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Analysis of Acrylamide in Mainstream Cigarette Smoke and Effects of Total Nitrogen and Reducing Sugars on Acrylamide Content

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ABSTRACT : Acrylamide has been found in many foods. Acrylamide in foodstuffs were analyzed by a GC/MS after bromination of acrylamide or by a LC/MS for underivatized acylamide. Time consuming and laborious clean up procedures is applied for the purification of the extract, in these methods. In this study, a simple and fast method without clean up step for the analysis of acrylamide in mainstream cigarette smoke was developed by using liquid chromatography-tandem mass spectrometry (LC/MS/MS) and the effects of tobacco leaf constituents on acrylamide content was observed. The analysis of acrylamide in mainstream cigarette smoke started to collect TPM (total particulate matter) from smoking and to extract by 0.1 % acetic acid solution and then to detect by liquid chromatography tandem mass spectrometry using electrospray in the positive mode. The recovery of acrylamide in 2R4F reference cigarette was 98 % and the reproducibility was 2.5 % and the limit of detection was 1.6 ng/mL. Reducing sugars and amino acids are considered to be main precursors of acrylamide in foodstuffs. Cut tobacco contain substantial amounts of reducing sugars and amino acid which may be explained the occurrence of acrylamide in mainstream cigarette smoke. The effects of reducing sugars and total nitrogen studied in an experiment with a various tobacco types. This result indicated that reducing sugars are not limiting factor for acrylamide formation, but the level of acrylamide in cigarette smoke was significantly correlated with the total nitrogen contents.

Key words **: Mainstream cigarette smoke, acrylamide, reducing sugar, total nitrogen**

In early 2002, Tareke *et al.* (2002) published that some heat-treated starch rich food contained high levels of acrylamide. After the initial finding, acrylamide has been found in many foods. Heat-treated potato and cereal products and coffee are major sources of intake. Little information was known about the possible effects of this compound on human consumers but it had been classified as "probably carcinogenic to humans" (group 2A) by the International Agency for Research on Cancer (IARC, 1994).

Acrylamide is produced in foods during cooking processes at high temperature such as roasting, baking or frying. Investigations on cooked food have led to several pathways for its formation. The major pathway involves the

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Maillard reaction between amino acids and carbonyl compounds like reducing sugars (Govaert *et al.*, 2002). Besides, the occurrence of acrylamide in cigarette smoke indicated that acrylamide is formed during incomplete combustion or heating of organic matters (Tareke *et al.*, 2002).

In foods, it is now well-established that free amino acid and reducing sugars are important precursors and that processing conditions, such as time, temperature, water activity, and matrix, will influence its formation and degradation. However, the factors that would influence the acrylamide content in cigarette smoke was not reported.

Acrylamide is a solid compound with a molecular weight of 71 Da and stable at room temperature. Chemical structure includes a polar amide function giving rise to high water-solubility and a vinyl function allowing polymerization. Due to the small molecular weight of acrylamide, GC/MS has historically been used for its determination (Castle *et al.*, 1991). However, this requires the time consuming step of derivatization with bromine. In order to avoid time consuming preparation and to obtain rapid methods destined for high sample throughputs, liquid chromatography coupled to tandem mass spectrometry was alternatively investigated (Rosen and Hellenas, 2002). Many procedures based on this analytical approach were then developed.

The purpose of this study was to develop a simple and fast method for the determination of acrylamide in mainstream cigarette smoke and to observe the effects of reducing sugars and total nitrogen on acylamide contents in mainstream cigarette smoke.

MATERIALS AND METHODS

Materials

Acrylamide, fructose, glucose and sucrose were supplied by Sigma Chemical Company (St. Louis, MO, USA). D_3-Acrylamide was purchased from polymer Source Inc. (Dorval, Quebec, Canada). Methanol (HPLC Grade) and formic acid was obtained from Merck (Darmstadt, Germany). The water was used Mili-Q water (Millipore, Bedford, MA, USA). All stock solutions and calibration standards were prepared in water and kept at 4 ℃ until use. Calibration standards were 0.2~5.0 μg/mL for acryamide with 0.1 μg/mL D_3-acrylamide. The 2R4F reference cigarette were obtained from the University of Kentucky, Tobacco Health Research Institute. In order to study the effects of tobacco leaf constituents on acrylamide content in cigarette smoke, the thirteen prototype unblended cigarette was manufactured. The specification of prototype cigarette was shown in Table 1.

