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Quantitation of mutagenic/carcinogenic heterocyclic aromatic amines in food products☆

G. A. Gross* and A. Grüter

NESTEC Ltd., Research Centre, Vers-chez-les-Blanc, Post Office Box 44, CH-1000 Lausanne 26 (Switzerland)

ABSTRACT

A method for screening genotoxic heterocyclic aromatic amines in cooked foods using solid-phase extraction and high-performance liquid chromatography with ultraviolet and fluorescence detection is described. Solid-phase extraction includes basic extraction on diatomaceous earth (Extrelut) and subsequent purification on propylsulphonic acid silica gel. This convenient procedure separates the analytes into a polar group and an apolar group. We have identified the following components in the two groups. The polar group contains aminoimidazoazaarenes *i.e.* 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline, 2-amino-3-methylimidazo[4,5-*f*]quinoline, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline, 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine, and glutamic acid pyrolysates, *i.e.* 2-amino-6-methyldipyrido[1,2-a:3′,2′-*d*]imidazole and 2-aminodipyrido[1,2-a:3′,2′-*d*]-imidazole. The apolar group consists of five carbolines: 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole, 3-amino-1-methyl-5H-pyrido-[4,3-*b*]indole, 2-amino-9H-pyrido[2,3-*b*]indole, 9H-pyrido[3,4-*b*]indole and 1-methyl-9H-pyrido[3,4-*b*]indole. The extraction efficiencies range from 45 to 90%, and the detection limits are in the low nanogram per gram range. The method was applied to the analysis of heterocyclic aromatic amines in pan-fried, oven-cooked and barbecued salmon.

INTRODUCTION

Frying or broiling of meat and other protein-rich foods may generate several heterocyclic aromatic amines (HAAs; for structures, see Fig. 1), *i.e.* amino acid pyrolysates or aminoimidazoazaarenes (AIAs) [1]. All the HAAs tested so far induce tumors at multiple sites in rodents and may be potential human carcinogens [2,3]. Therefore, accurate quantification of heterocyclic amines in cooked foods is essential for human risk assessment.

Most HAAs have shown potent mutagenic activity in the Ames test using *Salmonella typhimurium* frame shift trains TA98 or TA1538 and metabolic activation [4,5]. This assay successfully operates even with fairly crude or partially purified materials which are too dirty for liquid or gas chromatographic analysis. Many HAAs were isolated by bioassay-directed purification using the Ames test as a detection method.

Older methods for isolation or quantitative determination of HAAs included extensive fractionations in multiple steps (reviewed in ref. 1). These methods, therefore, were not suitable for routine screening. Specific monoclonal antibodies (MAbs) allowed the purification of MeIQx, IQ or PhIP (for abbreviations, see Table I) to be simplified [6–8], but these MAbs were not made commercially available. For food quality control the need for a more universal but also sensitive and convenient clean-up method still exists.

We previously reported two solid-phase extraction methods for the analysis of many HAAs [9]. Both methods relied on combined extraction and adsorption on coupled columns (tandems) of diatomaceous earth and a cation-exchange resin, *i.e.* propylsulphonic acid silica (PRS) or copper phthalocyanine-bound Sephasorb HP. However, neither of these procedures allowed the analysis of all HAAs.

☆ This paper has been dedicated to the memory of Hans-Ulrich Aeschbacher.

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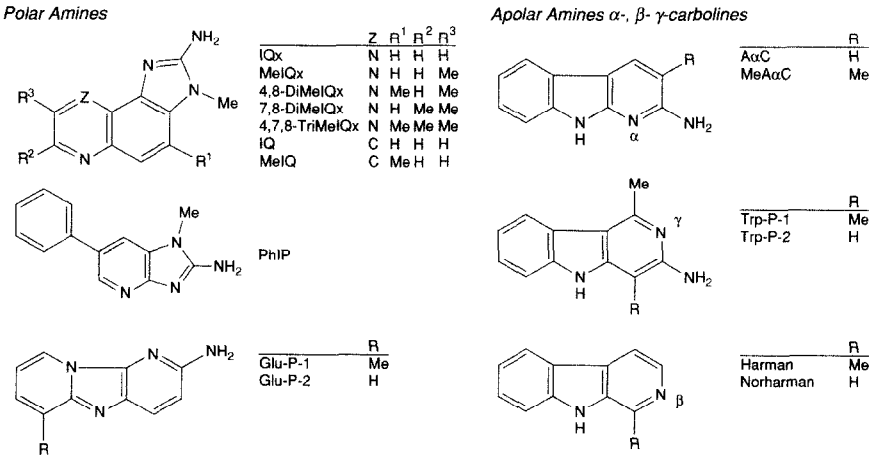

Fig. 1. Chemical structures of heterocyclic aromatic amines. Me = Methyl.

