission wavelengths used for fluorescence detection): 1 = IQ; 2 = 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx); 3 = MeIQ; 4 = MeIQx; 5 = 7,8-DiMeIQx; 6 = DiMeIQx; 7 = 2-amino[1,2*a*:3′,2′-*d*]imidazole (Glu-P-2) (360/450); 8 = Glu-P-1 (360/450); 9 = norharman (300/440); 10 = harman (300/440); 11 = Trp-P-2 (263/410); 12 = PhIP (315/390); 13 = 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1) (263/410); 14 = AαC (335/410). A 20-μl volume of a mixture of *ca.* 5 ng per compound was injected.

amines is somewhat dependent on the surrounding matrix and, therefore, difficult to predict precisely for different foods. Standard addition quantification is the method of choice for assessing individual recovery rates for each analyte. Triplicate or quadruplicate extractions with one or two samples spiked with a mixture of reference standards to obtain sets of concentration data for each analyte is

adequate. Linear regression analysis using the added concentration of reference standard (x) as dependent variable and the measured concentration (y) as independent variable enables calculation of individual extraction efficiencies and, therefore, to make accurate corrections of incomplete analyte recovery. Standard errors of slope and intercept give a good estimation of the achieved analytical precision. The necessity of performing replicate extractions may also be seen as a handicap lowering the sample throughput. Stable isotope dilution quantification using selected ion monitoring (SIM) LC–MS or GC–MS would be more economic in terms of sample extractions.

3.2.3. Validation, detection limits

Checking the reproducibility of any method by independent laboratories is crucial if the method is to be used routinely. In a 1991 study, four independent laboratories in the USA and Europe used the diatomaceous earth–PRS tandem extraction method to determine heterocyclic amine content in a meat extract. Analyte recoveries as observed in the four laboratories ranged from 41 to 85% for MeIQx, and from 43 to 83% for 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx). Recovery correction was done using the standard addition quantification model and lowered the interlaboratory variations almost threefold. A similar validation study will be completed in Europe in 1992.

The detection limits of heterocyclic amines purified through diatomaceous earth–PRS tandem extraction were variable and depended on the food sample investigated. With meat extracts or fried meat not processed at temperatures above about 250°C, detection limits may be as low as 1 ng/g using UV detection and below 1 ng/g with fluorescence detection. The question of limits of detection are crucial since many food products investigated to date did not show any detectable levels of heterocyclic amines. Limits of detection can be verified individually using low level spiking as shown in Fig. 5 for a commercial precooked meat product. Samples processed at high temperatures, *e.g.*, by grilling or flame-broiling, usually showed higher levels of interfering peaks. The detection limits then worsened to about 5 ng/g of food.

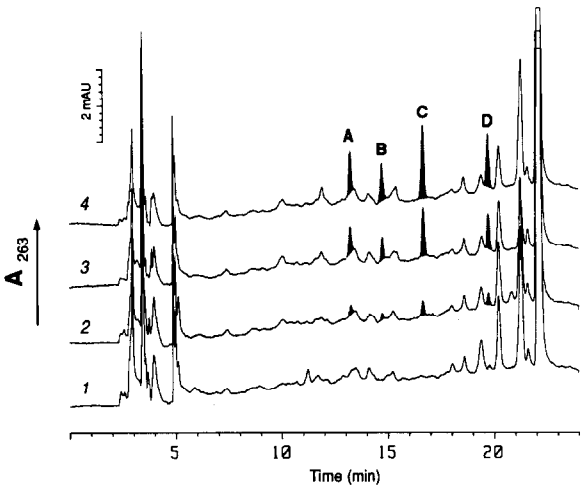

Fig. 5. Evaluation of heterocyclic amine detection limits through spiking with low levels of reference standards. A meat-containing industrially-prepared food product was extracted in quadruplicate using the Extrelut–PRS tandem extraction method and each extract analyzed by HPLC. The figure shows chromatograms of the four polar extracts (0.8 gram equivalents injected). Sample 1 was unspiked, whereas samples 2–4 were spiked with 0.25, 0.75 and 1.2 ng per gram heterocyclic amine reference standards IQ (A), MeIQ (B), MeIQx (C) and 4,8-DiMeIQx (D). Chromatographic conditions as in Fig. 4.

