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Analysis of heterocyclic aromatic amines in wine by high-performance liquid chromatography–electrospray tandem mass spectrometry

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Abstract

High-performance liquid chromatography–electrospray tandem mass spectrometry (HPLC–ESI-MS–MS) was used for the analysis of heterocyclic aromatic amines (HAAs) in 25 wines from various regions. In 24 wines under study HAAs were detected in the low ng/l range. 2-Amino-3,4-dimethyl-3H-imidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC) were found to be the most widely distributed HAAs in the wines under study. There was a difference in the presence of HAAs in white and red wines. In the latter, higher concentrations were found, with MeAαC in amounts ranging from <7.5 to 107 ng/l. Compared to the quantities of HAAs evaluated in meat and meat products, the amounts found in wines are 100–1000-fold lower. © 1997 Elsevier Science B.V.

Keywords: Wine; Amines, heterocyclic aromatic

1. Introduction

Since the first detection of the heterocyclic aromatic amines (HAAs) as a new category of mutagenic/carcinogenic substances by Sugimura and coworkers [1,2], several classes of HAAs have been identified in thermally treated foods [3,4] as well as in pyrolysis products of amino acids and proteins [5]. As HAA precursors amino acids, creatine, creatinine and, in some cases, sugars have been postulated [3,6]. Later, the more widespread occurrence of HAAs in foods has been demonstrated. For instance, HAAs have been detected in fermented foods such as soy sauce [7] and vinegar [8]. Most surprisingly, PhIP (2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]-pyridine) has been identified recently in a number of beer and wine samples [9]. The latter data encouraged us to perform a more extended study of wines using our newly developed sensitive and selective analysis of HAAs by high-performance liquid chromatography–electrospray tandem mass spectrometry (HPLC–ESI-MS–MS) [10].

In the past, LC–MS analysis based on thermospray ionization required selected ion monitoring (SIM) in order to obtain reasonable sensitivities [11,12]. Recently, the development of a soft ionization method like electrospray mass spectrometry combined with selected reaction monitoring (SRM) offered a highly sensitive tool for the analysis of HAAs as trace compounds. For instance, ESI-MS–

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MS has successfully been applied to the detection of PhIP and MeIQx in human urine [13] and for the characterization of several HAA metabolites [14,15]. In this paper, the results of the analysis of fourteen HAAs in wines are presented.

2. Experimental

2.1. Wine samples

Different brands of wines were purchased at a local supermarket: twelve white wines, from Spain (S 1, S 2, S 3), France (F 1, F 2, F 3), Italy (I 1, I 2, I 3) and Germany (G 1, G 2, G 3); and twelve red wines, from Spain (S 4, S 5, S 6), France (F 4, F 5, F 6), Italy (I 4, I 5, I 6) and Germany (G 4, G 5, G 6).

2.2. Chemicals

IQ (1), MeIQ (2), IQx (3), MeIQx (4), 4,8-DiMeIQx (5), 7,8-DiMeIQx (6), 4,7,8-TriMeIQx (7), PhIP (8), Glu-P-1 (9) (as hydrochloride), Glu-P-2 (10) (as hydrochloride), Trp-P-1 (11) (as acetate), Trp-P-2 (12) (as acetate), AαC (13), MeAαC (14), [$^2H_3$]PhIP (d_3-PhIP) (15) and [$^2H_3$]MeIQx (d_3-MeIQx) (16) were purchased from Toronto Research Chemicals (Ontario, Canada). Ammonium acetate (10 *M*) was from Aldrich (Deisenhofen, Germany). Water, methanol, acetonitrile, dichloromethane, all of HPLC-grade, and trifluoroacetic acid (spectroscopic-grade) were from Merck (Darmstadt, Germany). [$^2H_5$]Trp (d_5-Trp) was from Promochem (Wesel, Germany). All other chemicals were of analytical-reagent grade quality. Cation-exchange material, Dowex 50W-X8, was from Serva (Heidelberg, Germany). Other organic solvents were distilled before use. Isolute PRS (propylsulphonic acid silica; 200 mg, 3 ml) cartridges were from ICT International (Basel, Switzerland). The cartridges were each conditioned with 2 ml of dichloromethane before use.

2.3. Synthesis of [$^2H_4$]norharman

[$^2H_4$]Norharman (17) was synthesized via [$^2H_4$]1,2,3,4-tetrahydro-3-carboline-carboxylic acid [16] using L-tryptophan ([$^2H_5$]indole) as described by Harvey et al. [17].