We now report an improved fractionation method for purifying the entire range of HAAs on PRS resin.

EXPERIMENTAL

Chemicals and solvents were high-performance liquid chromatography (HPLC) or analytical grade. Water was from a Milli-Q water purification system (Millipore, Bedford, MA, USA). MeIQx, 2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline(4,7,8-TriMeIQx), IQ, MeIQ, PhIP and 2-amino-9H-pyrido[2,3-*b*]indole [amino-α-carboline (AαC)] were from Toronto Research Chemicals (Toronto, Canada). 3-Amino-1,4-dimethyl-5H-pyrido[4,3-*b*]-indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), Glu-P-1 and Glu-P-2 were from Wako (Neuss, Germany). 1-Methyl-9H-pyrido[3,4-*b*]indole (harman) and norharman were from Aldrich (Steinheim, Germany). 4,8-DiMeIQx and 7,8-DiMeIQx were synthesized in house [10]. 2-Amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx) was a gift from Professor J. Felton, Lawrence Livermore National Laboratory, University of California. Reference standards were used without further purification. Their purity was checked by HPLC and, where available, by comparison of extinction coefficients with values from the literature [11]. Stock mixtures of 5 ng/μl MeIQx, 4,8-DiMeIQx, IQ, MeIQ, PhIP, Glu-P-1, Glu-P-2, Trp-P-1, Trp-P-2, amino-α-carboline, norharman and harman in methanol (mixture A) and 5 ng/μl TriMeIQx in methanol (mixture B) served as internal standard solutions. Extrelut extraction cartridges (20 ml) and refill material were from Merck (Darmstadt, Germany). Bond-Elut PRS (500 mg) and C_{18} (100 mg and 500 mg) cartridges and stopcocks were from Analytichem International (ICT, Basle, Switzerland). Cartridge-coupling adapters were from Supelco (Gland, Switzerland). Connections between cartridges and the peristaltic pump were made with Luer-lock male connectors (Econo column system, Bio-Rad, Glattbrugg, Switzerland). Fine Luer-lock injection needles (25G × 5/8 in.) served to reduce flow-rates during extraction with dichloromethane (DCM). Purified extracts were stored in 1.1-ml Chromacol gold microvials (Infochroma, Zug, Switzerland).

Instrumentation

A Minipuls-2 peristaltic pump (Gilson, Viller-le-Bel, France) and a Supelco Visiprep SPE vacuum manifold were used for manipulations with solid-phase extraction cartridges. Fried fish samples were mixed with a Bamix ESGE M122 household blender (Menotec, Lausanne, Switzerland) and homogenized with an Ultra-Turrax Typ TP 18/10 high-speed blender (Jahnke & Kunkel, Staufen, Germany). HPLC was performed using a Merck L-6200 pump, a Gilson 231-401 automated sample processor and injector and Hewlett Packard Model 1040A diode array UV and Model 1046A programmable fluor-

escence detectors, both driven by an HP9000 Series 300 workstation (Chemstation). The column was a TSK gel ODS80 (Toyo Soda), 25 cm × 4.6 mm I.D. (5 μm particle size), protected by a Supelguard LC-8-DB (Supelco) precolumn which was attached via a "direct-connect" column coupler (Alltech, Lausanne, Switzerland). The mobile phase was as follows: solvent A, 0.01 *M* triethylamine adjusted with phosphoric acid to pH 3.2; solvent B, same as A but adjusted to pH 3.6; solvent C, acetonitrile. The linear gradient program was: 0–10 min, 5–15% C in A; 10–10.1 min, exchange of A with B; 10.1–20 min, 15–25% C in B; 20–30 min, 25–55% C in B; 30–55 min, column rinse and re-equilibration. Separations were carried out at ambient temperature. Peak identification was achieved by comparison of retention time and on-line recorded UV spectra with library entries.

