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THERMOSPRAY LIQUID CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF MUTAGENIC SUBSTANCES PRESENT IN TRYPTOPHAN PYROLYSATES

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SUMMARY

During protein pyrolysis, as can occur when broiling meat or fish, mutagenic substances are formed, as shown by *in vitro* mutagenicity assays. Some of the most active compounds have been shown to originate from tryptophan (Trp). Hundreds of grams of Trp had to be used previously to study the formation of these compounds by classical separation and detection methods. Studies have been made of the formation of two active heterocyclic amines, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), by heating Trp at different temperatures and for different periods at time. Advantage was taken of the high selectivity and sensitivity of thermospray liquid chromatography–mass spectrometry coupling, which permitted the use of much smaller amounts (10 g) of starting material. These conditions permit a more accurate control of the pyrolysis temperature and the method of extraction can be shortened and simplified. The results show that Trp-P-1 and Trp-P-2 were already formed at 250°C. These substances were detectable in the low ppb range, *i.e.*, less than the threshold levels necessary to elicit a positive response in the Ames test under standard conditions.

INTRODUCTION

As early as 1964 aromatic hydrocarbons known to be carcinogenic were detected in broiled meat[1]. The overlapping of mutagenicity and carcinogenicity, although controversial, is now widely accepted[2]. Therefore, the introduction of the Ames test in 1976 (ref. 3) provided a rapid method of isolating potential carcinogens in food on the basis of their mutagenic activity. Such testing has revealed the presence of mutagenic heterocyclic amines in broiled fish and meat[4,5]. These amines, which account for most of the mutagenic activity, were isolated, identified, subsequently synthesized and found to be carcinogenic in long-term animal studies[6]. In broiled fish and meat, several classes of mutagens were discovered, including IQ (see abbreviations in Table I) and its methylated derivative MeIQ[7,8] and MeIQx[9]. These compounds are thought to be formed from amino acids, creatinine and sugars on heating[10]. Trp-P-1 and Trp-P-2 have been identified in broiled food and are believed to originate from the tryptophan (Trp) present in the proteins[11], as they have also been

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TABLE I

ABBREVIATIONS OF HETEROCYCLIC AMINES

Compound	*Abbreviation*
2-Amino-3-methylimidazo[4,5-*f*]quinoline	IQ
2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline	MeIQ
2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline	MeIQx
3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole	Trp-P-1
3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole	Trp-P-2

isolated from pure Trp pyrolysates[12]. To our knowledge, no reaction scheme has been proposed for the transformation of Trp into its mutagenic derivatives.

These mutagens are readily formed on cooking food on grills or on a direct flame and possibly also in certain industrial processes. It is therefore of prime importance to minimize their formation as they may represent a potential health hazard on chronic consumption. As these compounds are present only in trace amounts in very complex mixtures[13], their detection and quantitation represents an analytical challenge. Further, in laboratory experiments, direct flame pyrolysis as commonly used makes it difficult to control the temperature, especially when large amounts of starting material have to be used.

In this study, a model system was used to investigate and quantitate the formation of Trp-P-1 and Trp-P-2 from pure Trp that had been heated at different temperatures for different periods of time in a fluidized bath.

The high specificity and sensitivity of thermospray liquid chromatography–mass spectrometry (TS-LC–MS) coupling permitted the use of small amounts of Trp and hence better temperature control.

EXPERIMENTAL

Chemicals

Tryptophan, triethylamine, acetonitrile, methanol, ammonium acetate and harman were purchased from Fluka (Buchs, Switzerland), Trp-P-1 and Trp-P-2 from Wako (Osaka, Japan) and Blue-cotton from Funakoshi Pharmaceutical (Japan).

Liquid chromatography

The LC system consisted of a Waters Assoc. (Milford, MA, U.S.A.) 6000A pump, an Altex 210 (20-μl loop) injector and a Supelcosil LC-18DB column from Supelco (Bellefonte, PA, U.S.A.), 25 cm × 4.6 mm I.D., packed with 5-μm particles. The mobile phase was a mixture of 60% 0.1 *M* ammonium acetate and 40% acetonitrile–methanol (4:1) to which triethylamine (0.01 *M*) was added. It was adjusted to pH 7.5 for optimal separation and peak shape. The flow-rate was 1.3 ml/min.