3.2.4. Peak identity confirmation

Peak confirmation is a crucial problem when working with such low levels of heterocyclic amines since co-elution with other co-extracted compounds can occur. The most convenient and accessible instrument to identify heterocyclic amines on-line during an HPLC separation is the UV photodiode array detector. Commercial software included with most instruments allows the recording of interpretable UV spectra even at low nanogram levels using background subtraction. A photodiode array detection system efficiently prevents false peak identifications as shown in Fig. 6 for a peak with the retention time of Trp-P-2 but not having the proper UV spectrum.

3.2.5. Solid-phase extraction with TSK CM650 for more complex samples

Some foods showed increased levels of chromatographic interferences, such as high-temperature processed meat and fish (see Fig. 7), and also some industrial flavorings, (so called process flavors), and, therefore, required a more thorough sample clean-up [14].

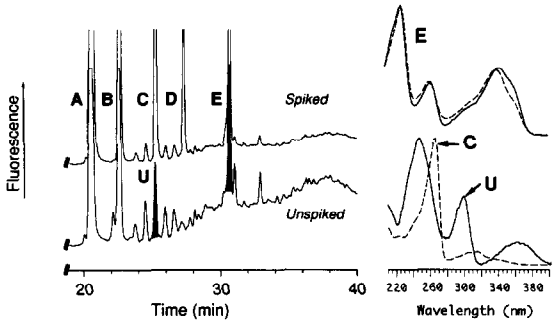

Fig. 6. Peak identification of heterocyclic amines in non-polar extracts of barbecued hamburgers. The left side shows expanded chromatograms of extracts from both spiked (0.8 gram equivalents injected) and unspiked (2 gram equivalents injected) barbecued hamburger samples. Heterocyclic amines identified through retention time matching in the unspiked sample included norharman (A), harman (B), Trp-P-2 (C/U), Trp-P-1 (D), and amino-α-carboline (E). However, as shown on the right with the aid of on-line recorded UV spectra the identification of Trp-P-2 (U) had to be rejected. Chromatographic conditions as in Fig. 4.

A weak cation-exchange material was used as a sorbent with different selectivity than used in the previous purification steps. TSK CM650 has superior affinity and selectivity for heterocyclic amines

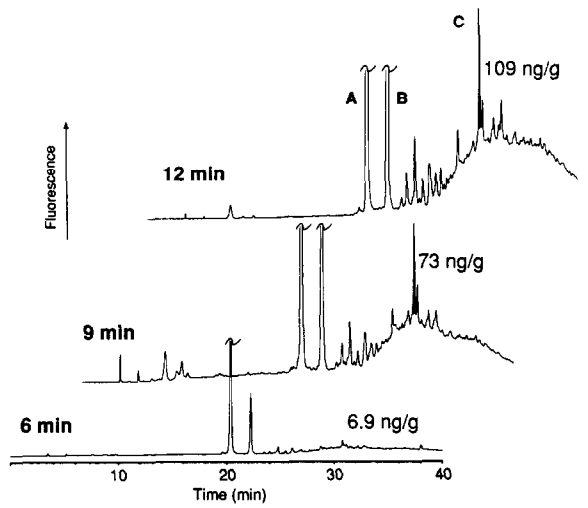

Fig. 7. Prolonged processing at high temperatures increases the level of chromatographic interferences. The graph shows non-polar extract chromatograms from salmon barbecued for 6, 9 or 12 min per side at 270°C. Identified heterocyclic amines include norharman (A), harman (B) and amino-α-carboline (C). Starting at 9 min processing time a dramatic increase in interference levels was clearly visible. Chromatographic conditions as in Fig. 4.

