Heterocyclic aromatic amines in cigarette smoke, chemical and biological assessments

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Objectives : Developing biological and chemical analyses in order to evaluate Heterocyclic Aromatic Amines (HAA) individual toxicity combined with inhibitory effect of mainstream cigarette smoke condensate mutagenicity.

Chemical Assessment - Abstract

Chemical assessment consist in the development of liquid chromatography tandem mass spectrometry (HPLC/MS/MS) method for the screening of HAA (AαC, MeAαC, PhIP, IQ, MeIQ, TrP-P-1, TrP-P-2, Glu-P-1, Glu-P-2) in smoke condensate applied to Ky-2R4F reference cigarette.

For the first time, HPLC/MS/MS is applied to Heterocyclic Aromatic Amine analysis in smoke condensate. A purification step is required and consists in Blue Rayon following by Molecular Imprinted Polymer NNAL solid phase extractions. Clean-up step needs further investigations for Glu-P-1 and Glu-P-2 assessment.
The quantification of AαC, MeAαC, TrP-P-1 and Trp-P-2 is performed by internal standard calibration which has been correlated with Spike Calibration as slight matrix effect was identified. AαC, MeAαC, TrP-P-1 and Trp-P-2 Ky-2R4F contents are assessed respectively to 28, 2.5, 0.7 and 1.5 ng/cig.
IQ, MeIQ, PhIP contents seem to be lower than 0.2 ng/cig as fortified condensate at this level have been detected. The high toxicity of individual compounds especially IQ and MeIQ will involved method adjustment to enhance detection limits.

According to these results, Ky-2R4F AαC , MeAαC, TrP-P-1 and Trp-P-2 contents fit in the published cigarettes ranges. However, IQ, MeIQ and PhIP contents are at a surprising low level , under the published cigarettes ranges.
This HPLC/MS/MS method is promising to achieve HAA screening for biological assessment correlation

Biological Assessment - Abstract

The effect of HAA on the mutagenic activity of 2R4F Smoke Condensate is assessed with Ames test using Salmonella typhimurium (TA98) in presence of metabolic activation.

The HAA tested are IQ and MeIQ.
Two Ames procedures have been compared :
- o Direct Incorporation
- o Pre Incubation

In both conditions, MeIQ is more mutagenic than IQ when the HAA are tested alone.
When HAA at 0.3 or 3 ng/plate are combined with 2R4F Condensate (CSC) at 0.25mg/plate, a strong interaction between HAA and CSC was observed.

These results lead to two further questions:
- o *Are the interactions due to a toxic effect of the HAA?*
- o *Do the HAA or their metabolites react with the CSC in order to reduce the mutagenic activity?*

Ky-2R4F Screening by liquid chromatography tandem mass spectrometry

Screening is focus on Heterocyclic Aromatic Amine previously identified in mainstream smoke condensate : AαC, MeAαC, PhIP, IQ, MeIQ, TrP-P-1, TrP-P-2, Glu-P-1, Glu-P-2 (Figure 1) in 2R4F Kentucky reference cigarette.

Liquid chromatography tandem mass spectrometry [1] has been reported to take over the first limited factor in HAA analysis [2] : the full and sensitive detection of HAA based on standard responses.

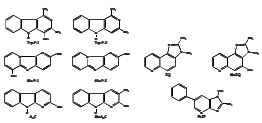


Figure 1 : Chemical structure of heterocyclic aromatic amine

Applied to Cigarette Smoke Condensate acid extract, liquid chromatography tandem mass spectrometry detection appears to be not specific enough against matrix smoke interferences. However, the highest content AαC is detected.

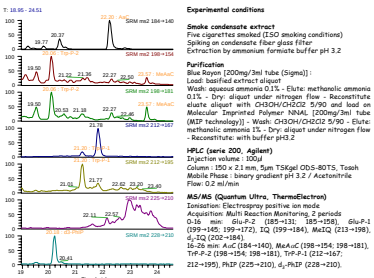
As HAA properties are very heterogeneous (basicity, polarity), purification is focused on molecular recognition: Blue Rayon followed by Molecular Imprinted Polymer (MIP) NNAL solid phase extractions.
Blue Rayon allows to eliminate the non three condensate rings components and MIP NNAL seems to be a discriminant support against resulted matrix interferences even if it is dedicated to the analysis of nicotine metabolites in urinary sample.
Quantification is performed by internal standard calibration with the two commercially available labelled compounds, d3-IQ and d3-PhIP.

