Gas Chromatographic–Mass Spectrometric Analysis of Acrylamide and Acetamide in Cigarette Mainstream Smoke after On-Column Injection

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Abstract

A method is described for the simultaneous determination of two short-chained amides, acrylamide and acetamide (classified by the International Agency for Research on Cancer as probable and possible human carcinogens, respectively), in total particulate matter using gas chromatography-on-column injection and mass spectrometric detection. Sample preparation is kept to a minimum, and the proposed analytical procedure proves to be fast, sensitive, and precise. Validation studies show good linearity with a regression coefficient of r^2 =1.000 for both compounds. Quantitation limits are 32 ng/mL for acrylamide and 70 ng/mL for acetamide. In the particulate phase of mainstream smoke from the University of Kentucky Reference Cigarette 2R4F, 2.3 µg/cig acrylamide and 4.7 µg/cig acetamide are found; no acetamide and only .0074 µg/cig acrylamide is found in the gas phase. Possible mechanisms of formation in cigarette smoke are discussed.

Introduction

The International Agency for Research on Cancer (IARC) currently classifies acrylamide (2-propenamide) and acetamide (acetic acid amide), two short-chained primary amides that are reported to be present in cigarette smoke, as probably carcinogenic to humans and possibly carcinogenic to humans, respectively (1).

In general, amides are derivatives of organic acids, and acrylamide and acetamide are used as chemical intermediates (2,3). They are solid at room temperature and soluble in water and in polar organic solvents.

The toxicological properties of acrylamide are well-studied and reviewed (2,4,5). Acrylamide and its carcinogenic metabolite glycidamide (an epoxide) form covalent adducts with hemoglobin in rats (6) and humans (7). Whereas acrylamide has been reported as not genotoxic and as marginally genotoxic (8), depending on the genotox assay, glycidamide has been reported to cause DNA damage (9). In addition, acrylamide induces gene mutations and chromosomal aberrations (3), and has neurotoxic effects (10). Acrylamide was the focus of much attention in toxicological food chemistry after publications from Tareke et al. (11) and Rosèn and Hellenäs (12). Tareke et al. (11) postulated that a major source of acrylamide comes from cooking food, and Rosèn and Hellenäs (12) demonstrated the presence of acrylamide in potato chips, crispbread, biscuits, and cereals. Dybing et al. (5) gave a detailed overview of acrylamide levels in food. The main categories of food appear to be fried potato products, breakfast cereals, baked goods, brewed coffee, and breads. In studies conducted in Australia, Europe, and the United States, the mean total intake per day ranged from 0.1 to 1.4 µg/kg body weight (5). Understanding the formation and reduction of acrylamide in foodstuffs is a major concern in the food industry and has been reviewed by Taeymans et al. (13). The wide variations observed in levels of acrylamide in different food categories, as well as in different brands of the same food category, appear to result not only from the amounts of precursors, but also from variations in processing conditions. No acrylamide has been reported in unheated or boiled food (9). It is generally agreed that acrylamide is formed by the Maillard reaction from the condensation of asparagines with reducing sugars (e.g., glucose, fructose) at temperatures above 120ºC (9). The reaction of acrylic acid with ammonia can generate acrylamide (14,15). Yasuhara et al. (16) showed that acrolein can form acrylamide in the presence of ammonia under certain model conditions. Industrial baking trials indicate that acrylamide formation is increased when ammonium carbonate is used as a baking aid (13). Other possible intake sources of acrylamide for humans are cigarette smoking and snuff tobacco consumption. This was shown by Schumacher et al. (17), who found acrylamide in the water-soluble portion of total particulate matter, and White et al. (18) and deBethizy et al. (19), who reported acrylamide yields for a 1R4F Kentucky Reference Cigarette of 1.38 µg/cig and 1.1 µg/cig, respectively, using the same analytical method. Pérez and Ostermann-Golkar (20) found up to 34

