Acrylamide Analysis in Tobacco, Alternative Tobacco Products, and Cigarette Smoke

Serban C. Moldoveanu and **Anthony R. Gerardi**
R.J. Reynolds Tobacco Co., 950 Reynolds Blvd., Winston-Salem, NC 27105

Abstract

A new liquid chromatography-tandem mass spectrometry (LC–MS–MS) method for the analysis of acrylamide in tobacco, alternative tobacco products (wet snuff, snus, orbs, strips, and sticks), and cigarette smoke was developed and evaluated. Besides the LC–MS–MS method, two additional procedures for acrylamide analysis were also developed with the purpose of comparison. A thorough evaluation of the LC–MS–MS technique was performed for typical characteristics of an analytical method such as selectivity, precision, accuracy, range of measurable levels, robustness. The method was found perfectly fit for the analyses of acrylamide in the tobacco, tobacco products, and smoke matrix. Although not essential, the sensitivity of the method was further increased by using a concentration step on a graphitized carbon solid phase cartridge, allowing the measurement of as low as 300 pg/mL and detection of 90 pg/mL acrylamide in solution. Several tobacco samples, alternative tobacco products, and smoke from several cigarettes including commercial cigarettes from the US market, 2R4F, and 3R4F Kentucky reference cigarettes were analyzed. The levels of acrylamide in tobaccos were about 100 ng/g, or lower. The levels found in snus and one brand of wet snuff was also below 100 ng/g. A wintergreen wet snuff had about 180 ng/g, strips had about 126 ng/g and the sticks about 367 ng/g acrylamide. The cigarette smoke had levels of acrylamide around 1 µg/cig or higher.

Introduction

The analysis of acrylamide in tobacco, alternative tobacco products, and cigarette smoke became an important subject since this compound was targeted as a toxicant in food and other consumer products (1–3). As an example, typical levels of acrylamide are 0.6 to 2 µg/g in potato crisps, 0.3 to 0.7 µg/g in French fries, and 50 to 250 ng/g in breakfast cereals (3). Acrylamide is "reasonably anticipated to be a human carcinogen" based on sufficient evidence of carcinogenicity in experimental animals (4), and is classified as a probable human carcinogen (Group B2) by the U.S. Environmental Protection Agency (EPA). Several studies on biomarkers for acrylamide in humans (5–7) showed that smoking is an important source of acrylamide exposure.

*Author to whom correspondence should be addressed: email moldovs@rjrt.com

A very limited number of reports is available regarding acrylamide analysis in cigarette smoke (8,9), and only one peer review paper is published on this subject (8), the paper reporting results only for 2R4F cigarette. Reports on acrylamide levels in tobacco and in alternative tobacco products (e.g., wet snuff, snus, orbs, strips, and sticks) are not available in peer reviewed publications. A considerable number of analytical techniques were described for acrylamide analysis in food (1–7,10–40).

For the selection/development of a method for acrylamide analysis in tobacco, tobacco products, and cigarette smoke, the main published analytical procedures applied for acrylamide analysis in other matrices than tobacco were reviewed. These procedures used a variety of extracting solvents (8–11), different pH values of the extracting solution (12,13), cleanup procedures (14–22), derivatization (9,19,23–25), GC or LC separation (8,11,14,15,19,26), EI+ (18,27), CI+ (15), electron capture (ECD) (28,29) for detection in GC, MS (14,30), MS–MS (13,18,31–33), or ultraviolet (UV) (26,34) for detection in LC. The calculation of results has been done using calibration curves (26), response factors (14), or using the labeled acrylamide only for the evaluation of recovery (15). Some studies used ^{13}C labeled acrylamide (14,27), and other used $^{2}H_3$ labeled compound (15) as internal standard. Since ^{13}C single labeled acrylamide has a MW = 72, and the ion 72 is also present in acrylamide mass spectrum (3.3%) some error is carried from the sample to the standard when using this internal standard. The critical evaluation of all these procedures, provided guidance for the development of an original LC–MS–MS method for the analysis of acrylamide in tobacco, alternative tobacco products, and cigarette smoke. In the present study, $^{2}H_3$ labeled compound was used as an internal standard and the quantitation was based on a response factor.

Experimental

Sampling and sample preparation

For tobacco analysis, about 20 g of tobacco (previously cut leaf or from cigarettes) were ground using a coffee grinder and used as a starting material. For orbs and sticks alternative tobacco products that contained tobacco in a non-tobacco matrix (about 2–3 g) were also ground. From the ground material 1.0 g ± 0.1 mg of sample was weighed and used for analysis. The strips were

used without any mechanical processing and 1.0 g ± 0.1 mg strips were taken for each analyzed replicate. No randomization was performed for orbs, sticks and strips, the material being assumed homogeneous. For the moist snuff samples, a composite sample of about 10 g was made from three different freshly opened pucks and 1.0 g ± 0.1 mg of material was used as a sample. All the alternative tobacco materials and the tobacco samples were analyzed without drying.

For smoke samples, the smoke was collected using a Borgwaldt RM20 CSR smoking machine (Borgwaldt, Schnackenburgallee 15, D-22525 Hamburg, Germany). The machine was initially tuned for conditions similar to those recommended for linear smoking machines by U.S. Federal Trade Commission (FTC) (41). The smoking was performed under two regimens, one using 35 mL puff volume, 2 s puff and 60 s puff interval (indicated as 35/2/60) [similar to that used in other studies on cigarette smoke (42)] and the other using 60 mL puff volume, 2 s puff and 30 s puff interval (indicated as 60/2/30). The cigarettes did not have the ventilation blocked. Then the smoking machine was tuned for ISO conditions (indicated as ISO) (43,44). Particulate phase smoke from 10 cigarettes for each sample was collected on a 92 mm Cambridge pad. For 2R4F and 3R4F Kentucky reference cigarettes, two additional smoking regimens were applied. The first regimen known as Massachusetts Department of Public Health puffing regimen (indicated as MDPH) consists of 45 mL puff, 2 s puff duration once every 30 s, and 50% blockage of filter ventilation holes (45). The second regimen known as Health Canada Intensive (indicated as HCI) consisting of 55 mL puff, 2 s puff duration once every 30 s, and 100% vent blockage of filter ventilation holes (46). Only acrylamide in the particulate phase was analyzed in this study, since only a very small percentage of acrylamide is present in vapor phase smoke (8). Various smoking regimens recommended for sampling cigarette smoke were developed for mimicking as well as possible the human smoking (47). However, for analytical purposes, these regimens are just a sampling protocol. Secondary effects related to different smoking regimens, such as reentry of a minute amount of sidestream smoke through filter ventilation holes when these are not blocked are not excluded, but such effects are an intrinsic part of the specific sampling protocol (48).