For the first time :
- HPLC/MS/MS is applied to Heterocyclic Aromatic Amine analysis in smoke condensate
- Molecular Imprinted Polymer NNAL is used in smoke condensate purification

AαC, MeAαC, TrP-P-1 & Trp-P-2
(Figure 2)

AαC, MeAαC, TrP-P-1 and Trp-P-2 Ky-2R4F contents are assessed at 24, 2.3, 0.7 and 1.5 ng/cig respectively.

Standards and spiked matrix external calibration slopes were compared to evaluate matrix effects after purification step. They consist in ion suppression of 16% for AαC, 30% for MeAαC, 13% for TrP-P-1, 23% for Trp-P-2 and 32% for PhIP (assimilated to d3-PhIP) : d3-PhIP Internal Standard calibration corrects matrix effect for MeAαC but leads to a slight underestimate of TrP-P-1 (10%), AαC (15%) and Trp-P-2 (20%).
Spike calibration was carried out and confirmed these results.
As matrix effect depends on cigarette composition additional labeled internal standards (using 13C, 15N or D labeling) are being studied.



Experimental conditions

Smoke condensate extract
Five cigarettes smoked (ISO smoking conditions)
Spiking on condensate fiber glass filter
Extraction by ammonium formiate buffer pH 3.2

Purification
Blue Rayon [200mg/3ml tube (Sigma)] :
Load: basified extract aliquot
Wash: aqueous ammonia 0.1% - Elute: methanolic ammonia 0.1% - Dry: aliquot under nitrogen flow - Reconstitute eluate aliquot with CH3OH/CH2Cl2 5/90 and load on Molecular Imprinted Polymer NNAL [200mg/3ml tube (MIP technology)] - Wash: CH3OH/CH2Cl2 5/90 - Elute: methanolic ammonia 1% - Dry: aliquot under nitrogen flow - Reconstitute: with buffer pH3.2

HPLC (serie 200, Agilent)
Injection volume : 100µl
Column : 150 x 2.1 mm, 5µm TSKgel ODS-80TS, Teash
Mobile Phase : binary gradient pH 3.2 / Acetonitrile
Flow: 0.2 ml/min

MS/MS (Quantum Ultra, ThermoElectron)
Ionisation: Electrospray positive ion mode
Acquisition: Multi Reaction Monitoring, 2 periods
0-16 min: Glu-P-2 (185→131; 185→158), Glu-P-1 (199→145; 199→172), IQ (199→184), MeIQ (213→198), d_3-IQ (202→184).
16-26 min: AαC (184→140), MeAαC (198→154; 198→181), TrP-P-2 (198→154; 198→181), TrP-P-1 (212→167; 212→195), PhIP (225→210), d_3-PhIP (228→210).

Figure 2 : detection of AαC, MeAαC, Trp-P-1 and Trp-P-2
After purification step Blue Rayon and Molecular Imprinted Polymer NNAL

PhIP, IQ, MeIQ, Glu-P-1 and Glu-P-2

50% Glu-P-1 and Glu-P-2 are lost during MIP NNAL washing step. Thus, it prevents us from detecting their trace level.

As PhIP, IQ, MeIQ, were not detected, investigations on fortified condensates (Figure 3) are carried out with three spiked levels 0.2, 0.4, and 0.6 ng/cig. Results (Table I) highlight the detection of the 3 levels and suggest lowest levels contents.
Even if contents are lower than 0.2 ng/cig, the high toxicity of individual compounds involved method adjustment to enhance detection limits.

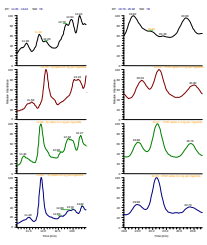


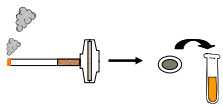
Figure 3: IQ and PhIP fortified condensate.
IQ (left side) and PhIP (right side) detection : unspiked (black), spiked with 0.2 (red), with 0.4 (green) and 0.6 ng/cig (blue).

Fortified* condensate	IQ	MeIQ	PhIP
0,20	0,27 ± 0,02	0,24 ± 0,14	0,25 ± 0,04
0,40	0,44 ± 0,01	0,39 ± 0,08	0,43 ± 0,02
0,60	0,67 ± 0,09	0,69 ± 0,04	0,59 ± 0,01

Table I : fortified 2R4F condensate Results
* in nanogrammes per cigarette

Ky-2R4F IQ, MeIQ, PhIP contents seem to be lower than 0.2 ng/cig.