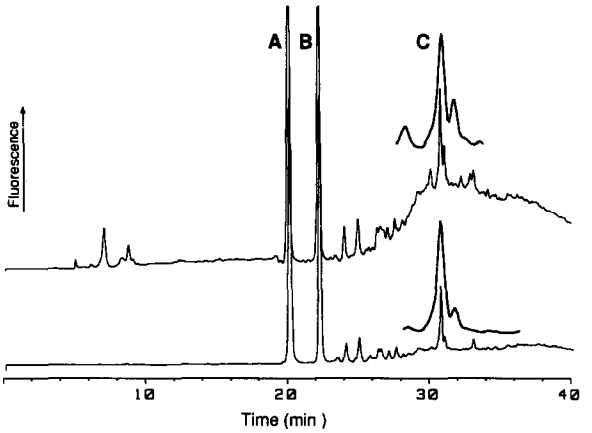

Fig. 8. Solid-phase extraction with TSK CM650 resin lowers chromatographic interferences. Both traces show chromatograms of a non-polar extract of salmon slices barbecued for 12 min per side (0.7 gram equivalents injected). The three major peaks are norharman (A), harman (B) and amino-α-carboline (C). The upper trace is from an extract purified by tandem extraction with Extrelut–PRS, the lower trace was obtained after additional solid-phase extraction using 250 μl TSK CM650 resin and illustrates the background removal effect. Amino-α-carboline peaks are enlarged. Chromatographic conditions as in Fig. 4.

compared to silica-based weak cation-exchange resins. The recovery of analytes from the TSK CM650 resin is close to 90% and its selectivity is impressive. TSK CM650 clean-up was applied to purify extracts of process flavors [17] and high-temperature-treated meat and fish. Interfering peaks in extracts prepared by diatomaceous earth–PRS extraction were significantly lowered (Fig. 8) using this treatment.

The cartridge solid-phase extraction–HPLC method has been used on a variety of samples including meat extracts, cooked meats and process flavors. Although complex samples are the subject of ongoing research, this method is currently being tested in multiple-laboratory collaborative studies which is an important step toward its widespread use.

3.3. Derivatization GC–MS

The most sensitive approach for heterocyclic amine analysis is that devised by Murray *et al.* [18]. Using cooked meat samples spiked with heavy-isotope-labeled standards, samples are dissolved in di-

