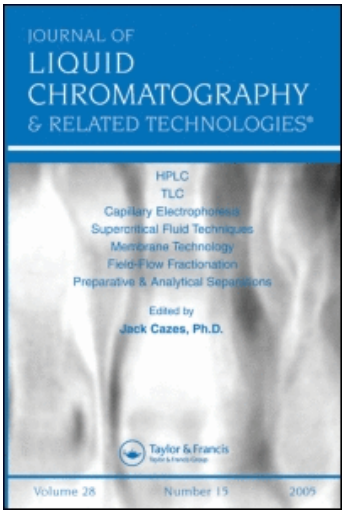

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Analysis of Carcinogenic Heterocyclic Amines by High Performance Liquid Chromatography

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ANALYSIS OF CARCINOGENIC HETEROCYCLIC AMINES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Separation and determination of mutagenic and carcinogenic heterocyclic amines by a reversed phase isocratic HPLC method is here in described. Five of them, namely 2-amino-3-methylimidazo[4,5-f] quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f] quinoxaline (4,8-DiMeIQx), 2-amino-3,4,7,8-tetramethylimidazo[4,5-f] quinoxaline (4,7,8-TriMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) were encountered in this study. Separation was accomplished on a reverse phase HPLC column of Supelcosil LC-8 equipped with a Supelguard LC-8 precolumn, at a flow rate of 2.0 mL/min. IQ, MeIQx and 4,8-DiMeIQx were resolved using 15% (v/v) acetonitrile in 50 mM triethylamine-phosphate buffer, pH 3.2, whereas 4,7,8-TriMeIQx and PhIP using 30% (v/v) acetonitrile in the same buffer. Detection of the eluted heterocyclic amines was performed at 263 nm for IQ, MeIQx and 4,8-DiMeIQx and at 254 nm for 4,7,8-TriMeIQx and PhIP.

Reproducibility tests measuring peak areas gave a relative standard deviation of 1.8-4.4%. The calibration graphs for all five amines injected into the column were linear up to approximately 2.0 μmol and the detection limits (signal-to-noise ratio 2:1) ranged from 10 to 30 pmol. The high sensitivity of the proposed method permits the accurate and reproducible determination in mixtures containing only a few ng/mL of such heterocyclic amines

INTRODUCTION

Heterocyclic amines belong to a group of compounds which contain three aromatic rings with one or more nitrogen atoms in their ring system. Usually they are found in the urine of healthy individuals eating a normal diet containing heat-processed food, such as broiled meat or grilled chicken.[1,2] Heterocyclic amines can be synthesized in heat-processed food from amino acids, protein pyrolysates and carbohydrates. During the last decade, it has been recognized that some members of aromatic amines, including the family of heterocyclic amines, may induce cancer in humans.[3]

There are five types of such amines isolated from cooked food: aminoimidazoquinoline, aminoimidazoquinoxaline, aminoimidazopyridine, aminodiazopyridoindole and aminopyridoimidazole. Compound 2-amino-3-methylimidazo[4,5-f] quinoline (IQ), which belongs to the first type, 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f] quinoxaline (4,8-DiMeIQx) and 2-amino-3,4,7,8-tetramethyl-imidazo[4,5-f] quinoxaline (4,7,8-TriMeIQx), belonging to aminoimidazoquinoxaline and 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) that belongs to aminoimidazopyridine group, constitute heterocyclic amines that are usually found in various cooked foods.[4,5] These amines are able also to induce mutagenesis and carcinogenesis when fed to mice and rodents.[6] The chemical structures of these mutagenic amines are shown in Figure 1.

The necessity for the development of analytical methods with high sensitivity and selectivity, so that mutagenic and carcinogenic heterocyclic amines reliably and accurately can be identified and determined, is obvious.