Sample preparation

A commercial meat extract and fresh salmon steaks of 150 g per slice served as samples. Salmon steaks were pan-fried over a gas flame, cooked in a hot-air oven or barbecued while measuring the frying temperature with a Newport 267B-T2 thermocouple (Seyffer, Zürich, Switzerland). After frying, the bone was removed and the salmon was mixed with a household blender.

Solid-phase extraction

To prepare the meat extract, 3-g samples were solubilized in 12 ml of 1 *M* sodium hydroxide and loaded individually onto Extrelut columns. In the case of fried fish, 10 g per determination of fried fish mix were homogenized in 20 ml of 1 *M* sodium hydroxide using a high-speed blender. Aliquots were thoroughly mixed with Extrelut refill material with the aid of a spatula and used to fill empty Extrelut columns.

Bond-Elut PRS cartridges were fitted with coupling adapters, and with fine needles for flow reduction. The cartridges were quickly rinsed with 2 ml of DCM under positive pressure, and another 2 ml were pipetted into each cartridge. The Extrelut columns were then filled with DCM. The PRS cartridges were coupled as soon as the liquid levels reached the bottom of the Extrelut. The extraction was stopped at 30 ml of DCM by separating the Extrelut and PRS cartridges. Extrelut cartridges were discarded and the PRS cartridges placed on a Visiprep and dried for at least 4 min under maximum vacuum. The PRS cartridges were then connected to a peristaltic pump and successively rinsed at about 1–2 ml/min with 6 ml of 0.1 *M* hydrochloric acid, 15 ml of methanol–hydrochloric acid 0.1 *M* in various proportions as specified in the Results section and 2 ml of water. The 0.1 *M* hydrochloric acid wash was discarded and the methanol–hydrochloric acid and water eluates (apolar amines) were collected into empty Extrelut column reservoirs fitted with stopcocks. PRS cartridges were then coupled to Bond-Elut C_{18} cartridges (100 mg), previously conditioned with 1 ml of methanol and 10 ml of water. These tandems were rinsed with 20 ml of 0.5 *M* ammonium acetate adjusted to pH 8 with ammonia solution. The PRS cartridges were then discarded. The C_{18} cartridges were rinsed with 2 ml of water and dried by applying strong positive nitrogen pressure. The adsorbed polar amines were carefully eluted into microvials using 0.8 ml of methanol–concentrated ammonia solution (9:1, v/v). The eluates containing apolar amines were neutralized by adding 500 μl of ammonia solution and diluted with water to less than 20% methanol. These mixtures were passed at 4–5 ml/min through Bond-Elut C_{18} cartridges (500 mg) conditioned with 2 ml of methanol and 10 ml of water. The C_{18} cartridges were rinsed with 2 ml of water, dried by applying strong positive nitrogen pressure, and adsorbed *apolar amines* carefully eluted into microvials using 1.2 ml of methanol–ammonia solution (9:1, v/v). Apolar and polar extracts were concentrated under nitrogen and redissolved in 40 μl (unspiked samples) or 100 μl (spiked samples) of internal standard solution B. For HPLC, 20-μl aliquots were injected into a 50-μl loop.

Measure of extraction efficiencies, quantitative determinations of HAAs

The standard addition method was used to measure HAA extraction efficiencies (quadruplicate determinations with two unspiked samples and two samples spiked with 50 μl of HAA standard mixture A) as well as to quantitatively determine HAAs (triplicate extractions with two unspiked samples and one spiked with 50 μl of standard mixture A). The extraction efficiencies were thus calculated for each analyte as the slope of the linear regression line