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nmol/g acrylamide in water-extracted Swedish snuff portion bags. Another interesting publication from Bergmark (7) investigated the level of acrylamide adducts in blood samples from smokers and non-smokers working with polyacrylamide gels for electrophoresis. He found twice the levels of hemoglobin adducts in blood samples from smokers compared with non-smokers. A high background of acrylamide adducts in the non-smoking control group was unexpected and was not explained by the authors. The indirect explanation for these background levels was uncovered a few years later with the publications on acrylamide in food. In a recent publication from Urban et al. (21), the authors concluded from their investigations on mercapturic acids of acrylamide as biomarkers that smoking is a major source of acrylamide exposure. After Rosèn and Hellenäs (12) published their results on the acrylamide content in food, the food industry and other institutions made large efforts to investigate the precursors and mechanisms of acrylamide formation and ways to prevent acrylamide formation during the production process. When these groups began to analyze acrylamide in food, several methods based on liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (MS) became available in the literature and were reviewed (22,13,23). From the GC–MS-based methods, the bromination of acrylamide to produce 2,3-dibromopropionamide is very common, thereby increasing the volatility of the acrylamide and the selectivity of the GC–MS procedure. Silylation of the amide resulting in *N,O-bis*(trimethylsilyl) acrylamide has also been described (24). A disadvantage of derivatization is the time-consuming clean-up step of the derivative. In the GC–MS methods listed by Wenzl et al. (22), nearly all extracts from food samples were cleaned-up with solid-phase extraction procedures or liquid–liquid extraction. GC–MS determinations without time-consuming derivatization steps are not widely distributed. In the reviews on analytical methods for the determination of acrylamide from Wenzl et al. (22) and Zhang et al. (25), only a few authors have reported using a GC on-column injection technique, even though split/splitless injection may lead to the formation of artifacts during vaporization in the injection port. This may be the cause for an overestimation of acrylamide in foodstuffs, compared with the approach using derivatization and GC–MS analysis or LC–MS (23, 26). Another drawback of GC–MS analysis of acrylamide in foodstuffs without derivatization may be the lack of characteristic ions (26). Still, Dybing (5) compared inter-laboratory studies and concluded that between-sample variability is by far the largest source of variability, including the comparison of LC–MS and GC–MS methods.

The toxicological properties of acetamide have been reviewed by IARC (3) and Kennedy (27). Human exposure data for acetamide are scarce. In general, acetamide intake could occur during production or further use as a chemical intermediate. Acetamide may be released into the environment in wastewater during its production and use. Acetamide has been identified as a minor metabolite of paracetamol in man (28). Another route of acetamide intake is cigarette smoking. Acetamide in cigarette smoke was found by Johnson et al. (29), by Schumacher et al. (17), White et al. (18), and deBethizy et al. (19). Whereas Johnson et al. (29) were the first to report acetamide to be present in cigarette mainstream smoke, White et al. (18) and deBethizy et al. (19) were the first to quantitate acetamide in cigarette mainstream smoke: 3.97 µg/cig and 2.2 µg/cig for a 1R4F Kentucky Reference Cigarette.

No data are available on studies relating acetamide to cancer in humans, but acetamide was tested for carcinogenicity in rats and mice (3). Acetamide is not mutagenic, either with or without metabolic activation, in the *Salmonella typhimurium* mutation assay (Ames Assay) (3). Acetohydroxamic acid, a metabolite of acetamide, showed genotoxic activity (30) and may be converted into hydroxylamine, which is an ultimate hepatocarcinogen in humans. Therefore, it was suggested that DNA adduct formation by hydroxylamine is involved in acetamide-induced carcinogenesis (31). Methods for the determination of acetamide can be found in an Environmental Protection Agency-sponsored survey on the status of ambient measurement methods for hazardous pollutants; however, the method described is only a "potential" method, published by the Occupational Safety and Health Administration with GC and nitrogen phosphorous detector detection, and only partially validated (32,33). Ramirez et al. (34) determined acetamide with GC and flame ionization detection, but few method details and no validation data were given. Lindström et al. (35) published a GC–MS method for the determination of acetamide as a metabolite of dimethylacetamide in whole blood. No LC–MS method was found in the literature.