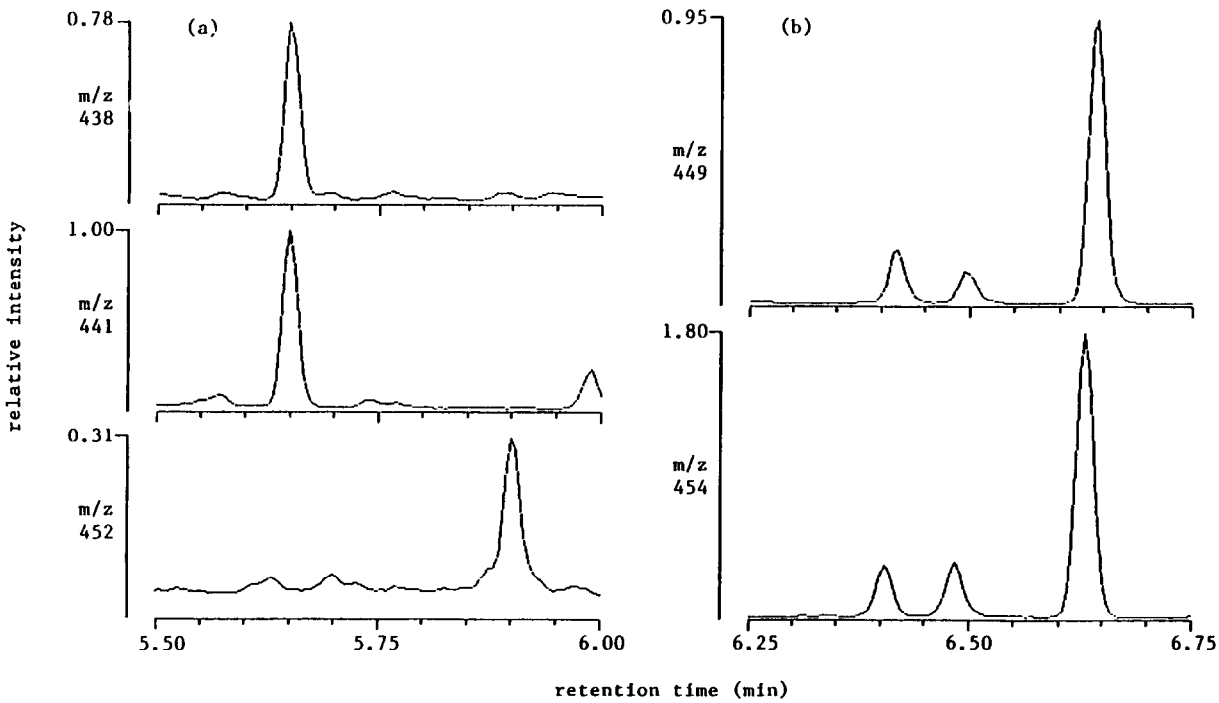

Fig. 9. Selected ion monitoring traces for the analysis of (a) MeIQx and DiMeIQx and (b) PhIP in fried beef. Retention times of the dibistrifluoromethylbenzyl derivatives of MeIQx (*m/z* 438) and [^{13}C, $^{15}N_2$]MeIQx (*m/z* 441) are 5.65 min, of DiMeIQx (*m/z* 452) 5.90 min, and of PhIP (*m/z* 449) and [$^{2}H_5$]PhIP (*m/z* 454) 6.65 min.

lute HCl, washed with dichloromethane to remove oils and fats and then extracted into ethyl acetate after pH adjustment to an alkaline condition.

Dried extracts are then derivatized with 3,5-bis-trifluoromethylbenzyl bromide at room temperature, washed with hexane and extracted with ethyl acetate. The total yield for these extraction steps is reported to be about 40% as determined by radioactivity measurements with [^{14}C]MeIQx [18].

Analysis is by GC–electron-capture negative ion chemical ionization MS. Recovery for extraction and derivatization can be calculated from the internal standard. The specificity of single ion monitoring does not require the degree of sample purification that is needed for the LC detectors.

Analysis of derivatized heterocyclic amines uses the high chromatographic efficiency of capillary GC. The chemical ionization and negative ion detection give a reported 1 pg detection limit using SIM.

Fig. 9 shows the results of the analysis of MeIQx, DiMeIQx and PhIP in fried beef by Murray *et al.* Monitoring of appropriate masses yields reasonably clean traces. Calculated results show 3.5 ng MeIQx, 1.2 ng DiMeIQx and 20.3 ng PhIP per gram of cooked beef [19].

Fig. 10 shows analysis of a beef stock cube. MeIQx, DiMeIQx and PhIP were detected at 0.6, 0.3 and 0.3 ng/g, respectively. The limits of detection for the complete liquid–liquid extraction–derivatization GC–MS assay of fried meat and beef stock cubes are 0.05, 0.1 and 0.2 ng/g for MeIQx, DiMeIQx and PhIP, respectively [19].

The sensitivity of this method has also been shown in the analysis of human urine in the determination of MeIQx with a detection limit of 5 pg/ml of urine. About 5% of the MeIQx ingested in beef hamburgers was recovered unchanged in urine [20].