Mass spectrometry

A Nermag (France) R10-10C quadrupole system was coupled to the LC system via a thermospray interface (Vestec, U.S.A.). The working temperatures were 183°C

for the thermospray tip and 280°C for the ion source. The mass spectrometer was operated in the positive mode, filament off at an ion pressure of 0.25 Torr.

Pyrolysis

In each experiment, 10 g of Trp were heated in a fluidized bath (Model FB-07, Techne, U.S.A.) in order to minimize temperature differences around the flask. The samples were heated at 250, 300 or 350°C for 15 or 30 min.

Extraction of heterocyclic amines

The extraction scheme is shown in Fig. 1. The amines were purified from the very complex material by acid–base partition and silica gel column purification. To achieve a highly selective recovery of these fused-ring compounds, a specific adsorbent, Blue-cotton, was used. It consists of cellulose cotton bearing covalently linked trisulpho-copper-phthalocyanine residues (10 μmol of copper phthalocyanine per gram of Blue-cotton). To improve the recovery from the small sample sizes, small glass columns packed with 0.5 g of Blue-cotton were used instead of adding Blue-

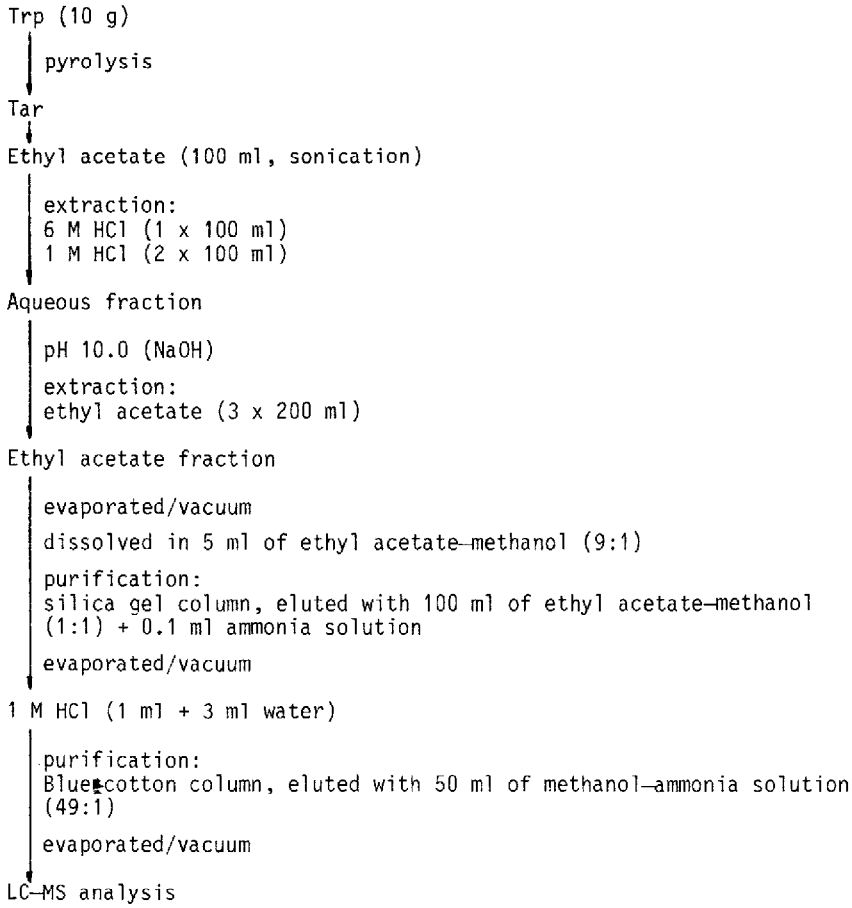

Fig. 1. Scheme of heterocyclic amine extraction.

cotton to the amine-containing aqueous solution. The final residues were then dissolved in 500 μl of a mixture of methanol and the mobile phase (2:3) and 20-μl aliquots were injected onto the LC column.