Water was found to be a very good solvent for tobacco type samples. However, methanol is a better solvent for particulate matter of tobacco smoke. For this reason, the extraction was performed involving a difference for tobacco (or tobacco containing) samples, and for smoke pads. The tobacco type sample (1.0 g in a 125 mL or 250 mL flask) was treated first with 10 mL water and allowed to soak for about 10 min. A volume of 10 mL methanol was then added and 40 µL of a solution of internal standard. The internal standard consisted of 20 µg/mL $^2H_3$-acrylamide in water. The final solution contained 40 ng/mL $^2H_3$-acrylamide. The extraction continued with mechanical agitation on a wrist action shaker for 30 min.

For the smoke pads that contained the particulate phase smoke from 10 cigarettes, the pads were initially soaked in 10 mL methanol. After this step, 10 mL water was added to the pads, and 40 µL of an internal standard solution that consisted of 200 µg/mL $^2H_3$-acrylamide in water. The final solution contained 400 ng/mL $^2H_3$-acrylamide. The extraction continued with mechanical agitation on a wrist action shaker for 30 min.

Methanol Optima (Fisher-Scientific, Suwanee, GA) and Chromosolv Plus for HPLC water (Sigma-Aldrich, St. Louis, MO) were used for the extraction. The deuterated acrylamide was from CDN Isotopes (Pointe-Claire, Canada). Acrylamide was obtained from Sigma-Aldrich.

About 4–5 mL solution were taken from each extract for further processing and were filtered through a 0.45-µm pore size PVDF filter (Whatman Inc., Florham Park, NJ). Sample cleanup was performed using C18 SPE cartridges processed with a SPE vacuum manifold. For this purpose, 2 mL of solution (50:50 methanol–water containing the sample and the internal standard) were passed through the SPE cartridge within about 1 min. The cartridges were previously conditioned with 2 mL water then with 2 mL acetone and then with 2 mL methanol, followed by air drying under mild vacuum for 10 min. The drying is necessary for not modifying the sample concentration by dilution with the remaining solvent in the cartridge. The cartridges were Bond Elute C18, 500 mg solid phase in 3 cc format (Varian, Walnut Creek, CA). The collected sample was used as is for the analysis for the case of tobacco samples. The extracted smoke samples were further diluted ten times with water. For this purpose, 0.2 mL sample was diluted with 1.8 mL water. Small variation in sample concentration during sample cleanup was not relevant for final analysis since the results were calculated based on the level of the internal standard.

Sample analysis

The clean sample solutions were analyzed using a LC–MS–MS technique. The HPLC separation was performed on two Gemini-NX 5u C18 150 × 2 mm columns with a guard cartridge C18 TWIN (Phenomenex, Torrance CA 90501, USA) in series, in isocratic mode using a solvent that contained 5% methanol in water and 0.1 % formic acid. The HPLC was a 1100 HPLC system (Agilent, Wilmington, DE) consisting of a degasser, a binary pump and a WPALS autosampler. The injection volume was 2 µL/min and the columns were kept at room temperature. The retention time for $^2H_3$-acrylamide was 3.65 min and for acrylamide was 3.68 min. The total run time for the chromatogram was 5 min.

During experimenting with different elution conditions, it was found that a higher level of methanol in the LC effluent decreases the peak intensity during detection. The isocratic elution selected in this study has the advantage that uses a constant, low percentage of methanol. This choice did not affect the column performance, but still may pose the risk that some components from the sample are not eluted. As a precaution, after every 100 samples the chromatographic column was flushed for about 10 min with 90% methanol in water, without sending the effluent to the MS system, followed by reconditioning the column with the mobile phase 5% methanol in water and 0.1% formic acid for about 10 min.

The measurement of acrylamide was done using a Micromass Quattro Ultima system (Waters, Milford, MA) with an atmospheric pressure ionization electrospray (Z Spray type) working in positive ion mode MRM (multiple reaction monitoring). The conditions involved a capillary potential of 3 kV, source temper-

ature of 120°C, dissolution temperature of 375°C, dissolvation gas of 764 L/h, cone gas 43 L/h, and collision energy 12 V. The parent ion for acrylamide was *m/z* = 72 and the daughter ion was *m/z* = 55, and for $^2H_3$-acrylamide the parent was *m/z* = 75 and the daughter ion m/z = 58. The confirmation daughter ions *m/z* = 44 for acrylamide and *m/z* = 47 for $^2H_3$-acrylamide were present in the mass spectra, but were not used in the quantitation. The extracted ion chromatogram for the peak corresponding to acrylamide in a tobacco sample from a 3R4F Kentucky reference cigarette obtained under the previously described conditions is shown in Figure 1, and the chromatogram for the smoke sample from a 3R4F Kentucky reference cigarette is shown in Figure 2 (acrylamide peak eluting at 3.68 min). Peak area is also indicated on the chromatograms. The smoking was performed using (35/2/60) protocol. The traces were smoothed using a 5 point smoothing procedure.

The quantitation was done using the ratio of peak area of the analyte vs. that of the internal standard using the following formula:

$$C_{\text{acrylamide}}\ (\text{ng/mL}) = \frac{\text{Peak area analyte}}{\text{Peak area IS}} C_{IS} \times F \qquad \text{Eq. 1}$$

where C_{IS} is the concentration of the internal standard (40 ng/mL) and *F* is a response factor for nondeuterated/deuterated acrylamide. This factor was determined by generating calibration curves (compound level) vs. (measured area counts) in the range 5 ng/mL to 320 ng/mL of standard solutions for both acrylamide and $^2H_3$-acrylamide using seven concentration levels. The slopes ratio of calibration lines for acrylamide and $^2H_3$-acrylamide gave the response factor nondeuterated/deuterated acrylamide *F* = 1.1397.