There were only two publications found on the analysis of acrylamide and/or acetamide in cigarette mainstream smoke. White et al. (18) published a multidimensional GC method coupled with an MS. However, the technical requirements (column switching system), the instability of column efficiency (the authors recommended routinely removing a short part of the precolumn), the greater possibility of MS leaks by using of two columns connected together, and the high number of cigarettes (100) smoked made this method unsuitable for routine analysis. In addition, the authors reported a drastic shift of the chromatographic characteristics of the analytical column when samples from cigarette smoke were compared with calibration samples. This indicates that the system is not very robust. The second method, published by Johnson et al. (29), is a GC method using a thermal conductivity detector.

The purpose of the present work was to verify the presence of acrylamide and acetamide in cigarette mainstream smoke. Analytical methods for both compounds exist in the literature, but they are not suitable for routine analysis. An LC–MS method may not be feasible for acetamide analysis due to its low molecular weight. Therefore, the focus was on a method using GC–MS but without complex derivatization steps, which may lead to additional, time-consuming sample clean-up steps, and an increase in assay variability. The prevention of possible artifact formation was achieved by using the on-column injection technique. No thermal decomposition, better reproducibility, and no discrimination of nonvolatiles are the major advantages of on-column injection, compared to split-splitless injection. For example, Gertz and Klostermann (36) reported decomposition of an acrylamide standard solution after eight to ten injections in a split–splitless injection port, possibly due to reactive sites in the inlet of the programmable temperature vaporizer injector.

Experimental

Materials and reagents

Acrylamide (99.9%) was purchased from Merck (Darmstadt, Germany), acetamide (99%) was purchased from Sigma-Aldrich (Deisenhofen, Germany), and acrylamide-d_3 and acetamide-d_3 were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and were labeled as 99.3% and 99.1% pure, respectively. Acetone (99.5 %) was purchased from Merck.

The test material was the University of Kentucky Reference Cigarette 2R4F. This reference cigarette is from a series of reference cigarettes developed for research purposes in a joint effort by the National Cancer Institute of the National Institute of Health, the Agriculture Research Service of the United States Department of Agriculture, and the University of Kentucky Tobacco and Health Research Institute. The 2R4F, which is a remake of the 1R4F, is a standard blended reference representative for conventional commercial cigarettes of similar total particulate matter (TPM) yield (37). The design of these cigarettes is 84 mm length, 24.8 mm circumference, and 35 mm butt length. Yields of TPM are 11.6 mg/cig for the 2R4F and 10.8 for the 1R4F, and nicotine yields are 0.85 mg/cig for the 2R4F and 0.8 mg for the 1R4F (38,39). The cigarettes were stored and conditioned according to ISO standard 3402 (40). Stock solutions were prepared in acetone at concentrations of 10 µg/mL for acrylamide and 31.9 µg/mL for acetamide. Internal standard stock solutions in acetone contained 62.3 µg/mL acrylamide-d_3 and 199 µg/mL acetamide-d_3. From these stock solutions, standard solutions were prepared for an internal standard calibration. A stock solution of 2.477 µg/mL acrylamide was prepared as a control sample.

Equipment

An Agilent (Palo Alto, CA) 6890 GC equipped with an Agilent 6890 autosampler was coupled to an Agilent 5973 mass selective detector. The GC was equipped with an Agilent on-column injector and fitted with a 30-m J&W Scientific free fatty acid phase fused silica capillary column with 0.25 mm i.d. and 0.25 µm film thickness. For data analysis, the chromatographic peak areas were determined automatically by the Agilent ChemStation Integrator program in the Agilent Enhanced ChemStation software (version D.00.00.38).

Smoke generation and collection

Mainstream smoke was generated in basic conformity with ISO 3308 (41) on a 20-port rotary smoking machine (Borgwaldt, Hamburg, Germany) with a 35 ± 0.5 mL puff volume and 2.0 ± 0.1 s puff duration every minute. TPM was collected on Cambridge filter pads, which trap cigarette smoke particles with 99.9% efficiency for particles larger than 0.1 µm in diameter. Ten cigarettes were smoked for each sample and five replicate samples were generated.