Advantages of this method are the use of isotopically labeled internal standards for accurate quantitation and recovery determination in a single chromatographic run, the unequaled sensitivity of

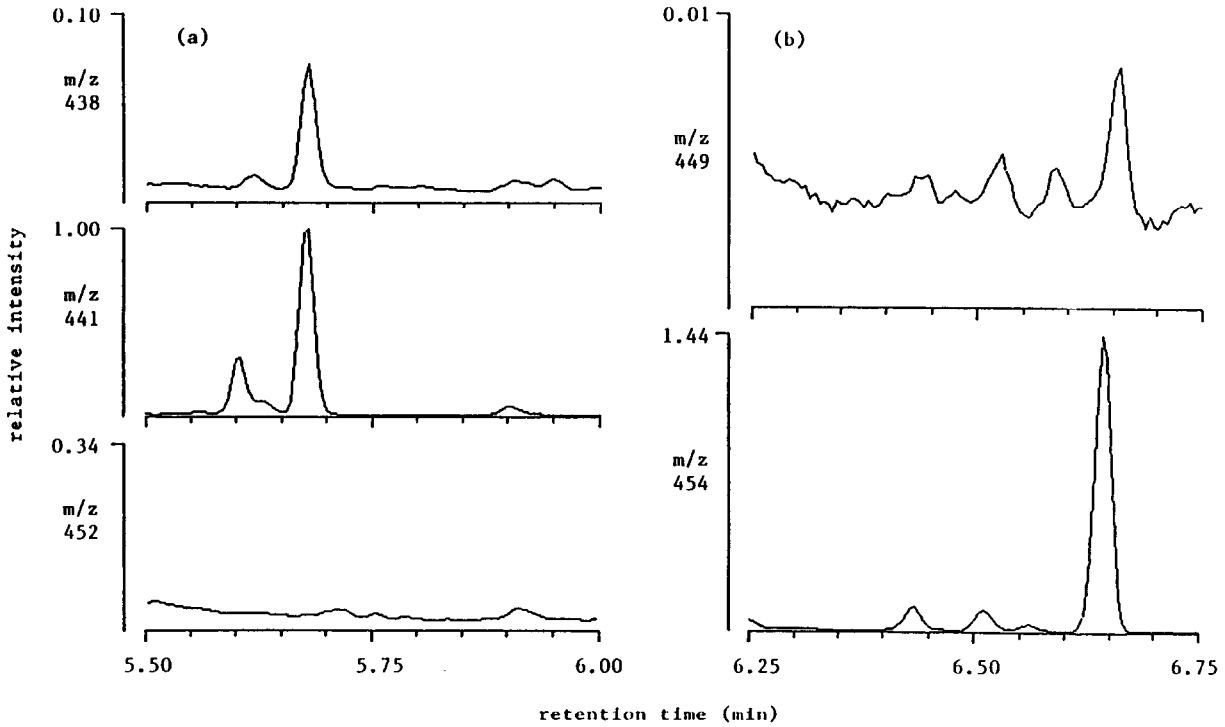

Fig. 10. Selected ion monitoring traces for the analysis of (a) MeIQx and DiMeIQx and (b) PhIP in a beef stock cube. Retention times of the dibistrifluoromethylbenzyl derivatives of MeIQx (*m/z* 438) and [^{13}C,$^{15}N_2$]MeIQx (*m/z* 441) are 5.70 min, of DiMeIQx (*m/z* 452) 5.90 min, and of PhIP (*m/z* 449)) and [$^2H_5$]PhIP (*m/z* 454) 6.65 min.

derivatization and negative ion detection, the simplified extraction scheme, and the peak confirmation possibilities using additional ion signals.

Disadvantages include the expensive and specialized instrumentation, the possible uncertainty of the derivatization reactions, especially at low quantities of reactants, and the instability of GC columns used at temperatures from 200 to 320°C. Heavy-isotope-labeled internal standards are needed, although some are available commercially. At present, the specific protocols have been developed only for the analysis of MeIQx, DiMeIQx and PhIP in foods with this method.