2.3.1. [$^2H_4$]1,2,3,4-Tetrahydro-3-carboline-carboxylic acid

To a solution of 0.5 g (2.5 m*M*) L-tryptophan ([$^2H_5$]indole) in 8 ml water 0.5 *M* sulfuric acid (2.5 ml) and formaldehyde solution (37%) (2.5 ml; 0.04 m*M*) was added. The mixture was stirred for 1.5 h and, after adding a slight excess of ammonia, was allowed to stand overnight. The mixture was then refluxed by boiling with water–ethanol (1:1, v/v) and sufficient ammonia. The alcohol and ammonia were boiled off and the crystalline product precipitated. After recrystallization 321.5 mg (1.6 m*M*) [$^2H_4$]1,2,3,4-tetrahydro-3-carboline-carboxylic acid was obtained.

2.3.2. [$^2H_4$]Norharman

A solution of 321.5 mg (1.6 m*M*) [$^2H_4$]1,2,3,4-tetrahydro-3-carboline-carboxylic acid in 250 ml boiling water was treated with 10% potassium dichromate solution (30 ml) and glacial acetic acid (6 ml). Boiling was continued for 1.5 min, the solution cooled and the excess of oxidising agent removed by sulphur dioxide. The mixture was rendered alkaline with sodium carbonate solution and, after continuous liquid–liquid extraction (45°C; 15 h) with diethyl ether, the product was purified by cation-exchange chromatography [rinsed with 5% ammonia, eluted with methanol–ammonia (9:1, v/v)]. After evaporation to dryness and lyophilisation 38 mg [$^2H_4$]norharman as white needles was obtained. Identification was performed using HPLC-MS with TSQ 7000 (Finnigan MAT, Bremen, Germany): $[M+H^+]=173.3$; product ion spectrum (CID: −45 eV; Ar=253 mPa): 171.2; 143.4; 119.3; 117.9; 92.1; 68.0. The most abundant product ion m/z 117.9 is resulting by the cleavage of the pyridyl moiety ($[M+H]^+-C_3H_5N$). NMR-data were recorded on a Bruker WM 400 spectrometer (Bruker, Karlsruhe, Germany). ^{1}H NMR data were in agreement with literature data [18].

2.4. Sample preparation

The concentrate obtained from 200 ml of wine ([$^2H_3$]PhIP and [$^2H_4$]norharman were added as

standards and the wine adjusted to pH 10 using 10 *M* NaOH) by continuous liquid–liquid extraction (45°C; 15 h) with dichloromethane and evaporation under vacuum to 20 ml was subjected to SPE at 1995 Pa (Visiprep SPE vacuum manifold system; Supelco, Bellefonte, PA, USA) using a PRS cartridge. Elution was performed with 1 ml MeOH–NH_3 (9:1, v/v) [19]. The PRS eluate was evaporated under a gentle stream of nitrogen and redissolved in 50 μl of water containing 25% methanol–acetonitrile (1:2, v/v) and [$^2H_3$]MeIQ as external standard (see Fig. 1).

2.5. Analytical procedure

Analysis was performed on a triple-stage quadrupole TSQ 7000 LC–MS–MS system with electrospray (ESI) interface (Finnigan MAT). Data acquisition and mass spectrometric evaluation were carried out on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT). For HPLC an Applied Biosystems dual syringe pump Model 140 B (Bai, Bensheim, Germany) was used. For injection a Spark Holland Triathlon autosampler (SunChrom, Friedrichsdorf, Germany) was used, the injection volume was 5 μl using the μl pick-up mode. Separations were carried out on a LiChrospher 60-RP select B column (100×2.0 mm I.D.; 5 μm; Knauer, Berlin, Germany) using a linear gradient. Solvent A was 0.05% trifluoroacetic acid in water; solvent B was methanol–acetonitrile (1:2, v/v). The separation of the fourteen HAAs in one unique chromatographic run was possible [20], but to reach higher sensitivity in MS detection, three SRM experiments with two different HPLC gradients were performed as follows. For compounds 1–10 (''polar'' HAAs), 15–16 (deuterated standards), the gradient program was as follows: 0–1 min, 20% B; 1–6 min, 20–80% B; 6–8 min, 80% B. The gradient program for compounds 11–14 (''apolar'' HAAs), 16–17 (deuterated standards), was 0–1 min, 30% B; and 1–9 min, 30–70% B. The flow-rate was 200 μl/min and the injection volume was 5 μl. The mass spectrometer was operated in the SRM mode as reported previously [10]. The SRM mode of the TSQ 7000 and the appropriate transitions were monitored during the region of elution of each analyte for the analysis of ''apolar'' and ''polar'' HAAs, respectively.