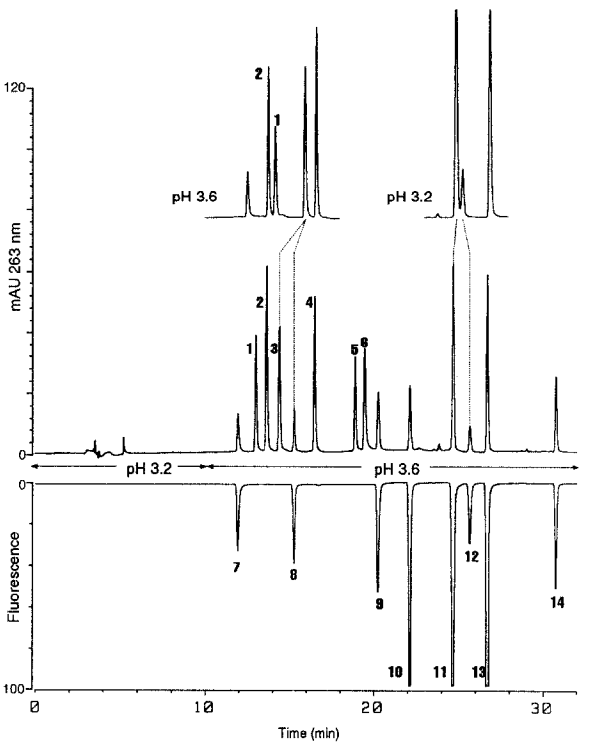

Fig. 2. HPLC separation of HAA standards. Complete chromatograms (UV and fluorescence, scaled to about equal peak heights) were obtained with a ternary gradient as specified in Experimental section. The two offset chromatogram extracts above show peak distribution with binary gradients at the indicated pH values. Peaks (excitation wavelength/emission wavelength): 1 = IQ; 2 = IQx; 3 = MeIQ; 4 = MeIQx; 5 = 7,8-DiMeIQx; 6 = 4,8-DiMeIQx; 7 = Glu-P-2 (360/450); 8 = Glu-P-1 (360/450); 9 = norharman (300/440); 10 = harman (300/440); 11 = Trp-P-2 (265/410); 12 = PhIP (315/390); 13 = Trp-P-1 (265/410); 14 = AαC (335/410).

"added analyte concentration (x) *versus* measured analyte concentration (y)".

Quantitative HAA measures were corrected for incomplete analyte recovery. Uncorrected results were calculated as the y-axis intercept of the regression line "added analyte concentration" *versus* "measured analyte concentration". The corrected result was obtained by dividing the intercept by the slope. The standard error of the corrected result therefore included contributions from both the intercept and the slope errors:

$$a = \bar{y} - b\bar{x}$$

$$\frac{a}{b} = \frac{\bar{y}}{b} - \bar{x}$$

$$\mathrm{Var}\left(\frac{a}{b}\right) = \mathrm{Var}\left(\frac{\bar{y}}{b}\right)$$

$$\mathrm{S.E.} = \sqrt{\mathrm{Var}\left(\frac{\bar{y}}{b}\right)} = \sqrt{\frac{1}{b^2}\,\mathrm{Var}\,(\bar{y}) + \frac{\bar{y}^2}{b^4}\,\mathrm{Var}\,(b)}$$

where x is the added concentration, y is the measured concentration, b is the slope, a is the intercept and S.E. is the standard error.

RESULTS

HPLC separation of HAAs

Baseline separation of all HAAs was a prerequisite for our study. Many reversed-phase silica columns were screened, but the TSK gel column showed the best peak symmetry and separation efficiency. Binary mobile phase gradients with acidic buffer between pH 3 and 4 and acetonitrile gave

TABLE I

EXTRACTION EFFICIENCY OF POLAR AMINES[a]

Substance	Abbreviation	Fish (%)	S.E. (%)	Meat extract (%)	S.E. (%)
2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline	MeIQx	77	11	69	8
2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline	4,8-DiMeIQx	85	9	84	6
2-Amino-3-methylimidazo[4,5-*f*]quinoline	IQ	73	10	68	4
2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline	MeIQ	60	5	72	6
2-Amino-6-methyldipyrido[1,2-a:3',2'-*d*]imidazole	Glu-P-1	63	8	83	5
2-Aminodipyrido[1,2-a:3',2'-*d*]imidazole	Glu-P-2	63	9	85	5
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine	PhIP	44	6	33	6

[a] Average from nine determinations with ratios of methanol to 0.1 *M* hydrochloric acid varied from 10 to 90% (see text).

good peak shapes, but above pH 3.2 Glu-P-1 and MeIQ co-eluted, and below pH 3.5 Trp-P-2 and PhIP were not baseline separated. A ternary gradient including pH switching from pH 3.2 to pH 3.6 during the run solved this problem (Fig. 2).

