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Gas Chromatography-Tandem Mass Spectrometry Method for Selective Detection of 2-Nitropropane in Mainstream Cigarette Smoke *

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SUMMARY

Although 2-nitropropane is a potentially harmful compound present in cigarette smoke, there are few fully-validated, modern methods to quantitate it in mainstream cigarette smoke. We developed an isotope dilution gas chromatography-tandem mass spectrometry (ID-GC-MS/MS) method for the detection of 2-nitropropane in mainstream cigarette smoke. The vapor fraction of mainstream cigarette smoke was collected in inert polyvinyl fluoride gas sampling bags and extracted with hexanes containing isotopically labeled internal standard, then purified and concentrated via solid-phase extraction using a normal phase silica adsorbent and a 100% dichloromethane eluant. This method is sensitive enough to measure vapor phase 2-nitropropane concentrations in the nanogram range, with a 19 ng per cigarette method limit of detection. Product variability estimated from the analysis of 15 cigarette products yielded relative standard deviations ranging from 5.4% to 15.7%, and estimates of precision from two quality control products yielded relative standard deviations of 9.49% and 14.9%. Under the Health Canada Intense smoking regimen, 2-nitropropane in machine-generated mainstream smoke from 15 cigarette products ranged from 98.3 to 363 ng per cigarette.

ZUSAMMENFASSUNG

Obwohl 2-Nitropropan eine potenziell schädliche Verbindung im Zigarettenrauch ist, gibt es nur wenige vollständig validierte, moderne Methoden, um es im Hauptstromrauch zu quantifizieren. Wir entwickelten eine Isotopen-verdünnungs-Gaschromatographie-Tandem-Massen-spektrometrie (ID-GC-MS/MS) zum Nachweis von 2-Nitropropan in Hauptstrom-Zigarettenrauch. Die Dampf-fraktion des Hauptstrom-Zigarettenrauchs wurde in inerten

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DISCLAIMER

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CONFLICT OF INTEREST

None

Polyvinylfluorid-Gasprobenentnahmebeuteln gesammelt und mit Hexan, welches den isotopenmarkierten internen Standard enthielt, extrahiert. Das Extrakt wurde gereinigt und mittels Festphasenextraktion unter Verwendung eines Normalphasen-Siliciumdioxidadsorptionsmittels und 100%-igem Dichlormethan als Elutionsmittel konzentriert. Dieses Verfahren ist empfindlich genug, um die Konzentration von 2-Nitropropan in der Gasphase im Nanogramm-bereich mit einer Nachweisgrenze von 19 ng pro Zigarette zu messen. Die aus der Analyse von 15 Zigarettenprodukten geschätzte Produktvariabilität ergab relative Standardabweichungen zwischen 5,4% und 15,7%, und Schätzungen der Präzision von zwei Qualitätskontrollprodukten ergaben relative Standardabweichungen zwischen 9,49% und 14,9%. Unter dem Health Canada Intense Smoking Regime lag 2-Nitropropan in maschinell erzeugtem Hauptrauch aus 15 Zigarettenprodukten im Bereich von 98,3 bis 363 ng pro Zigarette.

RESUME

Bien que le 2-nitropropane soit un composé potentiellement nocif présent dans la fumée de cigarette, il y a peu de méthodes modernes qui sont completement validees pour determiner sa quantité principale dans la fumée de cigarette. Nous avons développé une méthode de spectrométrie de masse en tandem avec chromatographie en phase gazeuse à dilution isotopique (ID-GC-MS / MS) pour la détection du 2-nitropropane dans la fumée principale de cigarette. La fraction vapeur de la fumée principale du cigarette a été collectee dans des sacs d’échantillonnage inertes de fluorure de polyvinyle et extrait à l’hexane contenant un étalon intern marqué isotopiquement, puis purifiée et concentrée par extraction en phase solide en utilisant un adsorbant de silice en phase normale et un éluant à 100% de dichlorométhane. Cette méthode est suffisamment sensible pour mesurer les concentrations de 2-nitropropane en phase vapeur dans la gamme des nanogrammes, avec une limite de détection de 19 ng par cigarette. À partir de l’analyse de 15 produits de cigarette, la variabilité estimée des produits a donné des écarts-types relatifs compris entre 5,4% et 15,7% et les estimations de la précision de deux produits de contrôle de la qualité ont abouti à des écarts-types relatifs de 9,49% et 14,9%. Sous le régime de tabagisme intense de Santé Canada, la concentration de 2-nitropropane présente dans la fumée principale générée par machine à partir de 15 produits de cigarette variait de 98,3 à 363 ng par cigarette.