3.4. LC–MS

Published methods for LC–MS for the analysis of heterocyclic amines in salmon, sardine and beef were reported by Yamaizumi *et al.* [21] and analysis of beef and beef extracts were reported by Turesky *et al.* [22].

Each procedure required multistep purification with cellulose trisulpho-copper-phthalocyanine (blue cotton) adsorption and acid–base partition. Heavy-isotope-labeled internal standards were used to determine extraction recovery and also for a chromatographic standard. For the published methods, different compounds were examined, IQ and MeIQ for Yamaizumi *et al.*, and MeIQx, DiMeIQx and IQ for Turesky *et al.* Thermospray MS was used and measurements of heterocyclic amines as low as 0.3 ng/g of food were reported by each group.

Fig. 11 shows the thermospray LC–MS analysis of fried beef for IQ, MeIQx and DiMeIQx, and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx) by Turesky *et al.* The traces monitored at the appropriate single ion for the (M +

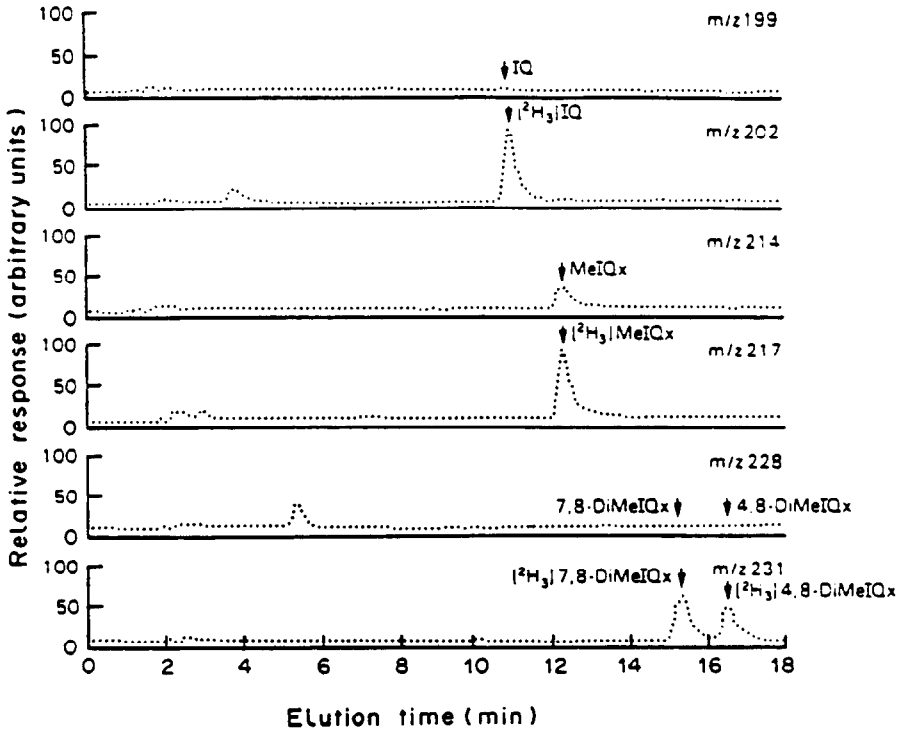

Fig. 11. Selected ion monitoring traces for the thermospray LC–MS analysis of fried meat with isotopically labeled internal standards. Separation was done on a gradient elution of a C_{18} column [22].

$H)^+$ ion are remarkably free of interfering peaks.

LC–MS offers the advantage of column stability compared to GC especially with complex food extracts. MS offers the selectivity of mass detection with confirmation by heavy-isotope-labeled standard coextraction and coelution.

Disadvantages include expensive instrumentation and the need for heavy-isotope-labeled internal standards. The method as published requires time-consuming sample preparation.