Solid-phase extraction method development

For the development of the extraction method a meat extract and fried salmon were used as samples. This allowed the simultaneous assessment of possible matrix effects on the extraction efficiency and selectivity. Purified extracts were analyzed by HPLC with UV and fluorescence detection. Briefly, alkaline sample preparations were loaded onto Extrelut columns. HAAs and other basic organosoluble materials were extracted with DCM and immediately retained on coupled PRS cation-exchange cartridges. The dried PRS cartridges containing adsorbed amines were re-equilibrated with 0.1 *M* hydrochloric acid. Retention of AIAs, especially of PhIP, was better when using 0.1 *M* than 0.01 *M* hydrochloric acid, as reported [9]. Subsequent rinsing of PRS with methanol–hydrochloric acid selectively desorbed carbolines (apolar extract). This acid extract was neutralized and diluted to 20% methanol for on-line concentration on C_{18} cartridges. The PRS cartridge was then coupled to a C_{18} cartridge and flushed with aqueous ammonium acetate buffer to release the polar amines as previously described [9].

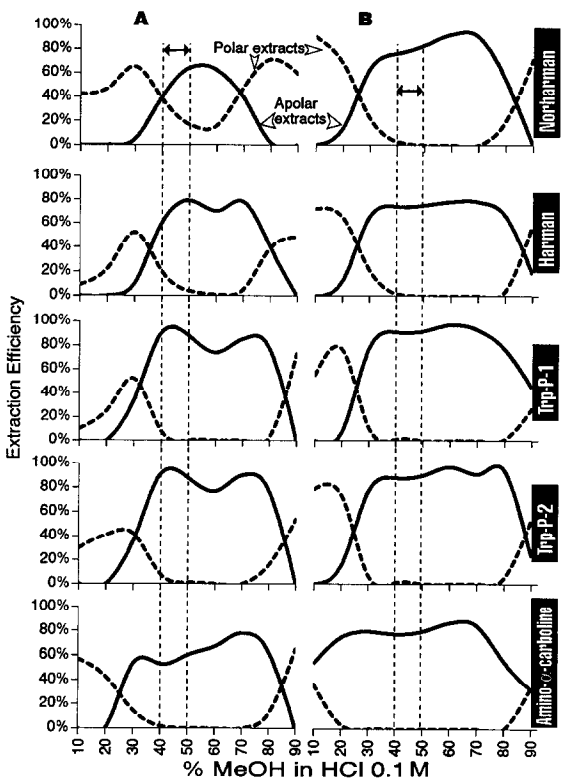

Fig. 3. Extraction efficiency of apolar amines. The apolar amines were partitioned in the apolar or the polar extract depending on the amount of methanol in the rinse phase. Extraction efficiencies from pan-fried fish (A) and meat extract (B) are shown. Smooth curves were plotted through each set of data points to illustrate solvent-dependent partition of carbolines in the polar (broken lines) or apolar extract (continuous lines) with maximal extraction efficiency in the apolar extract near 1:1 0.1 *M* methanol–hydrochloric acid (best range marked for each sample by a pair of vertical dashed lines).

The methanol–hydrochloric acid rinse step was studied in detail to optimize extraction efficiency and selectivity of apolar amines. Several determinations were carried out, varying the proportion of methanol to hydrochloric acid from 10 to 90% in steps of 10%. The volume was kept at a constant value in order to limit the number of determinations. Polar amines, including all AIAs, were so strongly bound to PRS that their extraction efficiencies remained remarkably constant at any proportions from 10 to 90% methanol in 0.1 *M* hydrochloric acid (see Table I). Matrix effects were minimal, as similar results from fish and meat extracts were observed.

The extraction efficiency of apolar amines showed a clear optimum with intermediate mixing ratios of methanol to hydrochloric acid (see Fig. 3). Theoretically, rinsing PRS with less than 20% or more than 80% methanol containing hydrochloric acid would allow the preparation of one unique, polar extract containing all amines. However, under such conditions the extraction efficiency was matrix-dependent (see Fig. 3) and co-eluting impurities interfered with low-level quantifications of AIAs, especially MeIQx. Therefore, the preparation of an apolar extract was advantageous. Elution with 40–70% methanol–hydrochloric acid gave an apolar extract with maximal extraction efficiency for most carbolines. However, below 50% methanol the apolar extracts were cleaner, therefore the best compromise between selectivity and recovery was 40–50% methanol–hydrochloric acid.