Several analytical methods, such as enzyme-linked immunosorbent assay (ELISA),[7] GC-MS[8,9] and HPLC-MS,[10,11] have been recently developed for this purpose. However, they either require very expensive equipment or, are of low selectivity, for the determination of individual heterocyclic amines. of selective determination for most of these compounds.

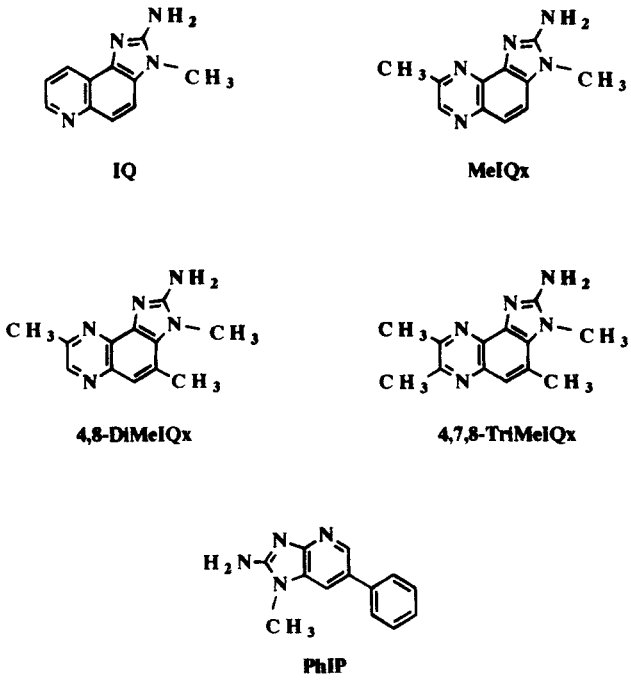

Figure 1. Chemical structures of 2-amino-3-methylimidazo[4,5-f] quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f] quinoxaline (4,8-DiMeIQx), 2-amino-3,4,7,8-tetramethylimidazo[4,5-f] quinoxaline (4,7,8-TriMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) encountered in this report.

Direct HPLC methods using UV[12] or electrochemical detection[13] offered the advantage. In the case of HPLC-UV technique, the gradient elution causes an increased baseline elevation during chromatography leading, to our experience, in difficult interpretation of the results when this method is applied on a routine basis.

In this paper, we report on an isocratic highly sensitive HPLC method by which the five most important heterocyclic amines (IQ, MeIQx, 4,8-DiMeIQx, 4,7,8-TriMeIQx and PhIP) are completely resolved from each other and, at the same time, are easily determined in the daily laboratory practice.

EXPERIMENTAL

Apparatus and Chemicals

For the determination of heterocyclic amines, a LDC system with a LDC III pump, a UV-vis detector LDC 1204A set at 263 or 254 nm with 8-μL flow cell and with a 50-μL loop injector was used. The analytical column is a Supelcosil LC-8, 5-μm, 250 x 4.6 mm I.D., stainless steel (Supelco) equipped with a Supelguard column, 20 x 4.6 mm I.D. (Supelco).

Studied compounds, as well as, unknown mixtures of known amounts of heterocyclic amines were prepared and generously offered to be tested by Dr. M. Rabache (C.N.A.M.-Conservatoire National des Arts et Metiers, Biochimie Industrielle et Agro-alimentaire, Paris, France). HPLC-grade acetonitrile, obtained from Merck (Darmstadt, Germany) and glass-distilled water constitute the eluant components. All other solvents and chemicals used were of analytical reagent grade.