Sample preparation

Twenty-five microliters of an internal standard solution containing acrylamide-d_3 and acetamide-d_3 were added to the filter pads, which were then extracted with 10 mL acetone, shaken for 1 min, and placed in an ultrasonic bath for 5 min at room temperature. After centrifuging, 500 µL of this extract were taken out and placed into 1-mL GC-autosampler vials. Finally, 0.5 µL of the cigarette smoke extract were injected on-column.

GC–MS analysis

The GC was equipped with an on-column injector, and the inlet pressure was maintained at 48 kPa in constant flow mode, resulting in an initial flow of 1 mL/min helium. The GC oven was started at 40°C, held for 4 min and heated at 5°C/min to 190°C, and then heated at 30°C/min to a final temperature of 250°C (held for 10 min). The total GC run time was 40 min. The MS was operated in electron impact (70eV) mode. The transfer line temperature was 250°C, and MS quadrupole and source heaters were maintained at 120°C and 250°C, respectively. Acrylamide and acrylamide-d_3 were quantitated using the ions at m/z = 71 and m/z = 74, respectively. Acetamide and acetamide-d_3 were quantitated using the ions at m/z = 59 and m/z = 62, respectively.

Results and Discussion

We developed a fast, reliable, and sensitive GC–MS method with on-column injection for the direct determination of acrylamide and acetamide in cigarette mainstream smoke. Acrylamide and acetamide, as well as their internal standards, were detected by using the $[M]^+$ ions (m/z = 71 for acrylamide and m/z = 74 for acrylamide-d_3, and m/z = 59 for acetamide and m/z = 62 for acetamide-d_3) (Figures 1 and 2). Qualifier ions for confirmation could not be used because of matrix effects.

A linear, 14-point calibration curve with a concentration range of 0.025 to 10 µg/mL for acrylamide and a 12-point calibration curve with a concentration range of 0.338 to 32.2 µg/mL for

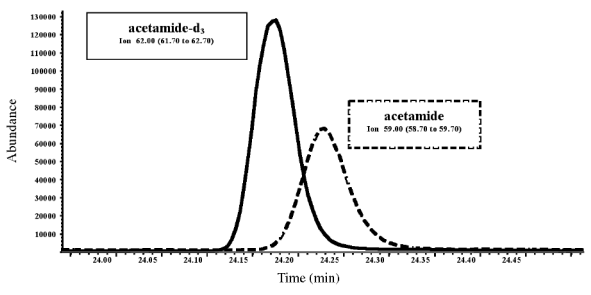

Figure 1. Typical GC–MS-SIM chromatogram of acetamide in TPM from the 2R4F.

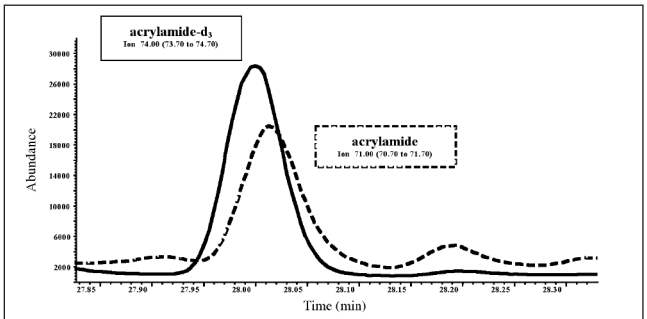

Figure 2. Typical GC–MS-SIM chromatogram of acetamide in TPM from the 2R4F.