Table 1. Specifications of the prototype cigarette

Parameter	Specifications
Filter type	Carbon dual filter
Filter length	27 mm
Circumference	24.5 mm
Column length	57 mm
Filter ventilation	0 %
Cut tobacco	Flue-cured produced in Korea in 2003 ~ 2004
	Burley produced in Korea in 2004
	Basma produced in Greece in 2002
	Krumovgrad in Bulgaria produced in 2002
	Izmir produced in Turkey in 2002

Sample preparation

Analytical procedure for acrylamide in mainstream smoke was shown in Fig. 1. Mainstream smoke collected in 44 mm Cambridge filter pad (CFP) from three cigarettes that was

smoked under ISO conditions (35 ± 0.2 cm^3 of puff volume, 2.0 ± 0.05 second of puff duration, every 60 ± 0.5 seconds of puff interval) by using the linear smoking machine (ASM 450, Cerulean, UK). After smoking, the CFP was transferred to a erlenmeyer flask containing 50 mL of 0.1 % acetic acid with 1.0 μg/mL of D_3-acrylamide. The sample was shaken for 30 min on a wrist action shaker and filtered by using a 0.45 μm syringe filter. Filtered sample was analyzed by LC/MS/MS.

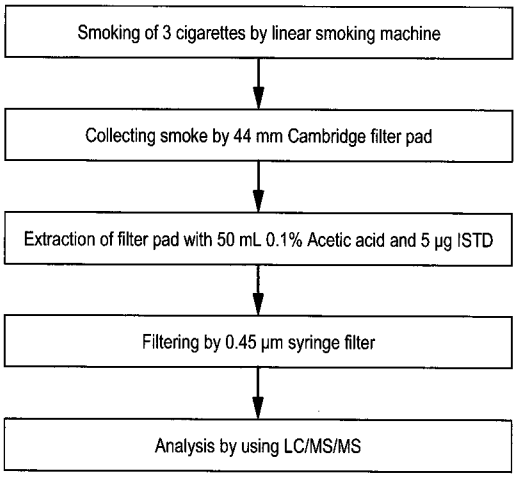

Fig. 1. Analytical procedure for acrylamide in mainstream cigarette smoke.

Instrumentation and chromatographic conditions

A quaternary pump system from Agilent Technologies (USA) model Series 1200 was coupled to an API 4000 (Applied Biosystems, Foster City, CA, USA) equipped with a turbo ionspray as ionization source and a triple quadrupole as analyzer. Data acquisition was carried out by Analyst 1.2 software. Optimal ionization source working parameters were as follows : electrospray voltage, 2.5 kV; nebuliser gas, 50 a.u.; curtain gas, 20 a.u.; turbo ionspray gas flow rate, 50 a.u.; turbo ionspray gas temperature, 400 ℃. Multiple reaction monitoring, using as precursor ion of the protonated molecular ions $[M+H]^+$ was used as data acquisition mode and the parameters were used given in Table 2.

The chromatographic separation of acrylamide was carried out by reversed-phase liquid chromatography using a Atlantis d_{18} column (Waters, Milford, MA, USA), with a particle size of 5 μm, 150 mm × 2.1 mm I.D. The column was eluted with 0.1 % acetic acid and 0.5 % methanol in water with a flow rate of 200 μL/min. The sample volume injected was 5 μL.

Table 2. MRM method parameters of LC/MS/MS

Analyte	MRM		
	Precursor → Product ion (m/z)	Collision Energy (V)	Declustering Potential (V)
Acrylamide	72 → 55	20	50
D_3-Acrylamide	75 → 58	25	50

RESULTS AND DISCUSSION

Method validation of acrylamide analysis

Chromatograms of the acrylamide standard of 0.1 μg/mL and 2R4F cigarette sample were shown in Fig. 2. The use of a labeled acylamide as an internal standard compensates for matrix suppression and variations in the MS-MS

Table 3. Validation data of the method based on five observations of 2R4F cigarette

Parameter	Value
Recovery	98 %
Reproducibility	2.5 %
Limit of detection	1.6 ng/mL
Linearity for calibration	0.999
Level in 2R4F cigarette	1.7 μg/cigarette