Chromatographic Conditions

The mobile phase used for the separation of the heterocyclic amines IQ, MeIQx and 4,8-DiMeIQx, designated as eluant 1, was 15% (v/v) acetonitrile in triethylamine-phosphate buffer, pH 3.2 and for the determination of 4,7,8-TriMeIQx and PhIP, designated as eluant 2, was 30% (v/v) acetonitrile in the same buffer. Triethylamine-phosphate buffer was prepared by diluting 1.4-mL triethylamine (Fluka, No. 90340) to 1000-mL of HPLC-grade water, followed by exact adjustment to pH 3.2 with 10% (v/v) phosphoric acid (Merck, No. 573). The flow rate for both determinations was 2.0 mL/min and the pressure approximately 1300 psi. The detection of IQ, MeIQx and 4,8-DiMeIQx was performed at 263 nm, whereas of 4,7,8-TriMeIQx and PhIP at 254 nm. The separation was performed at room temperature. Eluants used were degassed by vacuum filtration through a 0.2-μm membrane filter followed by agitation in an ultrasonic bath.

System Suitability

The column was equilibrated with each eluant separately at a flow rate of 2.0 mL/min. Once a stable baseline was obtained, the standard solutions were injected into the column and the peaks appeared over the increased retention time.

Table 1

High Performance Liquid Chromatographic Characteristics of Heterocyclic Amines

Compound	Retention Time/min (t_R)	Resolution (R_s)
IQ	5.2 ± 0.15^a	1.8
MeIQx	7.8 ± 0.20^a	2.3
4,8-DiMeIQx	13.4 ± 0.35^a	--
4,7,8-TriMeIQx	4.8 ± 0.20^b	--
PhIP	10.1 ± 0.40^b	2.1

[a, b] Values obtained using eluant 1 and eluant 2, respectively.

The resolution factors, R_s, are calculated between the chromatographic peak of 4,8-DiMeIQx and each individual peak of IQ and MeIQx and between the peak of 4,7,8-TriMeIQx and PhIP from the equation: $R_s = (t_2-t_1)/(W_1+W_2)$, where t_2 and t_1 are the retention times of the two peaks, while W_1, W_2 are the peak widths at the half height of the respective peaks. The resolution factors, R_s, are more than 1.8 in all cases, indicating complete separation between 4,8-DiMeIQx and IQ, MeIQx, as well as between 4,7,8-TriMeIQx and PhIP as illustrated in Table 1.

Selectivity

Chromatographic analysis of the various heterocyclic amines which were provided in methanolic solution and kept at 2-4°C until use, showed the presence of low amounts of impurities, which do not have any endogenous interference at the retention times of the separated amines. This fact indicates that the proposed method can be used in the determination of the five heterocyclic amines, avoiding the use of an internal standard.

Detection Limit

The detection limits for each heterocyclic amine is estimated as the quantity of these substances producing a signal of the peak height twice the

baseline noise. The minimum detectable amount in pmol injected into the column was estimated to be 10 pmol for 4,8-DiMeIQx and approximately 30 pmol for IQ, MeIQx, 4,7,8-TriMeIQx and PhIP.

Standard Calibration Graphs

Heterocyclic amines were dissolved in methanol and accurately weighed by the provider. Stock solutions of approximately 10 μg per g of solution were used in the present work. Standard solutions were prepared by serial dilutions of the stock solutions. Aliquots of 5 to 50 μL that were accurately weighed by us (N=12), were taken for HPLC analysis. Calibration curves were constructed by plotting the peak areas of heterocyclic amines against their concentrations expressed as ng/mL. Calibration graphs were evaluated for their linearity according to the standard method of van Trijp and Roos.

RESULTS AND DISCUSSION

Retention times of the heterocyclic amines tested, are reproducible under the chromatographic conditions used with a relative standard deviation of less than 0.8%. The mobile phases used enable good column performances for a long period of time. On the other hand, all five heterocyclic amines used are stable for at least several months when kept in methanolic solutions at 2-4°C, provided exclusion of light.

Repeated chromatographies of the heterocyclic amines under the separation conditions described, showed the presence of low amounts of impurities in all preparations. Since the detected impurities did not interfere with the determination of the amines tested, no further purification of the preparations was needed.