acetamide were produced. Correlation coefficients from the calibration curves, limit of quantitation (LOQ), intraday precision, day-to-day precision, and repeatability of injection are summarized in Table I. Within-day precision and day-to-day precision refer to the replication of the whole analytical procedure. The trapping efficiency was shown by the connection of a wash bottle behind the glass fiber filter. The wash bottle was filled with 10 mL acetone and cooled to –78°C with isopropanol/dry ice. One microliter of this solution was injected into the GC–MS. No acetamide was found in the wash bottle, but 74 ng/cig acrylamide was, which is only 3.2% of the yield trapped on the glass fiber filter (N = 5). Back-calculated concentrations of the calibration standards were within 4.7% of the expected value for acrylamide and within 6.0% of the expected value for acetamide. Standard addition experiments were performed to verify selectivity by adding two different concentrations of acrylamide and acetamide to the filter from ten 2R4F cigarettes (approximately 50% and 130% of the value found for 2R4F Reference cigarettes, respectively). The recovery was between 92% and 101% of the expected value for acrylamide, and between 97% and 103% of the expected value for acetamide. Experiments were repeated 5 times.

To investigate the stability of acrylamide and acetamide in TPM on glass fiber filters, one TPM-loaded glass fiber filter was extracted immediately after smoking and a second glass fiber filter was stored for 13 days at –18°C. The difference in acrylamide and acetamide yield was 7% (9.4% on a per mg TPM basis) between the glass fiber filters. Therefore, we recommend a maximum storage time of 14 days. A stock solution stored for 3 months at –78°C showed a loss in concentration of only 2.4%. The average yield of acrylamide in 2R4F cigarettes was 2.31 µg/cig and for acetamide 4.65 µg/cig.

Although formation pathways of acetamide and acrylamide in cigarette smoke were not specifically investigated in this work, there are several possibilities for the formation of these compounds, including the possibility that the formation is similar to what was observed in food chemistry.

The first possibility is the (reversible) reaction of ammonia with acrylic acid and acetic acid ($R\text{-}COOH + NH_3 \leftrightarrows RCONH_2 + H_2O$) which leads to the formation of acrylamide and acetamide. This reaction is described in the context of acrylamide formation in foodstuffs in, for example, Stadler et al. (14). Ammonia, acrylic acid, and acetic acid are all found in mainstream smoke from the Reference Cigarette 2R4F with yields of approximately 11 µg/cig for ammonia (42), approximately 12 µg/cig for acrylic acid, and approximately 53 µg/cig for acetic acid (unpublished results). The second possibility is the formation of acrylamide in the Maillard reaction from the condensation of asparagines with reducing sugars as described for food by Jägerstad and Skog (9). Asparagines and reducing sugars have also been described in tobacco (43,44), and the contribution of reducing sugars of the tobacco blend in the Reference Cigarette 2R4F was found to be 10.7% (39). Literature data on the formation of acetamide in the Maillard reaction could not be found. The third possibility is that under certain model conditions, acrylamide can be formed from acrolein. Acrolein can undergo oxidation to acrylic acid and then react with ammonia (16). Acrolein is an irritant in cigarette smoke (45) and was found at approximately 60 µg/cig in mainstream smoke from the Reference Cigarette 2R4F (42). The contribution of the acetamide formation from acetaldehyde (the yield is approximately 560 µg/cig [42]), which is oxidized to acetic acid, and the further reaction with ammonia may also be possible.

Table I. Method Performance, Expressed as Correlation Coefficient, LOQ, and Within- and Day-to-Day Precision

	Acetamide	*N*	Acrylamide	*N*
Correlation coefficient calibration curve	1.000	–	1.000	–
LOQ as S: N 10: 1	70 ng/mL	–	32 ng/mL	–
Within-day precision (RSD)	2.3%	5	3.1%	5
Repeatability	0.3%	10	1.3%	10
Day-to-day precision	5.8%	3	7.3%	3
Average yield 2R4F	4.65 (µg/cig)	5	2.31 (µg/cig)	5

Concluding Remarks

We have developed a fast, sensitive, and precise GC method with on-column injection and mass spectrometric detection for measuring acrylamide and acetamide in cigarette mainstream smoke. The application to the University of Kentucky Reference Cigarette 2R4F showed that the method is suitable for the evaluation of mainstream smoke for these compounds on a routine basis.

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