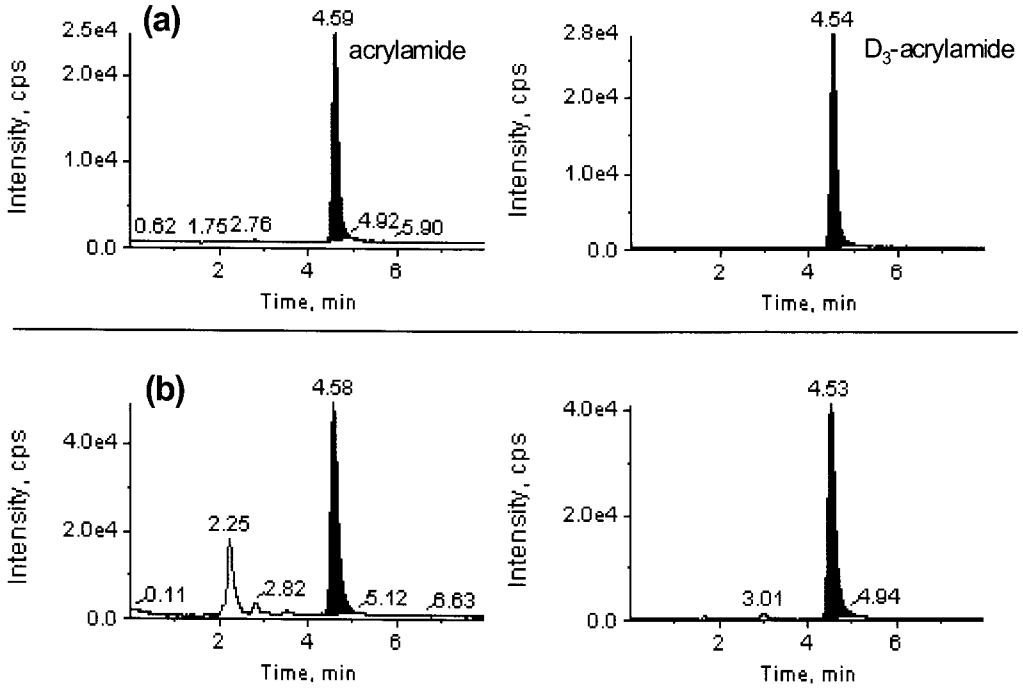

Fig. 2. Chromatogram of (a) a standard of 0.1 μg/mL acrylamide and a internal standard of 0.1 μg/mL D_3-acrylaimde and (b) a 2R4F cigarette sample.

Table 4. Acrylamide levels in cigarette smoke from unblended cut tabocco

Tobacco type	Grades	Acrylamide contents (ng/mg of TPM)	Reducing Sugars (%)	Total Nitrogen (%)
Flue-cured	AB3O	172.2	15.6	2.8
	AB4OR	225.2	13.8	3.0
	B1O	99.8	17.6	2.7
	B2O	97.1	18.3	2.5
	C1L	139.2	17.5	2.5
	C2L	119.0	18.5	2.4
	CD3L	130.3	17.7	2.3
	CD4L	272.2	7.6	2.7
Burley	B2T	558.4	0.4	4.8
	C1W	675.2	0.2	5.4
Oriental	Basma III	335.4	2.9	3.5
	Krumovgrad KP	464.4	7.6	3.7
	Izmir KP	142.6	12.4	2.4

response. As a result, the 4-point calibration curves from 0.2 to 5.0 μg/mL generally show a good correlation, mostly r^2 > 0.999. Recovery from 2R4F cigarette samples spiked with 0.1 μg/mL was 98 %, reproducibility based on five 2R4F cigarette samples was 2.5 % and the limit of detection (LOD) was 1.6 ng/mL. The acrylamide content in mainstream smoke of 2R4F cigarette was 1.7 μg/cigarette. Table 3 was shown the validation data for the method.

Contents of acrylamide in mainstream smoke

The acrylamide contents from cigarette smoke and the contents of reducing sugar and total nitrogen of 13 prototype cigarettes were shown in Table 4. Triple analysis was carried out for every cigarette sample, and calculated the mean value. The range of acrylamide contents in mainstream smoke were 97.1~272.2 ng/mg of TPM, 142.6~335.4 ng/mg of TPM and 558.4~675.2 ng/mg of TPM of flue-cured, oriental and burley tobaccos, respectively.