Validation of the LC–MS–MS method

The LC–MS–MS method used for the analysis of acrylamide in tobacco, alternative tobacco products, and cigarette smoke was validated following a typical set of steps (49). The selectivity of the LC–MS–MS procedure was in part assured by the selectivity of the MS–MS detection, with specified parent ions and specified daughter ions in MRM mode. However, the HPLC separation needed to be verified for a good separation. For this purpose, one and two Gemini-NX columns were used, the retention time extending with two columns compared to one column (by a factor of about 2.0, as expected). Also some variation in the content of methanol in the mobile phase was used, in the range of 3–15% methanol. An increase in the methanol content led to a reduction of the retention time. Also, it was noticed that the increase in the methanol content had an effect on the method sensitivity, peak areas for the same acrylamide concentration decreasing when the methanol content was higher. In addition to that, separation on a Synergy 4u Hydro-RP 80 A column, 250 × 4.6 mm from Phenomenex was performed. The separation conditions were the same as on the Gemini-NX columns, using the same solvent and 0.3 mL/min flow rate. These alternative separations were studied only for one tobacco sample (tobacco from 3R4F cigarette blend) and one smoke sample (3R4F cigarette smoke generated in 35/2/60 conditions). In all experiments, the ratio of the areas for the acrylamide and for the internal standard remained the same, indicating that no interference is present in the analyte peak. The confirmation daughter ions were present in the mass spectra of the peaks for the analytes, although not used for quantitation.

The precision of the LC–MS–MS method was found to be very good. The relative standard deviation RSD% for the analyzed tobacco samples varied between 0.8% and 6.9%, the one for alternative tobacco products varied between 0.7% and 5.2 %, and for smoke varied between 0.3% and 2.7%.

The linear range for the LC–MS–MS method was verified between 5 ng/mL and 320 ng/mL, although higher concentrations may still give a linear response (R^2 higher than 0.998 for both acrylamide and $^2H_3$-acrylamide). Since the quantitation was done using a ratio of peak areas (by formula 1), the importance of linearity of peak area dependence vs. concentration is diminished.

The values for the limit of detection (LOD) and for the limit of quantitation (LOQ) for the LC–MS–MS method were estimated by measuring ten times the 5 ng/mL sample followed by the calculation of the standard deviation (SD), and taking LOD = 3·SD and LOQ = 10·SD. The results were LOD = 0.6 ng/mL and LOQ = 2.1 ng/mL acrylamide in solution. For the tobacco samples, since 1 g material was extracted with 20 mL solution, these

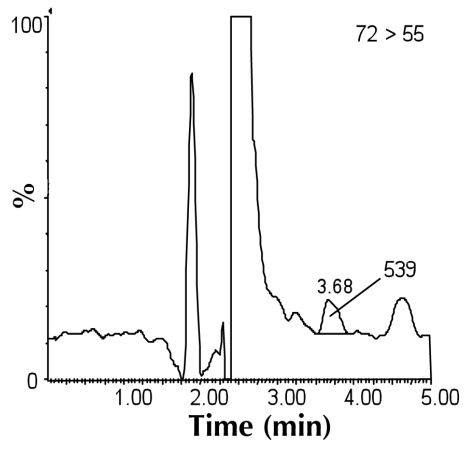

Figure 1. Trace for acrylamide (ion *m/z* = 55) obtained using LC–MS–MS in MRM mode for 3R4F tobacco (peak of acrylamide at 3.68 min).

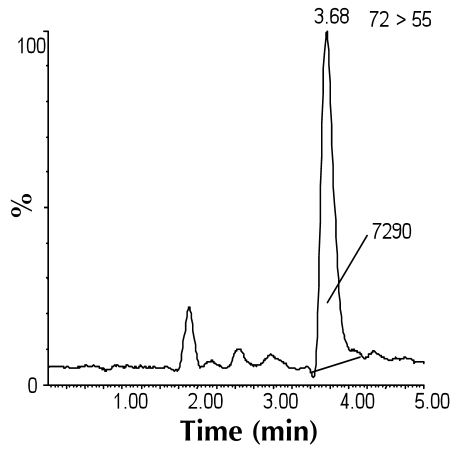

Figure 2. Trace for acrylamide (ion *m/z* = 55) obtained using LC–MS–MS in MRM mode for smoke from a 3R4F cigarette (35/2/60 smoking).

values indicate that at least 12 ng/g acrylamide can be detected in the sample, and at least 42 ng/g acrylamide can be quantitated. These LOD and LOQ values are below the measured levels of acrylamide in tobacco samples. For 10 cigarettes which were smoked per pad and extracted with 20 mL solution followed by ten times dilution, at least 12 ng acrylamide can be detected per cigarette, and at least 42 ng can be quantitated. These limits are much lower than the acrylamide levels detected in cigarette smoke (see Results and Discussion section).

The recovery study started with the evaluation of acrylamide loss when a solution in water/methanol containing 40 ng/mL was passed through the C18 SPE cartridge (for cleanup). The methanol content in the solution was varied between 20% and 80%. The acrylamide concentration in the final solution was measured using the calibration curve with the formula:

$$C \text{ (ng/mL)} = 5.2835 \text{ E-3} \cdot \textit{Peak Area} \text{ with } R^2 = 0.99848 \quad \text{Eq. 2}$$

The use of formula 1 for acrylamide analysis was not considered useful for recovery evaluation, since any loss of the analyte could be compensated by the loss of internal standard. The results for the recovery study are given in Table I. As seen from Table I, the recovery values for acrylamide through the SPE cartridge are above 90% for methanol at levels higher than 20%. A slightly lower recovery than 100% for all samples, was probably due to a slight dilution of the samples with the remaining solvent after cartridge conditioning. This part of the study showed that a 50 % methanol solution in water does not affect the level of acrylamide following SPE cleanup.

Further recovery efficiency for the sample preparation procedure for the LC–MS–MS technique was verified only for $^2H_3$-acrylamide. This did not require any additional experiment since it was possible to use the available data for tobacco or smoke analysis and calculate the level for $^2H_3$-acrylamide using the calibration curve with the formula:

$$C \text{ (ng/mL)} = 4.6359 \text{ E-3} \cdot \textit{Peak Area} \text{ with } R^2 = 0.99967 \quad \text{Eq. 3}$$

The results regarding the calculated levels with their relative standard deviation (RSD%), and the recovery % are given in Table II. As seen in Table II, the recovery % for all samples is very good. It can be inferred that similar recovery levels can be obtained for acrylamide.

Repeatability of the LC–MS–MS analysis was verified by generating results with good precision when replicates of the same sample were analyzed within a short period of time (several days). The repeatability was verified by analyzing one tobacco sample (tobacco from 3R4F cigarette) and one smoke sample (from 3R4F cigarette smoked in 35/2/60 conditions) for five times within a period of several weeks. The results are shown in Table III. Repeatability for alternative tobacco products was not verified. The difference in the matrix in which acrylamide is present, did not influence the response for $^2H_3$-acrylamide standard, and it can be inferred that is not influencing the response for acrylamide.