RESULTS

The thermospray mass spectra of Trp-P-1, Trp-P-2 and harman standards (Fig. 2) show $(M+H)^+$ ions as base peaks and no significantly important fragments. Single ion monitoring of the $(M+H)^+$ ions was therefore very suitable for quantitative analysis.

Calibration graphs were established by reporting the area ratio of the peaks corresponding to Trp-P-1 or Trp-P-2 and harman used as an internal standard. The graphs (Fig. 3) show good linearity (r = 0.992) from 0.1 to 200 ng of either Trp-P injected onto the column. However, as harman is also formed during Trp pyrolysis (see Fig. 4), we used an external standard method for the quantitative analyses of the pyrolysate extracts: the detection response for each sample was checked by injecting a standard solution containing an equivalent concentration of Trp-P-1 and Trp-P-2.

The mass chromatogram of a Trp pyrolyste extract (350°C, 30 min) is shown in Fig. 4 and the concentrations of Trp-P-1 and Trp-P-2 found in each sample are given in Table II.

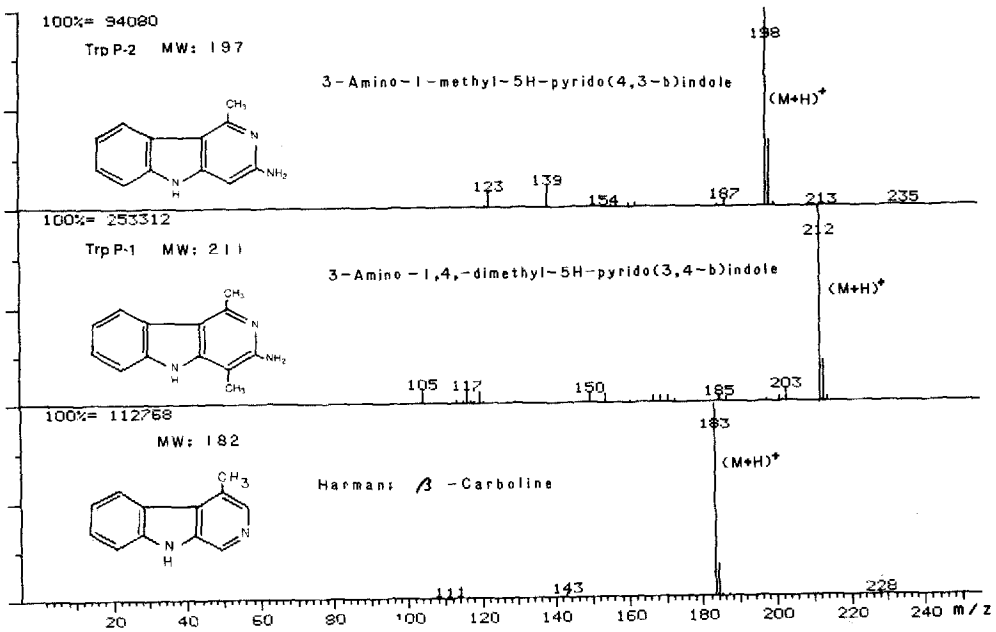

Fig. 2. Thermospray mass spectra of Trp-P-1, Trp-P-2 and harman.