Increasing the concentration of the organic modifier, acetonitrile, from 15% to 30% (v/v) results in higher and more reproducible peaks for the more retarded 4,7,8-TriMeIQx and PhIP, improving the sensitivity and accuracy of the described method for these two amines. As shown in Figure 2, the best resolution among the heterocyclic amines IQ, MeIQx and 4,8-DiMeIQx is obtained using 15% (v/v) acetonitrile (Fig. 2a). In higher acetonitrile concentration (30%), 4,7,8-TriMeIQx is completely resolved from PhIP, whereas MeIQx is coeluted with IQ (Fig. 2b).

Evaluation of the method's quality parameters (including linearity, detection limit and precision) was carried out using methanolic solutions for all of the described derivatives, under optimum separation conditions and

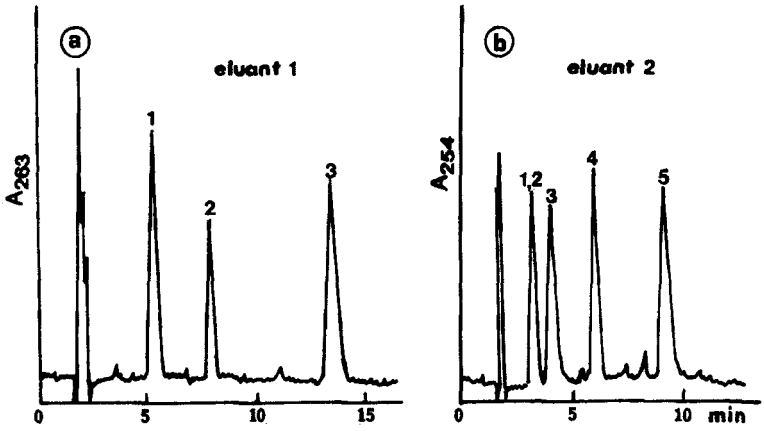

Figure 2. Typical HPLC chromatograms of IQ, MeIQx and 4.8-DiMeIQx (a) and 4,7,8-TriMeIQx and PhIP (b) under elution conditions described in the experimental section. IQ, MeIQx and 4.8-DiMeIQx were detected at 263 nm and 4,7,8-TriMeIQx and PhIP at 254 nm. Eluant 1= 15% (v/v) acetonitrile in triethylamine-phosphate buffer, pH 3.2, and eluant 2= 30% (v/v) acetonitrile in the same buffer. Flow rate was 2.0 mL/min.

concentrations ranging from 0.005 to 10 μg/mL. Calibration graphs obtained by plotting peak area versus concentration (ng/mL), exhibit excellent linearity to all heterocyclic amines tested (Fig. 3). The correlation coefficients of linearity graphs range from 0.9994-0.9999 (N=7-13) for MeIQx, 4,8-DiMeIQx, 4,7,8-TriMeIQx and PhIP, and 0.9957 (N=16) for IQ. Twelve 50 ng of consecutive analyses (10-μL injection from 5.0 μg/mL solution) of each heterocyclic amine were used to determine the precision of the method. The relative standard deviations (R.S.D., %) obtained were in the range of 1.8-4.4%. The detection limits, based on the signal-to-noise ratio of 2:1, ranged from 10 to 30 pmol, according to the retention times.

In order to test the applicability of the proposed analytical method, unknown mixtures, containing unknown heterocyclic amines at the level of 150 to 300 ng/g of solution, were prepared and provided by Dr. Rabache's laboratory, as previously mentioned. As shown in Fig. 4, the detected compounds were IQ, 4,8-DiMeIQx and PhIP. The estimated amounts after a two day analysis in triplicates were 217.0 ± 9.3, 214.75 ± 12.1 and 247.35 ± 5.8 ng/g, respectively. Obtained results show an excellent agreement with target values of 209.4, 202.5 and 257.0 for IQ, 4,8-DiMeIQx and PhIP, respectively.