Regarding the method robustness, the LC–MS–MS technique (and the alternative techniques) have no particular weaknesses. No solvent evaporation steps were involved that can lead to acrylamide losses, no partial retention on a solid phase which depends on the solute volumes was used. The SPE cleanup for the LC–MS–MS technique may lead to a slight dilution of the sample if the C18 SPE cartridge is wet when utilized. Since both the analyte and the internal standard are equally diluted, this does not influence the results when the acrylamide level is calculated using formula 1.

Table I. Results of the Recovery Study of 40 ng/mL Acrylamide in a C18 SPE Cartridge for Different Levels of Methanol in the Solution

Methanol conc. (v/v) in water	Recovered acrylamide ng/mL	Recovery %
80%	39.6	99.00
80%	36.8	92.00
50%	38.2	95.50
50%	39.4	98.50
20%	34.9	87.25
20%	37.3	93.25

Experimental conditions for GC–MS alternative measurement

Acrylamide analysis was also performed in this study using some other procedures applied with the purpose of comparing the results obtained by the LC–MS–MS procedure previously described. One such procedure used a GC–MS technique. The procedure was applied only for the analysis of cigarette smoke and was not found appropriate for acrylamide analysis in tobacco or tobacco products. Since water is not a good solvent to be injected directly into a GC column, the sample extraction must be performed for GC–MS analysis using a different solvent. Both methanol and acetone can be used for this purpose. However, comparing the chromatographic peak shape and sensitivity for standards in acetone and in methanol, better results were obtained when acetone was used as a solvent. For this reason, the extraction of smoke pads was performed using 20 mL acetone,

Table II. Recovery of $^2H_3$-acrylamide from Various Analyzed Samples Using the LC–MS–MS Technique

Number of samples	$^2H_3$-Acrylamide added (ng/mL)	Average $^2H_3$-acrylamide recovered ng/mL	RSD% of recovered	Recovery (%)
24	40	38.44	7.95	96.1
26	400	419.9	9.01	105.0

Table III. Repeatability study results for LC–MS–MS

Sample	Number of replicates	Average acrylamide	RSD%
Tobacco from 3R4F cig	5	60.7 ng/g	6.40
Smoke from 3R4F cig (35/2/60)	5	947.4 ng/cig	0.27

which were added to the smoke pads followed by extraction for 30 min on a wrist action shaker. The internal standard of $^2H_3$-µm pore size PVDF filter and analyzed directly by GC–MS. The GC–MS analysis was performed on a 6890 GC/5973 MS instrument (Agilent, Wilmington, DE 19808, USA) working in selected ion monitoring mode (SIM) and equipped with a DB-1701 column 60 m, 0.25 mm i.d., and 1 µm film thickness (J&W Scientific from Agilent). The liner used for the injection port of the instrument was double tapered with no glass wool and deactivated. The GC conditions included initial oven temperature 90°C, initial time 2.0 min, oven temperature first ramp rate 5°C/min, final oven temperature first ramp 180°C, final time first ramp 0 min, oven temperature second ramp rate 20°C/min, oven final temperature 260°C, final time 10 min. Inlet temperature was 260°C, splitless mode, injection volume 2.0 µL, carrier gas helium with constant flow, purge time 1.0 min, flow rate 1.1 mL/min, MSD transfer line 280°C. The MS working parameters included ion source temperature 230°C, EM offset 250 V, and solvent delay 10.0 min. The ions measured in the GC–MS procedure were m/z = 71 for acrylamide and m/z = 74 for $^2H_3$-acrylamide. The confirmation ions for acrylamide m/z = 55 and m/z = 44 and the corresponding ones for $^2H_3$ acrylamide were not used in the quantitation. However, their presence was verified in the acrylamide peak.

Under the conditions previously described, $^2H_3$-acrylamide elutes at 16.38 min and acrylamide elutes at 16.40 min. The extracted ion chromatogram for the smoke sample from a 3R4F Kentucky reference cigarette is shown in Figure 3. Peak area is also indicated on the chromatograms. The smoking was performed using (35/2/60) protocol.

The quantitation for the GC–MS analysis was performed using the ratio of peak area of the analyte vs. that of the internal standard following formula (1). In this case, the calibration was performed in the range 400 ng/mL to 4 µg/mL using four concentration levels. The value for the response factor F was determined by the same procedure as for the LC–MS–MS technique. The slopes ratio of calibration lines for acrylamide and $^2H_3$-acrylamide gave the response factor nondeuterated/deuterated acrylamide F = 0.8678.

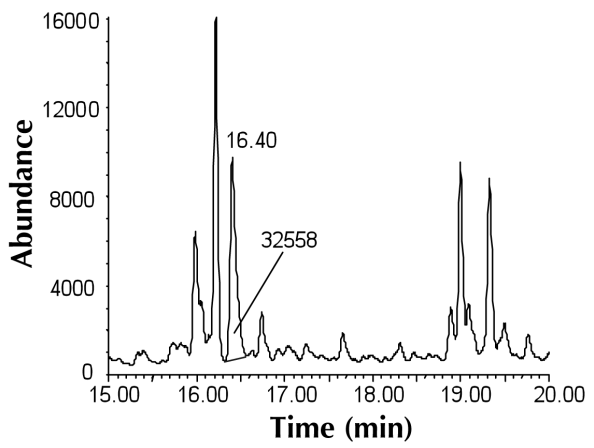

Figure 3. Trace for acrylamide (ion m/z = 71) obtained using GC–MS smoke from a 3R4F cigarette using 35/2/60 smoking protocol (peak of acrylamide at 16.40 min).

Validation of the GC–MS procedure

For the GC–MS alternative method, the separation was performed with several initial oven temperature (60°C, 80°C, 90°C, and 100°C), the other chromatographic conditions being maintained as previously indicated. Only the smoke sample was used to verify the selectivity of the GC separation, and the result of constant ratio for the peak areas of analyte vs. internal standard was achieved.

The precision of the GC–MS method were not verified with a particular experiment. The very good R^2 values for all the calibrations (amount vs. area count in the chromatograms) and the low relative standard deviations for the analyzed samples (see Results and Discussion section) indicated that the GC–MS method have good precision.