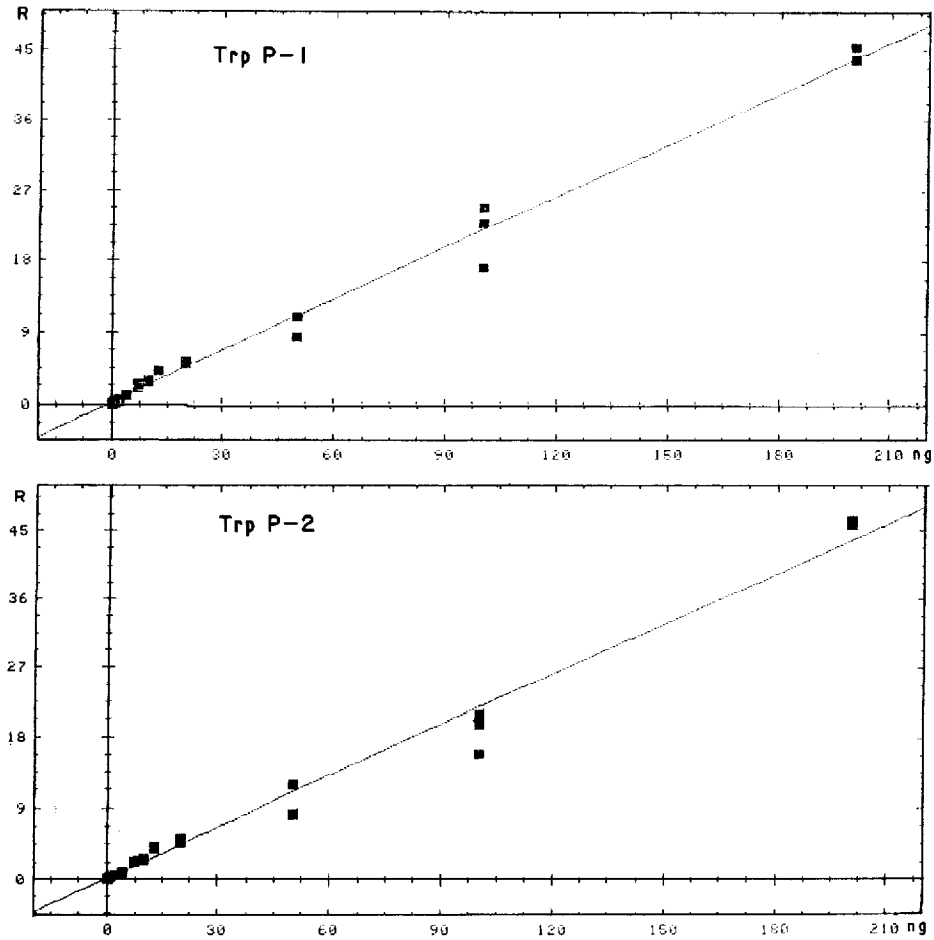

Fig. 3. Calibration graphs for Trp-P-1 and Trp-P-2 using harman as an internal standard.

TABLE II

YIELDS OF Trp-P-1 AND Trp-P-2 IN PYROLYSED Trp

Temperature (°C)	*Trp-P-1 (ng/g Trp)*		*Trp-P-2 (ng/g Trp)*	
	15 min	*30 min*	*15 min*	*30 min*
250	20	20	20	7
300	150	300	150	275
350	3700	7100	3300	6000