Correlation between acylamide levels and reducing sugar contents

The potentials of acrylamide formation were related to contents of reducing sugars and free amino acids. Therefore the relationship among acrylamide, reducing sugar and total nitrogen was studied. Fig. 3 showed the correlation between acylamide content in mainstream smoke and reducing sugar contents in the cut tobacco.

Interestingly, reducing sugar did not influence the acylamdie content. On the contrary, this result indicated that the level of reducing sugars in cut tobacco was reversely correlated to acrylamide contents in mainstream smoke. We think that the result was caused by tobacco leaf variety. Therefore, reducing sugar was added to cut tobacco and then the variation of acrylamide content was observed. Burley was chosen as the cut tobacco since it is known that have low

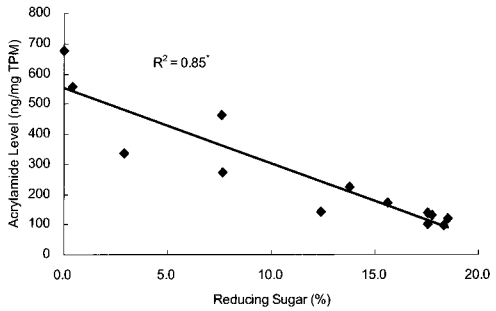

Fig. 3. Correlation between acylamide levels and reducing sugar contents.

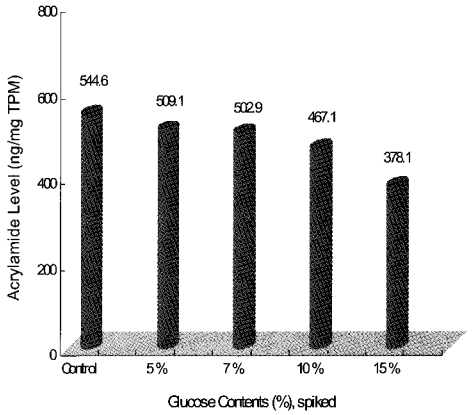

Fig. 4. Effect of added glucose on acylamide contents in cigarette smoke.

contents of reducing sugar. In the range of 5~15 % glucose was added to cut tobacco. However, added glucose did not seem to influence the acrylamide content. In this experiment, the results showed a unsignificant effects for added glucose or their interaction (Fig. 4). This result indicates that reducing sugars are not limiting factor for acrylamide formation.

Correlation between total nitrogen contents and acrylamide levels

Regression analysis did not showed a significant effects for the reducing sugar. Thereby, the

correlation between acrylamide contents and total nitrogen contents was monitored, because total nitrogen contents could estimate the amino acid levels. Significant correlation was observed between acrylamide and total nitrogen contents.

Fig. 5 showed the correlation between total nitrogen in cut tobacco and acrylamide level in mainstream smoke. This figure indicates that, even though reducing sugars is present, the contents of total nitrogen is the limiting factor in the generation of acrylamide in cigarette smoke.

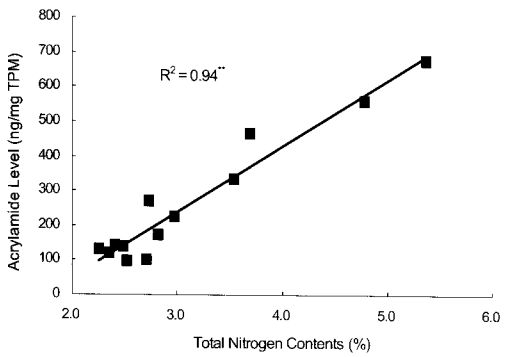

Fig. 5. Correlation between total nitrogen and acrylamide levels.

CONCLUSIONS

A simple and fast method without clean up step for the analysis of acrylamide in mainstream cigarette smoke was developed by using liquid chromatography-tandem mass spectrometry. Results obtained from this study indicate good linearity and reproducibility and low limit of detection. The range of acrylamide contents in mainstream smoke for 13 prototype cigarettes were 97.1~675.2 ng/mg of TPM. Also, this result indicated that reducing sugars are not limiting factor for acyrlamied formation and the level of acrylamide in cigarette smoke was correlated with the total nitrogen contents.

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