3.5. Other purification and detection methods

Electrochemical detection of heterocyclic amines was reported for beef extracts following acid–base partition, cellulose trisulpho-copper-phthalocyanine and silica adsorption [23,24]. Electrochemical detection offers increased detector specificity over UV absorbance detection, although the sensitivities of the two detector types are about the same. Peak confirmation with electrochemical detectors is not possible as it is with photodiode array detectors. Methods analyzing prepared foods with electrochemical detection have not been reported in the peer-reviewed literature, but results of food analysis with electrochemical detection were included in a review [25].

Immunoaffinity chromatography was used for the purification of IQ and MeIQx from heated beef products [26]. Following solubilization, samples were adsorbed on XAD-2 resin, eluted, and applied to affinity columns which had antibodies immobilized on them to IQ or MeIQx. Resulting eluents were chromatographed using HPLC and photodiode array detection. Specific antibodies are needed for each heterocyclic amine, but to determine only one marker compound, immunoaffinity column chromatography offers impressive specificity.

Monoclonal antibodies were developed for the direct analysis of heterocyclic amines in food extracts, but cross-reacting interfering substances made quantitation of heterocyclic amines in these complex samples impractical [27].

4. CONCLUSIONS AND FUTURE NEEDS

It appears that some type of chromatography will always be required for the analysis of heterocyclic amines from foods following rigorous sample clean-up.

The cartridge SPE scheme is a dramatic improvement over the liquid–liquid extractions and the use of large columns filled with XAD resin or cellulose trisulpho-copper-phthalocyanine adsorbent. It is probable that the cartridge extraction scheme could be used successfully with some of the other chromatographic separation and detection procedures described above.

The choice of a method depends on the scientific information needed and complexity of the sample. If only a single marker compound is necessary, extractions can be modified to optimize for a single analyte. For quantitation of all known heterocyclic amines, the general detection advantages of HPLC are required. More concentrated samples like bacterial or food-grade beef extracts could be analyzed by any of the methods presented. Meats and process flavors require extensive sample clean-up for HPLC analysis or the specificity of a MS detector. To detect very low concentrations of analytes (below 1 ng/g), the derivatization GC–MS instrumentation method is best suited.

The amounts of heterocyclic amines in cooked beef or fish published to date show ranges from 0.02–0.36 ng/g for IQ, 0.9 to 8.3 ng/g for MeIQx and 9.7 to 49 ng/g for PhIP [28]. These measurements are all from laboratory-cooked food, the range of amounts in a typical human diet has not been determined.

Validation for the methods discussed in this review should include the determination of the precision, accuracy, and ruggedness as used in other laboratories in comparative studies. To our knowledge, only the cartridge SPE–HPLC method is being used in interlaboratory comparisons. An important criteria for method accuracy is to demonstrate that the results obtained from one method are the same as those determined by a second and independent method. To our knowledge, the comparison has not been done for any of the published methods.

The cartridge SPE method is well developed for 12 heterocyclic amines. Still, new column materials could improve yields or reduce interfering peaks during chromatography, particularly for the more complex foods produced at higher temperatures. Improved columns may help resolve coeluting peaks or increase detector sensitivity for HPLC or GC. And for GC more stable separation columns, particularly those used at higher temperatures, would improve reproducibility.

For all of the analysis methods discussed, improved detector sensitivity or specificity would improve quantitation. The faster and less expensive computers now available for almost any detection system reduce the cost and time needed for data processing especially when large numbers of samples are analyzed.

The variety and complexity of foods and the small amounts of the heterocyclic amines present insures that the analysis of low nanogram levels of these compounds in such a complex matrix such as food will never be simple or inexpensive. Still, great progress has been made in heterocyclic amine analysis and a variety of chromatographic methods have been successfully used. The experience of the chromatographer and the instrumentation on hand will play a large role in the methods chosen. The analysis of foods is just beginning. The validation of the methods presented needs to be made by comparisons of food samples among different laboratories using various methods of analysis.

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