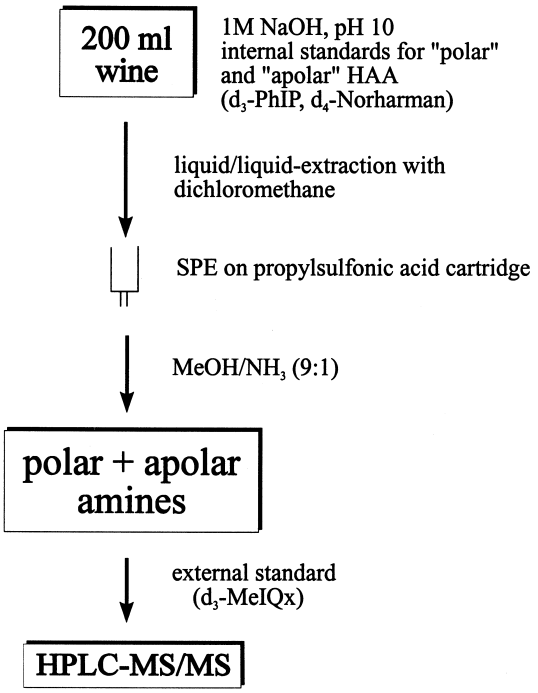

Fig. 1. Sample preparation procedure for the extraction of HAAs from wines.

The experiment list for the time depending SRM (experiment I A) was as follows (offset voltages in brackets): 0 min to 3.30 min, *m/z* 213.1/198.1 (−30 eV) for (2), *m/z* 185.1/78.2 (−45 eV) for (10); 3.30 min to 5.20 min, *m/z* 214.1/199.1 (−30 eV) for (4), *m/z* 217.1/199.1 (−30 eV) for (16), *m/z* 228.1/213.1 (−30 eV) for (5 and 6); 5.20 min to 8.00 min, *m/z* 225.1/210.1 (−30 eV) for (8), *m/z* 228.1/210.1 (−30 eV) for (15).

The experiment list for the time depending SRM (experiment I B) was as follows (offset voltages in brackets): 0 min to 2.40 min, *m/z* 200.1/185.1 (−30 eV) for (3), *m/z* 199.1/184.2 (−30 eV) for (1); 2.40 min to 3.40 min, *m/z* 200.1/185.1 (−30 eV) for (3), *m/z* 199.1/92.2 (−40 eV) for (9); 3.40 min to 4.50 min, *m/z* 199.1/92.2 (−40 eV) for (9), *m/z* 217.1/199.1 (−30 eV) for (16); 4.50 to 8.00 min, *m/z* 228.1/210.1 (− 30 eV) for (15), *m/z* 242.3/227.1 (−30 eV) for (7).

For experiment IA and IB positive ions were detected at a total scan duration of 0.5 s and dwell time of 0.002 s. The temperature of the heated capillary serving simultaneously as repeller electrode

(20 V) was 200°C; the electrospray capillary voltage was set to 4.3 kV; the multiplier was set to 2 kV. Nitrogen served both as sheath (50 p.s.i.; 1 p.s.i.= 6894.76 Pa) and auxiliary gas and argon served as collision gas at a pressure of 253 mPa.

The ion pairs for the SRM experiment (experiment II) for ''apolar'' HAAs were (offset voltages in brackets): m/z 198.1/181.1 (−25 eV) for (12 and 14), m/z 212.1/195.1 (−25 eV) for (11), m/z 217.1/199.1 (−30 eV) for (16), m/z 173.3/118.1 (−45 eV) for (17), m/z 184.1/167.1 (−25 eV) for (13). The mass spectrometer was operated in the SRM mode at a total scan duration of 1.0 s. The dwell time was 0.002 s. The temperature of the heated capillary (20 V) was 200°C; the electrospray capillary voltage was set to 4.8 kV. Nitrogen served both as sheath (70 psi) and auxiliary gas and argon served as collision gas at a pressure of 253 mPa.

Quantitative evaluations were carried out in duplicate using model solutions of HAAs (ranging from 0.1 ng/l to 200 ng/l) added to the fraction obtained from a HAA-free wine after sample preparation.