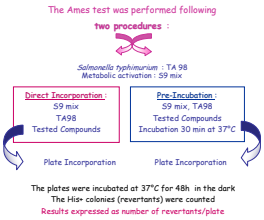
Mutagenicity assessment with AMES test

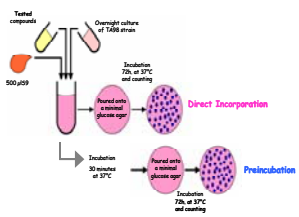


Cigarette smoke condensate (CSC) extracted from the cigarette 2R4F is dissolved in DMSO to make stock solutions (10 mg/ml) which were stored at -80°C prior to use.

AMES TEST METHODOLOGY in presence of S9 :

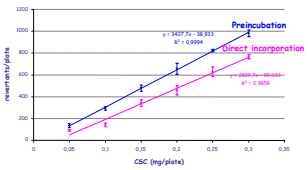
Mutagenicity was assessed by the AMES assay [3].
The bacteria used (TA98) are histidine-requiring mutants of *Salmonella typhimurium*.
In presence of mutagens, the bacteria can revert to a wild-type phenotype and restore the capacity to synthesize histidine (revertant). The revertants are detected by their ability to grow in the medium without histidine.
The number of revertants is related to the mutagenic activity of the tested product.



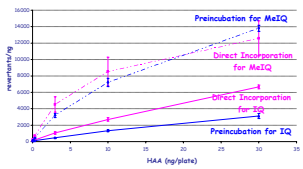


MUTAGENICITY OF EACH COMPOUND ASSESSED INDIVIDUALLY:

Mutagenic potentiality for 2R4F condensate was assessed in doses ranging from 0.05 to 0.3 mg/plate.



Mutagenic potentialities for both HAA were assessed in doses ranging from 0.03 to 30 ng/plate.



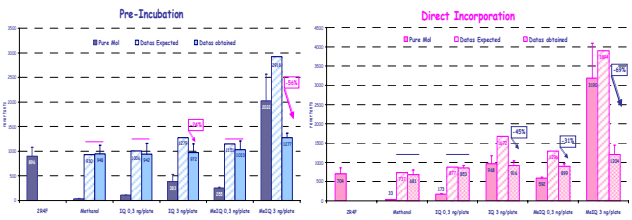
2R4F Results
Mutagenicity of 2R4F condensate is higher by Pre-Incubation compared to the direct Incorporation condition

IQ and MeIQ Results
Mutagenicity of both HAA is higher in direct Incorporation compared to the Pre-Incubation condition.
MeIQ is more mutagenic than IQ

EFFECT OF HAA ASSOCIATED TO 2R4F CONDENSATE:

2R4F CSC at the dose 0.25 mg/plate is combined with two doses of each HAA : 0.3 and 3 ng/plate.

Theoretical values were calculated by adding the number of revertants induced by 0.3 or 3 ng/plate of each HAA to the number of revertants induced by the CSC at 0.25 mg/plate.



In both conditions, a strong interaction is observed between CSC and the two HAA at dose 3 ng/plate (the observed mutagenicity is lower than the theoretical value).
In direct incorporation condition, this effect is also observed with the MeIQ at a lower dose (0.3 ng/plate).
These results lead to two further questions:
o Are the interactions due to a toxic effect of the HAA?
o Do the HAA or their metabolites react with the CSC in order to reduce the mutagenic activity?

References
[1] E. Barcelo-Barrachina, E. Moyano, L. Puignou, M.T. Galceran, Evaluation of different liquid chromatography-electrospray mass spectrometry systems for the analysis of heterocyclic amines, J. Chromatogr. A 1023 (2004) 67-78
[2] H. Kataoka, Methods for the determination of mutagenic heterocyclic amines and their applications in environmental analysis, J. of chromatogr. A, 774 (1997) 121-142
[3] D.M. Maron, and B.N. Ames, Revised methods for the Salmonella mutagenicity test, Mutation Res.,,(1983) 113, 173-215.

Abbreviations : HAA, Heterocyclic Aromatic Amine; ng/cig, nanogramme per cigarette; NNAL, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanol; CSC, Cigarette Smoke Condensate; DMSO, Dimethylsulfoxide;