INTRODUCTION

Application

We examined HAA formation during cooking of fish. Salmon slices were fried and extracted using the optimized extraction conditions as explained above (for the results, see Table II). Less than 6 min of pan broiling or oven cooking at about 200°C or barbecuing at 270°C produced no or low amounts of MeIQx, PhIP, AαC, norharman and harman. MeIQx and PhIP even showed an apparent decrease with time during pan broiling. We believe that unequal heating of the gas flame-heated pan was responsible for this effect.

Barbecuing samples for more than 6 min resulted in a ten-fold increase in levels of PhIP, AαC, norharman and harman. Surprisingly, MeIQx formation did not follow this trend, and less than 1 ng/g MeIQx was found in such samples. Fig. 4a and b shows typical chromatograms.

DISCUSSION

HPLC with UV and fluorescence detection proved to be a convenient method of analyzing HAAs. All the amines of interest could be baseline-separated by a ternary mobile phase gradient including pH switching on a suitable C_{18} reversed-phase silica column. A detection limit of about 1 ng/g in purified extracts was obtained, and for fluorescent HAAs this limit was slightly lower. Identification of HAAs was feasible using their typical UV spectra even at low nanogram per gram levels.

The improved PRS tandem extraction method allowed the screening of nanogram per gram levels of ten genotoxic HAAs and two co-mutagenic β-carbolines. Compared with our previous method, we introduced as the major modification the separation of analytes into two groups, *i.e.* a polar group including AIAs and glutamic acid pyrolysates, and an apolar group including α-, β- and γ-carbolines.

Tandem extraction combines the selectivities of basic organic solvent extraction on Extrelut and cation-exchange chromatography on PRS. The focus in this work was to optimize PRS clean-up by selectively releasing analytes while letting co-extracted impurities bound to the sorbent. Extraction efficiencies were more than 60%, except for PhIP. However doubling the DCM extraction volume on Extrelut to 60 ml improved its recovery to more than

TABLE II

FORMATION OF HETEROCYCLIC AROMATIC AMINES IN FRIED FISH

Sample	MeIQx[a]	S.E.[b]	PhIP	S.E.	AαC	S.E.	Norharman	S.E.	Harman[c]	S.E.
Pan-broiled at 200°C										
2 × 3 min	1.4	0.01	1.7	0.7	n.d.[d]		8	0.1	2	0.1
2 × 6 min	5	0.01	23	7	4.6	0.1	26	1.4	20	1.4
2 × 9 min	4.7	0.3	14	2.8	8	0.3	24	4.4	34	3.5
2 × 12 min	3.7	0.2	17	0.6	9	0.04	28	1.8	16	0.1
Oven-cooked at 200°C										
20 min	<1		n.d.		n.d.		2	0.9	<1	
30 min	4.6	0.3	18	0.1	n.d.		11	0.7	3	0.2
40 min	3.1	0.02	5.9	1.4	n.d.		15	0.9	3	0.1
Barbecued at 270°C										
2 × 4 min	<1		2	0.3	2.8	0.2	8	0.1	3	0.1
2 × 6 min	<1		6.2	0.6	6.9	0.5	44	0.2	13	0.1
2 × 9 min	<1		69	23	73	2	160	46	108	3
2 × 12 min	<1		73	0.45	109	22	184	95	130	34

[a] Values (ng/g fried fish) are corrected for incomplete recovery as specified in Experimental section.
[b] Standard error of corrected result.
[c] Initial temperature displayed on thermocouple.
[d] n.d. = not detected.