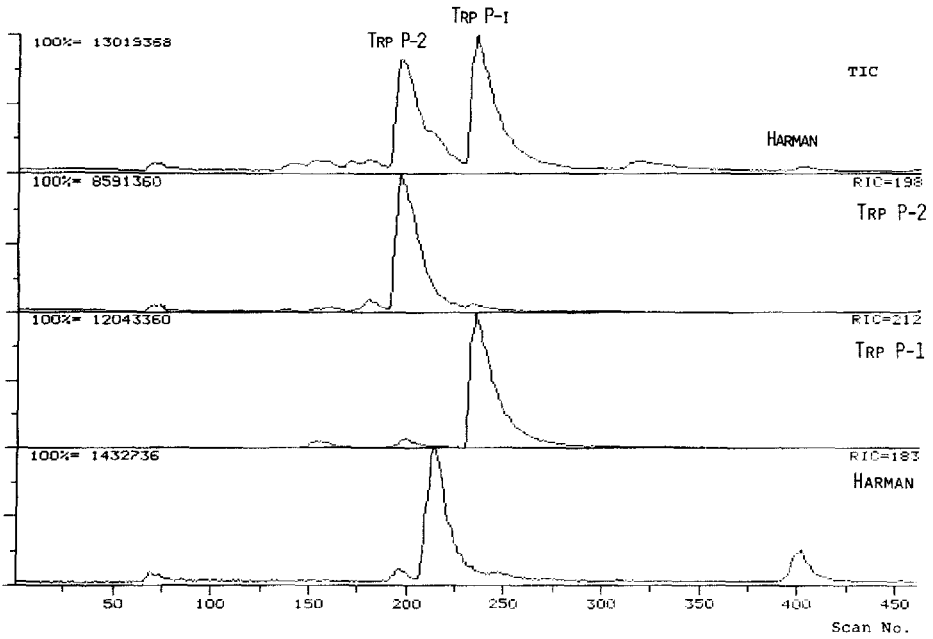

Fig. 4. Mass chromatogram of a Trp pyrolysate extract (for conditions, see Results). Retention times: Trp-P-2, 5 min 37 s; Trp-P-1, 6 min 42 s; harman, 11 min 25 s.

DISCUSSION

Analysis

Several techniques have been used to determine mutagens in cooked food, *e.g.*, gas or high-performance liquid chromatographic separation, followed by either off-line or on-line UV, electrochemical or MS detection. Two aspects are of importance: first, owing to the very low levels observed (ppb* range) and depending on the sensitivity of the detection method, very large amounts of starting material are required, *e.g.*, hundreds of grams for UV or off-line MS[14]. Secondly, as pyrolysis of food produces a huge number of substances, a laborious and time-consuming purification procedure is necessary before the final separation and detection steps can be performed[15]. Therefore, the combined use of Blue-cotton and LC–MS was proposed in order to permit a selective and efficient recovery of the compounds of interest, *i.e.*, fused aromatic rings[16].

Blue-cotton in a column facilitated the use of smaller volumes of extract and gave higher yields. The overall recovery was estimated by spiking the ethyl acetate extract from the first step of the clean-up procedure (see Fig. 1) with known amounts of Trp-P-1 and Trp-P-2 corresponding to the observed range of concentrations for the lowest pyrolysis temperature. Under these conditions, the recovery was found to be around 80%.

* Throughout this article, the American billion (10^9) is meant.

Trp-P-1 and Trp-P-2 yields

Even at the lowest temperature used (250°C), significant amounts of the heterocyclic amines were observed. This result was surprising, considering that the melting point of Trp is *ca.* 280°C. Apparently, some degradation of Trp occurred, even if the results are not very consistent, especially with increasing pyrolysis time. With increasing pyrolysis temperature, the yields increased very rapidly and correlated well with the pyrolysis time. The only value found in the literature (10 μg of Trp-P-2 per gram of Trp at 550°C) is in the range of our results, although no indication of the pyrolysis method or time was given[17].

Biological correlations

In order to establish the usefulness and the limitations of these determinations, correlations between the chemical analysis of these mutagens and their biological activity in the Ames test should be tentatively established.

The detection limit obtained under the present conditions (100 pg of either Trp-P injected onto the column) corresponds to 2.5 ng per gram of pyrolysed Trp (2.5 ppb). The sensitivity of the method could certainly be improved if necessary and further refined by the use of deuterated standards (not available to us). An Ames test performed with pure Trp-P-1 and Trp-P-2 showed that the activity threshold is 5 ng of Trp-P-1 per dish and 2.5 ng of Trp-P-2 per dish[18]. However, the mutagenic activity of food extracts assessed by summing the effects of purified isolated components may lead to erroneous results. Food may contain substances that can act as synergistic or inhibitory agents when assayed by bacterial mutation tests. Moreover, when food is ingested, metabolic reactions occur that cannot be simulated by mutation assays. In other words, determination of mutagen concentrations in food extracts alone is not sufficient to evaluate the potential risk of foods but is complementary, although necessary, information to that proved by mutagenic tests.

From the results of the present study, it can be said that the amounts of mutagens detectable by TS-LC–MS are lower than those corresponding to the activity thresholds detectable by the Ames test under standard conditions. TS-LC-MS was shown to be the method of choice for the detection and quantitation of these heterocyclic amines.

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