3. Results and discussion

Using HPLC–ESI-MS–MS analysis for the determination of HAAs in wine a method had to be chosen which allowed checking the efficiency of both the extraction method and efficiency of the ionization procedure. The ionization of analytes in the electrospray interface is influenced by different factors, in particular, the sample matrix, thus requiring the use of deuterated standards. These exhibit the same properties during sample preparation and MS analysis as the non-deuterated analytes (Fig. 1). For all HAAs to be determined deuterated standards were not available. Thus, for the groups of ''polar'' and ''apolar'' HAAs two deuterated standards, namely [$^2H_3$]PhIP and [$^2H_4$]norharman for ''polar'' and ''apolar'' HAAs, respectively, were added before the extraction. [$^2H_3$]MeIQx was used as an external standard to check the ionization process of the instrument.

Calibration curves were based on linear regression analyses with r^2=0.9841 to r^2=0.9066 evaluated by quantitative HPLC–ESI-MS–MS determinations (in duplicate) using a HAA-free wine sample with HAA model solutions. The limit of quantification ranged from 0.5 ng/l for MeIQx to 7.5 ng/l for MeAαC. Limits of detection were approximately 2–2.5-fold lower than these values. The recovery rates determined for the HAAs under study are summarized in Table 1. For MeAαC the recovery rate was determined at 51%. Comparable moderate recoveries for α-carbolines have already been reported by Gross et al. [21].

Using HPLC–ESI-MS–MS analysis quantitative determinations of fourteen HAAs in a number of commercial white and red wines from various countries were performed. The use of SRM mode guaranteed high selectivity and extreme sensitivity. As representative examples, in Figs. 2 and 3 the HPLC–ESI-MS–MS chromatograms of ''apolar'' and ''polar'' HAAs from a red wine extract are outlined. As shown from the graphs, apolar HAAs were present with MeAαC (107 ng/l). IQ was found as main polar HAA contaminant in this sample, occurring at 10 ng/l, together with Glu-P-1 as minor constituent.

In total, from the 25 wines under study 24 showed the presence of HAAs. The HAAs identified in white and red wines are summarized in Table 2. As shown from these data, a high variation in the distribution of HAAs was observed, except for MeIQx and MeAαC, which were found to be the most widely distributed HAAs in the wines under study. There was a difference in the occurrence of HAAs in white and red wines. Whereas in white wines HAAs were detected in the lower ng/l range, i.e., except for one sample (G1; PhIP: 83 ng/l), below the limit of determination, higher concentrations of HAAs were

Table 1
Recovery rates of HAAs found in wines

Compound	Recovery (%)
IQ	109±11
MeIQ	85±11
MeIQx	90±10
Glu-P-1	98±33
Glu-P-2	88±5
PhIP	78±1
4,7,8-TriMeIQx	80±8
MeAαC	51±9

Determination using standard concentrations of 25 ng/l (average values from double determinations given).