The linear range for the GC–MS alternative method was verified for the 400 ng/mL to 4 µg/mL. The dependence equation for acrylamide was:

$$C \text{ (ng/mL)} = 0.01247 \times \textit{Peak Area} \text{ with } R^2 = 0.99711 \qquad \text{Eq. 4}$$

and the dependence equation for $^2H_3$-acrylamide was:

$$C \text{ (ng/mL)} = 0.01437 \times \textit{Peak Area} \text{ with } R^2 = 0.99945 \qquad \text{Eq. 5}$$

The estimation of LOD and LOQ for the GC–MS method, was done using the same procedure as for the LC–MS–MS, but using a 500 ng/mL sample. The result was LOD = 19.5 ng/mL and LOQ = 65 ng/mL acrylamide in solution. The GC–MS method being applied for the analysis of cigarette smoke at these values were considerably lower than the typical analyte level in a smoke extract. This result also showed that tobacco samples cannot be analyzed using the GC–MS technique.

Further recovery efficiency for the sample preparation procedure for the GC–MS technique was verified only for $^2H_3$-acrylamide. This did not require any additional experiment since it was possible to use the available data for smoke analysis and calculate the level for $^2H_3$-acrylamide using formula (5). The results regarding the calculated levels showed a recovery 98.8% with an RSD% of the measurements of 11.22% for six replicates. For the GC–MS procedure, it can be inferred that similar recovery levels can be obtained for acrylamide.

Experimental conditions for LC–MS–MS alternative measurement

The acrylamide was also analyzed by LC–MS–MS utilizing a concentrating step using SPE. Acrylamide is a small molecule and its retention on most stationary phases is very poor. For the retention of acrylamide, the compound had to be present in saline water, since otherwise the analyte retention is only partial. For this reason, the sample is extracted with water (1.0 g sample in 20 mL water). To 10 mL filtered solution containing the sample of acrylamide, 0.2 g LiCl was added to generate a solution 2% in LiCl. A volume of 8 mL from this solution was passed through a graphitized carbon SPE cartridge (Enviro Clean Cucarb) containing 500 mg solid phase in a 6 mL format obtained from UCT (2731 Bartram road, Bristol, PA). The cartridges were previously conditioned with 2 mL water, followed by 2 mL acetone and 2 mL methanol, and dried under mild vacuum

for 10 min. After sample retention, the cartridge was dried for 10 min with air under mild vacuum and then the acrylamide was eluted with 1.0 mL of methanol. Less than 1 mL eluate was collected and further analyzed by the procedure previously described using a LC–MS–MS technique. This procedure is able to increase the concentration of the final solution to slightly more than 8 times. Any additional methanol in the extraction solution is impeding the complete retention of acrylamide on the SPE cartridge. This alternative procedure was applied only to tobacco samples. The trace for acrylamide obtained using LC–MS–MS in MRM mode of a 250 pg/mL acrylamide standard (in the presence of $^2H_3$-acrylamide at the same level) generated using 8 mL solution and graphitized carbon SPE concentration is shown in Figure 4.

The back calculation of the level of acrylamide for the 0.25 ng/mL sample using formula 1 (with the concentration of $^2H_3$-acrylamide 0.250 ng/mL) led to an expected 0.260 ng/mL level. However, the calculation of the level of acrylamide using formula (2) corresponded to 0.43 ng/mL in the recovered solution. This higher than expected calculated level of acrylamide by formula (2) can be explained by the fact that less than 1 mL eluting methanol was collected from the SPE cartridge. Part of the methanol remained absorbed in the cartridge but the fraction of the eluting volume of the solvent was sufficient for extracting all the acrylamide from the solid phase. In this way, a higher concentration of acrylamide (and $^2H_3$-acrylamide) was present in the eluate.

A rough estimation of the LOD and LOQ procedure using the graphitized carbon SPE concentration, was performed by measuring four times the 250 pg/mL standard followed by the SD calculation. The results indicated LOD = 91 pg/mL and LOQ = 302 pg/mL.

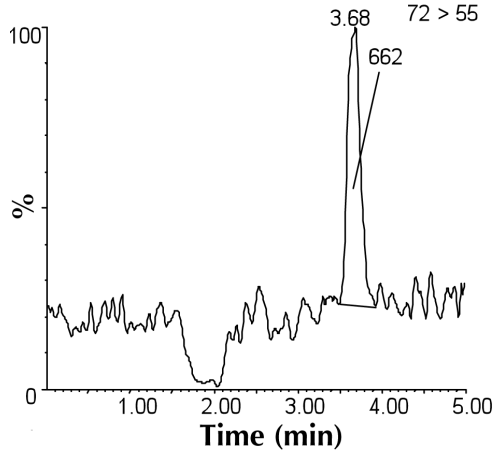

Figure 4. Trace for acrylamide (ion *m/z* = 55) obtained using LC–MS–MS in MRM mode from a 250 pg/mL acrylamide + 250 pg/mL 2H3-acrylamide standard after graphitized carbon SPE concentration of 8 mL solution.

Table IV. Levels of Acrylamide in Several Tobacco Samples Obtained by LC–MS–MS Technique (Triplicate Samples).

No.	Sample description	Acrylamide in ng/g	RSD%
1	Flue-cured leaf top stalk	49.7	4.35
2	Domestic burley leaf	86.6	6.91
3	Domestic flue-cured leaf	51.8	5.42
4	Flue cured green leaf (dry)	N.D.*	–
5	Mixed stem	45.8	0.78
6	Tobacco from Cigarette A	88.5	3.91
7	Tobacco from Cigarette B	50.3	4.17
8	Tobacco from Cigarette C	119.6	6.45
9	Tobacco from Cigarette D	84.4	5.65
10	Tobacco from 3R4F cigarette†	60.7	6.40
11	Tobacco from sticks Mellow	129.7	5.16
12	Tobacco from snus Robust	96.0	1.68

* N.D. = Not detected
† five replicates

Results and Discussion

Twelve tobacco samples were analyzed by the LC–MS–MS procedure described in this study (with no concentration step of the extract). These samples are described in Table IV which gives the levels of acrylamide as an average of triplicate samples and the corresponding RSD%. The calculation was performed using formula (1). The samples were analyzed without drying and some moisture can be assumed to be present (tobacco from cigarettes may have around 10% moisture).

Three of the tobacco samples were also analyzed (in triplicate) using the SPE concentration procedure. The results are given in Table V. This table also gives the difference compared to the direct analysis procedure. The results obtained by the two procedures are in good agreement.