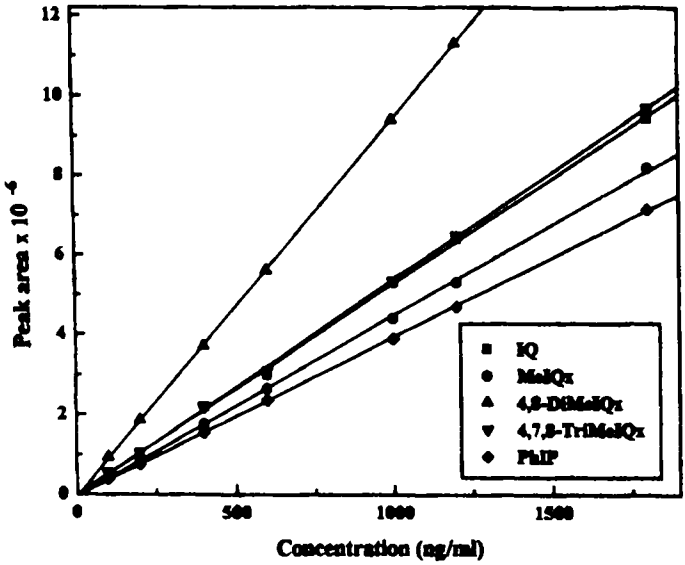

Figure 3. Linearity graphs of detector response, as peak areas vs concentration of heterocyclic amines in ng/mL. The detector response corresponds to the following equations: IQ, y= 2763.6 + 10281.5 x (sd= 95856.8); MeIQx, y= -16335.1+ 710.0 x (sd=14004.1); 4,7-DiMeIQx, y= -329592.7 + 14305.2 x (sd=852746.7); 4,7,8-TriMeIQx, y= -45012.5 + 3908.3 x (sd=215007.7) and PhIP, y= -233307.5 + 7257.8 x (sd=257854.5).

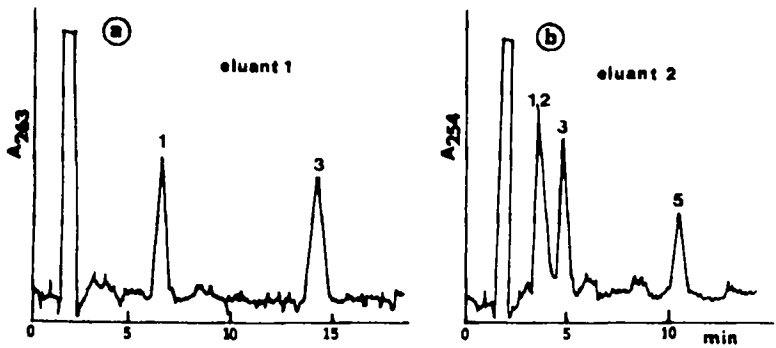

Figure 4. Analysis of mixtures containing unknown heterocyclic amines using eluant 1 (a) and eluant 2 (b). Compounds detected in chromatogram (a) are IQ (peak 1) and 4,8-DiMeIQx (peak 3) and in chromatogram (b) are IQ and/or MeIQx (peak 1, 2), 4,8-DiMeIQx (peak 3) and PhIP (peak 5). Eluants and conditions as in Fig.1. The injected amounts as estimated from the chromatogram and the linearity plots were 10.5 ng for IQ, 10.1 ng for 4,7-DiMeIQx and 12.85 ng for PhIP.

CONCLUSIONS

Identification of the type and chemical analysis of mutagenic and carcinogenic heterocyclic amines present in heat-processed food, are of great value in estimating the risk of these compounds to human beings. Description of a simple reversed phase isocratic HPLC-UV method for the separation and determination of five heterocyclic amines is presented in this report. Tests for linearity, sensitivity, precision and applicability of the method show, that this procedure can be reliably applied for the accurate and reproducible analysis of these constituents. Analysis of heterocyclic amines in heat-processed food can be performed after a successful extraction and clean-up procedure, according to the methods established by Galceran et al.[13] and Gross et al.[14]

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