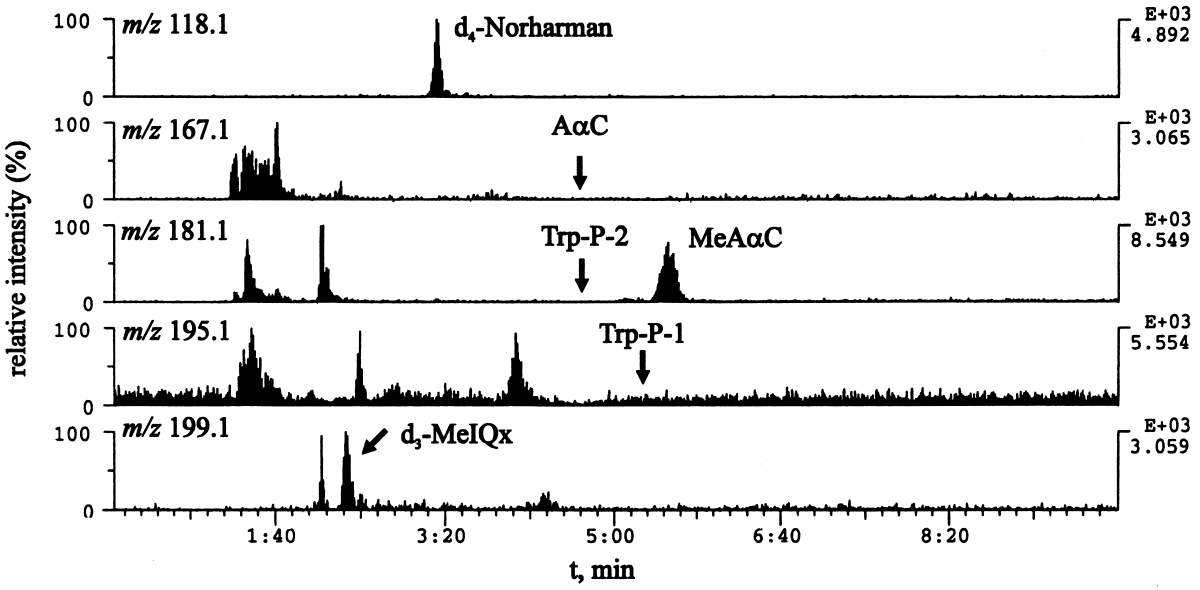

Fig. 2. HPLC–ESI-MS–MS chromatogram of apolar HAAs (experiment II, cf. Section 2.5) in a French red wine sample (F4) containing MeAαC as the main HAA contaminant (standards [$^2H_4$]norharman and [$^2H_3$]MeIQx).

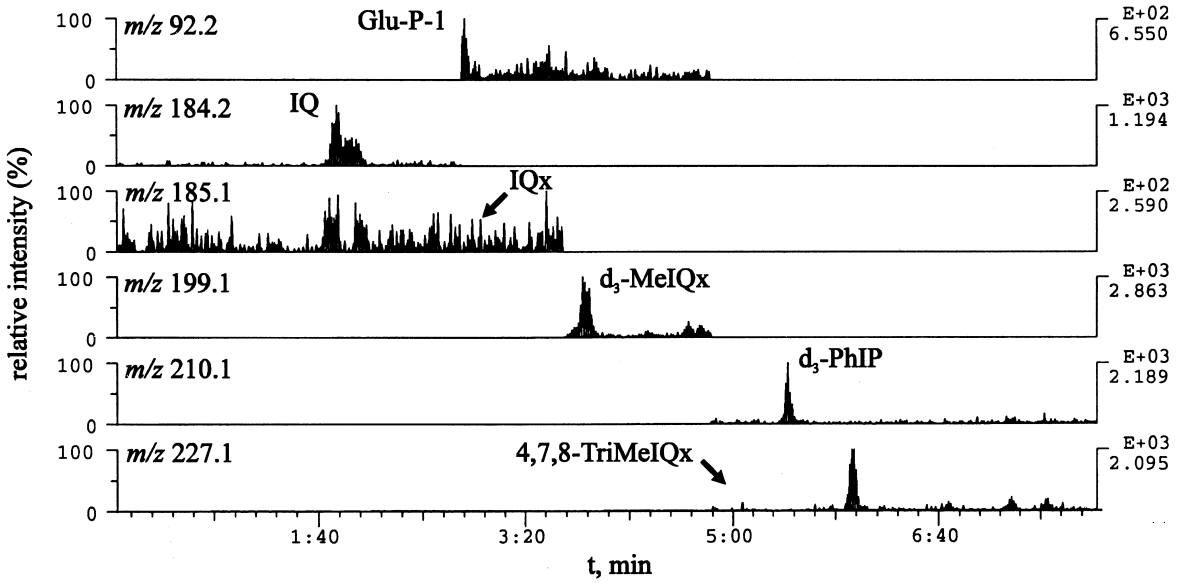

Fig. 3. HPLC–ESI-MS–MS chromatogram of the polar HAAs (experiment IB, cf. Section 2.5) in a French red wine sample (F4) containing IQ (10 ng/l) and Glu-P-1 (<4 ng/l) (standards [$^2H_3$]PhIP and [$^2H_3$]MeIQx). IQ was clearly identified at 1.40 min by symmetrical peak enhancement after standard addition and by its relative retention time compared to [$^2H_3$]MeIQx and [$^2H_3$]PhIP. 4,7,8-TriMeIQx (at 5.10 min) could not been identified amongst the HAAs in this sample. However, a peak of unknown identity was observed exclusively in sample F4 at 6.00 min.