Several alternative tobacco products were analyzed using the LC–MS–MS procedure. The results are given in Table VI (for triplicate samples). As seen from Table VI, except for the sticks

Table V. Levels of Acrylamide in Several Tobacco Samples Analyzed Using an 8× SPE Sample Concentration and LC–MS–MS Technique (Triplicate Samples)

No	Sample description	Acrylamide (ng/g)	RSD %	Difference from direct LC–MS–MS method
1	Domestic burley leaf	96.2	7.77	+ 11.1%
2	Domestic flue-cured leaf	58.8	8.52	+ 13.5%
3	Flue cured green leaf (dry)	N.D.	–	–

Table VI. Levels of Acrylamide in Several Alternative Tobacco Products by the LC–MS–MS Technique (Triplicate Samples)

No	Sample description	Acrylamide in ng/g	RSD%
1	Camel snus Frost	82.7	1.09
2	Camel snus Robust	69.9	1.20
3	Wet snuff Grizzly Natural	86.5	0.71
4	Wet snuff Grizzly Wintergreen	179.9	1.53
5	Strips Fresh	125.6	1.46
6	Sticks Mellow	366.7	1.12

Mellow, the level of acrylamide in the alternative tobacco products can be considered to be in the same range as for tobacco leaf. Only the sticks sowed a slightly higher level of acrylamide. The level of acrylamide in alternative tobacco products (except for sticks) being similar to that in tobacco, is an indication that this compound is not formed during the manufacturing of these products. For the case of sticks, some acrylamide is likely to be formed during processing.

Table VII. The Main Characteristics of the Cigarettes Analyzed in this Study

Descriptor	Cig A	Cig B	Cig C	Cig D	Cig E	Cig F	Cig G
FTC 'tar' (mg/cig)	10.6	14.9	10.6	10.5	16.2	5.0	10.7
Tobacco weight (g/cig)	0.657	0.668	0.609	0.654	0.753	0.657	0.610
Cigarette length (mm)	83	80	83	83	83	83	83
Filter length (mm)	27	24	32	27	21	27	32
Filter ventilation (%)	32	0	25	32	23	54	25
Blend type	US	US	US	US	US	US	US
Nicotine (mg/cig)	0.92	1.09	0.82	0.91	1.31	0.5	0.81
CO (mg/cig)	10.7	15.3	11.2	10.7	13.9	7.4	11.2
Smoke menthol (mg/cig)	N.D.*	2.87	N.D.	N.D.	N.D.	N.D.	N.D.

* Not detected.

Table VIII. Levels of Acrylamide in Several Cigarette Smoke Samples Obtained by LC–MS–MS Technique (Duplicate Samples)

No.	Sample description	Smoking conditions	WTPM mg/cig	RSD % WTPM	Acrylamide (ng/cig)	RSD% acrylamide
1	2R4F	35/2/60	11.18	4.94	802.8	1.75
2	2R4F	ISO	10.65	2.12	790.7	2.21
3	2R4F	MDPH	24.68	3.31	2099.3	2.43
4	2R4F	HCI	32.17	2.31	2887.3	1.19
5	3R4F*	35/2/60	11.18	1.08	967.4	0.27
6	3R4F	60/2/30	33.58	1.16	3184.9	2.7
7	3R4F	ISO	10.60	1.17	884.2	2.34
8	3R4F	MDPH	24.77	1.56	2111.6	2.48
9	3R4F	HCI	32.24	3.01	2801.1	3.44
10	Cigarette A	35/2/60	11.03	0.06	1281.9	1.27
11	Cigarette A	60/2/30	34.38	1.71	4341.9	0.14
12	Cigarette A	ISO	10.52	1.11	1173.1	1.14
13	Cigarette B	35/2/60	23.93	1.98	2976.5	1.81
14	Cigarette B	60/2/30	60.05	0.25	8019	1.03
15	Cigarette B	ISO	22.69	2.21	2728.2	1.51
16	Cigarette C	35/2/60	12.41	0.97	1175.9	1.46
17	Cigarette C	60/2/30	36.65	1.12	4168.8	0.14
18	Cigarette C	ISO	11.84	1.02	1053.7	1.24
19	Cigarette D	35/2/60	13.05	1.08	1248.2	1.8
20	Cigarette D	ISO	12.38	1.11	1127.1	2.01
21	Cigarette E	35/2/60	20.76	1.1	2069	1.04
22	Cigarette E	ISO	19.71	1.98	2023.1	0.05
23	Cigarette F	35/2/60	6.64	1.44	512.7	1.86
24	Cigarette F	ISO	6.30	2.22	497.1	0.71
25	Cigarette G	35/2/60	12.29	1.41	1126.6	1.55
26	Cigarette G	ISO	11.65	1.97	1027.2	1.34

* Five replicates.

Smoke from seven common commercial cigarettes on the US market, 2R4F and 3R4F Kentucky reference cigarettes was also analyzed in this study for acrylamide content. The cigarettes were smoked using different smoking protocols as described in the experimental section. The description of main characteristics for the commercial cigarettes used in the study is given in Table VIII. The level of wet total particulate matter (WTPM) and the level of acrylamide in the smoke are given in Table IX as an average of two samples. The RSD% for the duplicates are also listed in Table IX.

The alternative GC–MS analytical technique for the measurement of acrylamide in smoke was applied only to 2R4F and 3R4F cigarettes. The smoking was performed only using the (35/2/60) protocol. The samples were analyzed in triplicate. The results are given in Table IX The results obtained using the GC–MS are in good agreement with those obtained using the LC–MS–MS technique. Single factor ANOVA evaluation of the means showed that they are not different for the 2R4F cigarette with a P value of 0.716, and not different for 3R4F cigarette with a P value of 0.056. The agreement of the results generated by LC–MS–MS and by GC–MS, on the same sample indicates that, very likely, the two procedures are accurate.

The levels of acrylamide in cigarette smoke is significantly higher than that in tobacco (and alternative tobacco products). This result indicates that the main proportion of acrylamide in smoke is pyrosynthesized. A graph showing the correlation between the level of acrylamide with WTPM is given in Figure 5. The good correlation (R^2 = 0.975) between acrylamide and WTPM is an indication that probably more than one acrylamide precursor is present in tobacco.

The results for the measured level of acrylamide in 2R4F cigarette is not in good agreement with a previously reported result (8) that indicated a level of 2.31 µg/cig acrylamide with (35/2/60) smoking protocol. However, the accuracy of the measurements in the present study has been verified by using two completely different methods, and by extensive efforts to prove that no interference or losses take place in the LC–MS–MS method. The higher level of acrylamide previously reported in the smoke of 2R4F cigarette may be caused by a potential coelution of acrylamide with another compound able to generate a fragment with m/z = 71, ion which is very common in mass spectra. The finding that the correct level of acrylamide in the smoke of 2R4F and 3R4F Kentucky reference cigarettes is

Table IX. Levels of Acrylamide in 2R4F and 3R4F Cigarette Smoke Obtained by GC–MS Technique

No.	Sample description	WTPM mg/cig	RSD% WTPM	Acrylamide (ng/cig)	RSD% acrylamide
1	2R4F	11.13	5.03	808.3	3.43
2	3R4F	11.18	1.06	987.1	1.97

* Smoking performed using 35/2/60 protocol for triplicate samples.