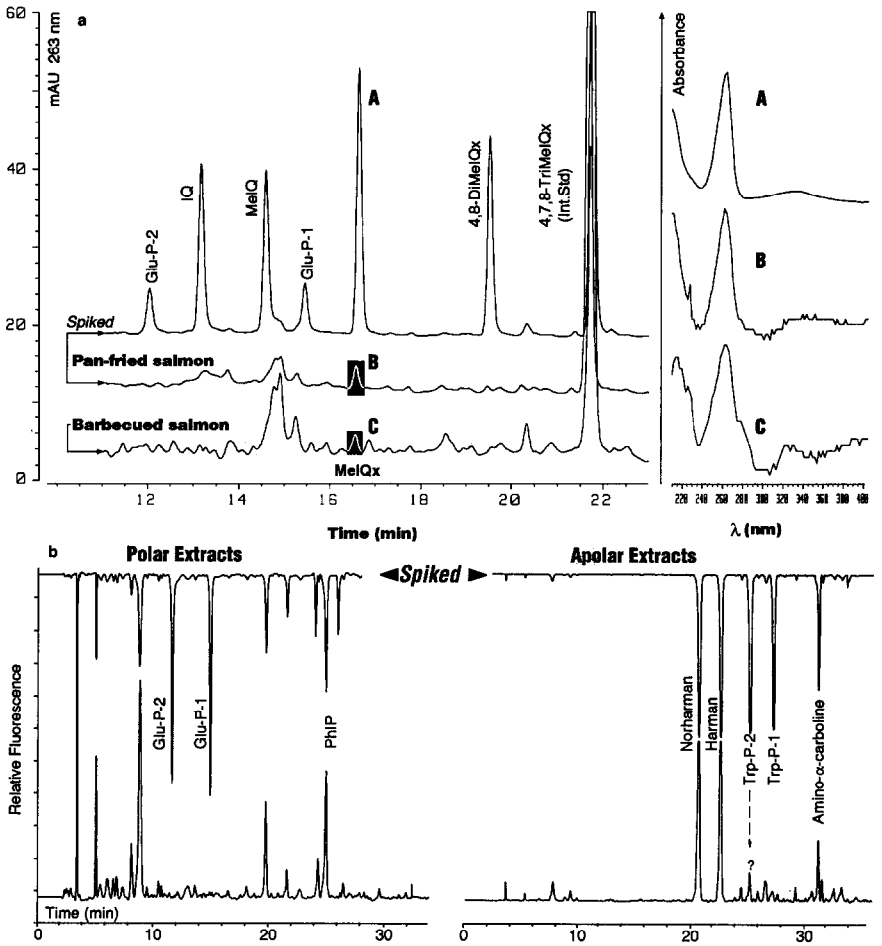

Fig. 4. (a) Polar extracts of fish pan-fried and barbecued for 9 min. Pan frying at 200°C produced more MeIQx (peak B) than barbecuing for the same time at 270°C (peak C). MeIQx peak C (< 1 ng/g) illustrates the detection limit of the method. On-line recorded UV spectra from MeIQx peaks are shown at the right. (b) Polar and apolar extracts of fish barbecued for 9 min per side at 270°C. These samples contained easily detectable PhIP in the polar extract (left), and AαC, norharman and harman in the apolar extract (right). A trace (< 0.1 ng/g) of Trp-P-2 might be present, but the peak (marked by "?") was too small to allow substance identification by a UV spectrum.

80% (data not shown). The absence of off-line transfer steps was beneficial for good analyte recovery and was also time-saving. Purifying a series of samples, typically four at a time, took only about 2 h of bench-work. The method may therefore be considered suitable for routine work.

In the kinetic study with fried fish we found MeIQx, PhIP, AαC and β-carbolines (norharman and harman) at levels which were in reasonable agreement with expectations from previous analysis and literature values (ref. 9 and references cited therein). The formation of PhIP, AαC and the β-carbolines was markedly influenced by cooking temperature and was clearly non-linear. However, more data are required to define accurately the relation between time and temperature and mutagen formation. Surprisingly, MeIQx formation did not follow the same trend but rather was triggered by the cooking method. These results require further investigation. Nevertheless, our data suggest that pan frying and oven cooking generate low levels of HAAs whereas prolonged barbecuing at high tem-

perature mainly facilitates formation of PhIP and carbolines.

In conclusion, the improved "PRS tandem extraction" is a simple, efficient and selective purification method for mutagenic/carcinogenic HAAs. The basic approach of extraction and purification on coupled cartridges should prove applicable to the determination of other related compounds.

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