Table 2
HAAs in wines found by HPLC–ESI-MS–MS analysis

Wine no.	IQ (1)	MeIQ (2)	MeIQx (4)	4,7,8-Tri-MeIQx (7)	PhIP (8)	Glu-P-1 (9)	Glu-P-2 (10)	MeAαC (14)
S 1	−	−	+	−	−	−	−	−
S 2	−	−	+	−	−	−	−	−
S 3	−	−	+	−	−	−	−	−
F 1	−	−	+	−	−	−	−	+
F 2	−	−	+	−	+	−	−	−
F 3	−	−	+	−	−	−	−	−
I 1	+	−	−	−	−	−	−	−
I 2	+	−	−	−	−	+	−	−
I 3	+	−	−	−	−	−	−	−
G 1	+	−	−	−	83 ng/l	−	−	−
G 2	−	+	−	−	−	−	−	+
G 3	−	+	−	−	−	−	−	−
S 4	−	−	+	−	−	−	−	−
S 5	−	−	+	−	−	−	−	22 ng/l
S 6	+	−	+	−	−	−	−	−
F 4	10 ng/l	−	−	−	−	+	8 ng/l	107 ng/l
F 5	+	−	−	−	−	+	−	+
F 6	+	−	+	−	−	−	−	+
I 4	−	−	−	−	−	+	−	−
I 5	−	−	−	−	−	+	−	+
I 6	+	−	+	−	−	+	−	53 ng/l
G 4	−	−	+	−	−	−	−	+
G 5	−	−	−	−	−	−	−	+
G 6	−	−	−	+	−	−	−	15 ng/l

S, F, I, G 1–3: White wines from Spain, France, Italy and Germany, respectively. S, F, I, G 4–6: Red wines from Spain, France, Italy and Germany, respectively.
−=Not detectable; +=below limit of quantification; single determinations using internal standards [$^2H_3$]PhIP/[$^2H_4$]norharman to establish efficiency of sample preparation.

found in red wines. In particular, MeAαC was determined in amounts ranging from <7.5 to 107 ng/l.

With these data, it was demonstrated that not only PhIP occurred in wines [9], but also other HAAs appeared to be common wine contaminants at trace levels. At present, the source of the HAA contamination is still unclear. Our preliminary studies showed that both molasses and yeasts may contain HAAs; thus, a ''carry over'' from these raw material into wines could be envisaged. In addition, as reported by Manabe et al. [22], PhIP is present in airborne particles and diesel exhaust. This means that at least PhIP is a ubiquitous environmental component. However, the lack of information about winemaking of the commercial wines under study does not allow to establish such a correlation to date. Further studies are required to clarify the origin of HAAs in wines.

Recently, the risk potential for HAA-containing meat and meat products has been set to 10^{-6} [23]. Compared to the amounts of the HAAs evaluated in these products, the impact of HAAs from wines should be negligible.

4. List of abbreviations

IQ= 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoline (CAS No. 76180-96-6) (1)
MeIQ= 2-amino-3,4-dimethyl-3H-imid-

azo[4,5-*f*]quinoline (77094-11-2) (2)

IQx= 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoxaline (108354-47-8) (3)

MeIQx= 2-amino-3,8-dimethyl-3H-imidazo[4,5-*f*]quinoxaline (77500-04-0) (4)

4,8-DiMeIQx= 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-*f*]quinoxaline (95896-78-9) (5)

7,8-DiMeIQx= 2-amino-3,7,8-trimethyl-3H-imidazo[4,5-*f*]quinoxaline (92180-79-5) (6)

4,7,8-TriMeIQx= 2-amino-3,4,7,8-tetramethyl-3H-imidazo[4,5-*f*]quinoxaline (132898-07-8) (7)

PhIP= 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (105650-23-5) (8)

Glu-P-1= 2-amino-6-methyldipyrido [1,2-α:3′,2′-*d*]imidazole (67730-11-4) (9)

Glu-P-2= 2-aminodipyrido[1,2-α:3′,2′-*d*] imidazole (67730-10-3) (10)

Trp-P-1= 3-amino-1,4-dimethyl-5H-pyrido [4,3-*b*]indole (75104-43-7) (11)

Trp-P-2= 3-amino-1-methyl-5H-pyrido-[4,3-*b*]indole (72254-58-1) (12)

AαC= 2-amino-9H-pyrido[2,3-*b*]indole (26148-68-5) (13)

MeAαC= 2-amino-3-methyl-9H-pyrido-[2,3-*b*]indole (68006-83-7) (14)

[$^2H_3$]PhIP= 2-amino-1-(trideuteromethyl)-6-phenyl-imidazo[4,5-*b*]pyridine (15)

[$^2H_3$]MeIQx= 2-amino-8-methyl-3-(trideuteromethyl)-3H-imidazo[4,5-*f*]-quinoxaline (16)

[$^2H_4$]norharman= 9H-pyrido[3,4-*b*]tetradeuteroindole (17)

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