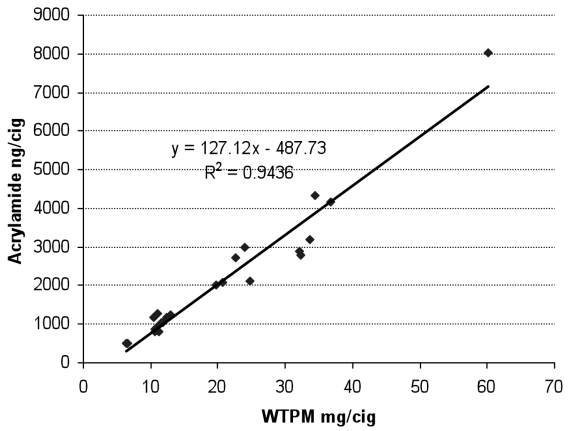

Figure 5. Variation in acrylamide level as a function of the WTPM from cigarette smoke.

around 1 µg/cig (for 35/2/60 smoking) is important since only these levels should be correlated with the results from studies on biomarkers for acrylamide exposure in humans.

Conclusions

A new LC–MS–MS method for the analysis of acrylamide in tobacco, alternative tobacco products, and cigarette smoke was developed and evaluated. Two alternative procedures for acrylamide analysis were also developed with the purpose of comparing with the results obtained by the LC–MS–MS procedure. A thorough evaluation was performed for typical characteristics of an analytical method such as selectivity, precision and accuracy, range of acrylamide levels where it is applicable, robustness, etc. The method was found perfectly fit for the analyses of acrylamide in the tobacco, alternative tobacco products, and smoke matrix. Several tobacco samples, six alternative tobacco products, and smoke from several cigarettes including commercial cigarettes from US market, 2R4F, and 3R4F Kentucky reference cigarettes were analyzed.

References

1. E. Tareke, P. Rydberh, P. Karlsson, S. Eriksson, and M. Törnqvist. Acrylamide: a cooking carcinogen. *Chem. Res. Toxicol.* **13:** 517–552 (2000).
2. Swedish National Food Admin. Information about acrylamide in food, April 24, 2002, http://www.slv.se
3. H. Lingnert, S. Grivas, M. Jägerstad, K. Skog, M. Törnqvist, and P. Aman. Acrylamide in food: mechanism of formation and influencing factors during heating of food. *Scandinav. J. Nutrit.* **46:** 159–172 (2002).
4. U.S. National Toxicology Program (NTP), 10th Report on carcinogens.
5. M. Urban, D. Kavvadias, K. Riedel, G. Scherer, and A.R. Tricker. Urinary mercapturic acids and a hemoglobin adduct for the dosimetry of acrylamide exposure in smokers and nonsmokers. *Inhalation Toxicol.* **18:** 831–839 (2006).
6. H. W. Vesper, J. T. Bernert, M. Ospina, T. Meyers, L. Ingham, A. Smith, and G.L. Myers. Assessment of the relation between biomarkers for smoking and biomarkers for acrylamide exposure in humans. *Cancer Epidemiol. Biomarkers Prevention* **16:** 2471–2478 (2007)
7. C.-C. J. Huang, C.-M. Li, C.-F. Wu, S.-P. Jao, and K.-Y. Wu. Analysis of urinary N-acetyl-S(propionamide)-Cysteine as a biomarker for the assessment of acrylamide exposure in smokers. *Environ. Res.* **104:** 346–351 (2007).
8. J. Diekmann, A. Wittig, and R. Stabbert. Chromatographic-mass spectrometric analysis of acrylamide and acetamide in cigarette mainstream smoke after on-column injection. *J. Chromatogr. Sci.* **46:** 659–663 (2008).
9. N. Qian, Q. Zha, and S.C. Moldoveanu. Analysis of acrylamide in mainstream cigarette smoke. 58th Tobacco Science Research Conference, Sept. 2004, Winston-Salem, NC, Paper #45.
10. Extraction and cleanup of acrylamide in complex matrices using accelerated solvent extraction (ASE) followed by liquid chromatography tandem mass spectrometry (LC–MS–MS). Dionex application note 358.
11. Fast determination of acrylamide in food samples using accelerated solvent extraction (ASE) followed by ion chromatography with UV or MS detection. Dionex application note 409.
12. S. Eriksson and P. Karlsson. Some analytical factors affecting measured levels of acrylamide in food products. In M. Friedman, D. Mottram, eds., Chemistry and Safety of Acrylamide in Food, Springer, 2005, pp. 285–291.
13. S. Eriksson and P. Karlsson. Alternative extraction techniques for analysis of acrylamide in food: Influence of pH and digestive enzymes. *LWT* **39:** 392–398 (2006).
14. B.L. Eberhart II, D.K. Ewald, R.A. Sanders, D.H. Tallmagde, and D.V. Zyzak. Quantitation of acrylamide in food products by liquid chromatography/mass spectrometry. *J. AOAC Internat.* **88:** 1205–1211 (2005).
15. M. Biedermann, S. Biedermann-Brem, A. Noti, K. Grob, P. Egli, and H. Mändli. Two GC-MS methods for the analysis of acrylamide in foodstuffs. *Mitt. Lebensm. Hyg.* **93:** 638–652 (2002).
16. S. Reiediker, R. H. Stadler. Analysis of acrylamide in food by isotope dilution liquid-chromatography coupled with electrospray ionization tandem mass spectrometry. *J. Chromatogr. A* **1020:** 121–130 (2003).
17. Health Canada Pub., http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/chem./index_e.html (Acrylamide)
18. J. Rosen and K.E. Hellenas. Analysis of acrylamide in cooked foods by liquid chromatography tandem mass spectrometry. *The Analyst* **127:** 880–882 (2002).
19. A.F. Lagalante and M.A. Felter. Silylation of acrylamide for analysis by solid-phase microextraction/gas chromatography/ion-trap mass spectyrometry. *J. Agric. Food. Chem.* **52:** 3744–3748 (2004).
20. L. Peng, T. Farkas, L. Loo, A. Dixon, J. Teuscher, and K. Kallury. Rapid and reproducible extraction of acrylamide in French-fries using a single SPE sorbent Strata X-C. Phenomenex application note TN-007.
21. Acrylamide analysis by gas chromatography. Restek application note #59485.
22. Detection and quantitation of acrylamide in foods. FDA draft method, http://www.cfsan.fda.gov/~dms/acrylami.html.
23. L. Castle, M.-J. Campos, and J. Gilbert. Determination of acrylamide monomer in hydroponically grown tomato fruits by capillary gas chromatography-mass spectrometry. *J. Sci. Food. Agric.* **54:** 549–555 (1991).
24. L. Castle. Determination of acrylamide monomer in mushrooms grown on polyacrylamide gel. *J. Agric. Food Chem.* **41:** 1261–1263 (1993).
25. E. Tareke, P. Rydberg, P. Karlsson, S. Eriksson, and M. Törnqvist. Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *J. Agric. Food Chem.* **50:** 4998–5006 (2002).
26. Y. Yuan, F. Chen, G. -H. Zao, J. Lu, H.-X. Zhang, and S.-S. Hu. A comparative study of acrylamide formation induced by microwave and conventional heating methods. *J. Food Sci.* **72:** C212–C216 (2007).

27. S. Nemoto, S. Takatsuki, K. Sasaki, and T. Naitani. Determination of acrlamide in food by GC–MS using 13C-labeled acrylamide as internal standard. *J. Food Hyg. Soc. Japn.* **43:** 371–376 (2002).
28. C. Gertz and S. Klostermann. Analysis of acrylamide and mechanisms of its formation in deep-fried products. *Eur. J. Lipid Sci. Technol.* **104:** 762–771 (2002).
29. J.H. Raymer, C.M. Sparacino, G.R. Velez, S. Padilla, R.C. MacPhail, and K.M. Crofton. Determination of acrylamide in rat serum and sciatic nerve by gas chromatography-electron capture detection. *J. Chromatogr.* **619:** 223–234 (1993).
30. R.A. Sanders, D.V. Zyzak, M. Stojanovic, D.H. Tallmadge, B.L. Eberhart, and D.K. Ewald. An LC/MS acrylamide method and it's use in investigating the role of asparagine. Annual Meeting of AOAC International, September 26, 2002.
31. J.A.G. Roach, D. Andrzejewski, M.L. Gay, D. Nortrup, and S.M. Musser. Rugged LC-MS/MS survey analysis of acrylamide in foods. *J. Agric. Food Chem.* **51:** 7547–7554 (2003).
32. A. Becalski, B.P.-Y. Lau, D. Lewis, and S.W. Seaman. Acrylamide in foods: Occurrence, sources and modeling. *J. Agric. Food Chem.* **51:** 802–808 (2003).
33. T. Delatour, A. Perisset, T. Goldmann, S. Riediker, and R.H. Stadler. Improvement sample preparation to determine acrylamide in difficult matrixes such as chocolate powder, cocoa, and coffee by liquid chromatography tandem mass spectroscopy. *J. Agric. Food Chem.* **52:** 4625–4631 (2004).
34. S. Cavalli, R. Maurer, and F. Hoefler. Fast determination of acrylamide in food samples using accelerated solvent extraction followed by ion chromatography with UV or MS detection. *LC-GC Internat.* 2003, 16–17.
35. L. Castle and J. Eriksson. Analytical methods used to measure acrylamide concentrations in foods. *J. AOAC Int.* **88:** 274–284 (2005).
36. Y. Zhang, G. Zhang, and Y. Zhang. Occurrence and analytical methods of acrylamide in heat-treated foods: Review and recent developments. *J. Chromatogr. A* **1075:** 1–21(2005).
37. D. Taeymans, J. Wood, P. Ashby, I. Blank, A. Studer, R. H. Stadler, P. Gonde, P. Van Eijck, S. Lalljie, H. Lingnert, M. Lindblom, R. Matissek, D. Muller, D. Tallmadge, J. O'Brien, S. Thompson, D. Silvani, T. Whitmore. A review of acrylamide: an industry perspective on research, analysis, formation, and control. *Crit. Rev. Food Sci. Nutr.* **44:** 323–347 (2004).
38. T. Wenzl, M.B. De La Calle, and E. Anklam. Analytical methods for the determination of acrylamide in food products: a review. *Food Addit. Contam.* **20:** 885–902 (2003).
39. J.A.G. Roach, D. Andrzejewski, M.L. Gay, D. Nortrup, S.M. Musser. Rugged LC-MS/MS survey analysis for acrylamide in foods. *J. Agric. Food Chem.* **51:** 7547–7554 (2003).
40. K. Mastovska and S.J. Lehotay. Rapid sample preparation method for LC–MS/MS or GC–MS analysis of acrylamide in various food matrices. *J. Agric. Food Chem.* **54:** 7001–7008 (2006).
41. H.C. Pillsbury, C.C. Bright, K.J. O'Connor, and F.H. Irish. Tar and nicotine in cigarette smoke. *J. AOAC* **52:** 458–462 (1969).
42. M. Kalaitzoglou and C. Samara. Yields of cadmium, tar, nicotine and carbon monoxide in maistraem smoke of Greek cigarettes; A comparative study. *Beitr. Tabak. Int.* **18:** (1999) 235–244.
43. ISO 3308: Routine analytical cigarette smoking machine- definitions and standard conditions; Reference number ISO 3308:1991 (E) International Organization for Standards, Geneva, Switzerland, 1991.
44. ISO 4387: Cigarette –Determination of total and nicotine free dry particulate matter using a routine analytical smoking machine; Reference number ISO 4387:1991 (E) International Organization for Standards, Geneva, Switzerland, 1991.
45. G.N. Connoly and H. Saxner. Memorandum; The Commonwealth of Massachusetts, Exec. Office of Health and Human Services, 19 August 1997.
46. Canada Government Tobacco Act: Tobacco Reporting Regulations, SQR/2000-273. registration June 26, 2000. Schedule 2: Official method for collecting emission data on mainstream smoke.
47. F.K. St.Charles, A.A. Kabbani, and M.W. Ogden. Smoking behaviour: How close to the tipping do consumers actually smoke? *Beitr. Tabak. Int.* **21:** (2005) 435–440.
48. M.E. Counts, M.J. Morton, S.W. Laffoon, R.H. Cox, and P.J. Lipowicz. Smoke composition and predicting relationships for international commercial cigarettes smoked with three machine smoking conditions. *Regulatory Toxicol. Pharmacol.* **41:** (2005) 185–227.
49. S.C. Moldoveanu and V. David. Sample Preparation in Chromatography. Elsevier, Amsterdam 2002.

Manuscript received October 12, 2009;
revision received January 14, 2010.