Keywords

2-Nitropropane; volatile organic compound; mass spectrometry; gas chromatography; tobacco

INTRODUCTION

Cigarette smoke comprises a complex cocktail of chemicals, including 93 substances recognized as harmful or potentially harmful compounds (HPHCs) by the United States Food and Drug Administration (FDA) (1). The vapor phase of mainstream cigarette smoke is primarily made up of air constituents, with a minority composition (7%) of tobacco-derived volatile organic compounds (VOCs) (2-6). Cigarette smoking is the most significant non-occupational route of human exposure to many harmful or potentially harmful VOCs. To date, many multianalyte VOC panels have been developed for the quantitation of these compounds in smoke; however, a few volatile HPHCs cannot be reliably quantified in broad-

spectrum multianalyte panels due to volatilization losses, chromatographic interferences, and/or poor sensitivity (7, 8).

A number of volatile nitro compounds occur in mainstream cigarette smoke as a result of *in situ* pyrosynthesis reactions between hydrocarbon radicals and nitrates during the combustion process (9). Among the nitro compounds known to be present in smoke, 2-nitropropane has been identified as a HPHC due to its toxicity and probable carcinogenicity (10-12). To the best of our knowledge, few methods have been reported in peer-reviewed publications for the quantitation of 2-nitropropane, to date. In 1968, Hoffmann and Rathkamp published a rather elaborate method that involved drawing the smoke from 20–200 cigarettes through water-filled impingers, adding the internal standard, then subjecting the samples to a complex series of acidifications, steam distillations, and liquid-liquid extractions in order to clean up and concentrate the samples prior to analysis by gas chromatography with flame ionization or electron capture detection (GC-FID or GC-ECD) (9). These experiments employed a structurally distinct internal standard, although the results were corroborated using an isotopically-labeled internal standard and gas chromatography-mass spectrometry (GC-MS) analysis. Given the limitations of instrumentation in the 1960s, the method was elegantly designed for its purposes; however, the method is quite complex and no longer suitable for the higher throughput demands of the modern laboratory. In 2011, a simpler method was reported by Gaworski *et al.* In this analytical technique, mainstream cigarette smoke was drawn through a silica solid phase extraction cartridge, and 2-nitropropane was quantified against a surrogate internal standard using gas chromatography-tandem mass spectrometry (13). While several publications appear to have made use of this method to quantitate 2-nitropropane, no data regarding the validation parameters for the method itself could be located (13-16). Most recently, Wang *et al.* reported a third method for the quantitation of 2-nitropropane in cigarette smoke (17). In this method, volatile mainstream smoke components were collected using two consecutive impingers filled with ethyl acetate. After collecting smoke from 20 cigarettes, the impinger extracts were combined, then a surrogate internal standard (2-methyl-2-nitropropane) was added. Samples were analyzed using heart-cutting multidimensional gas chromatography (GC-GC-MS) to separate analytes from interferents. This method is time intensive due to the need to collect smoke from 20 cigarettes per sample, the use of impingers, and the long analysis times necessitated by the use of multidimensional gas chromatography. Moreover, the extent of sample handling prior to the introduction of the internal standard and the use of a structurally distinct internal standard without a full evaluation of matrix interferences raise additional concerns about the possibility of matrix effects. Thus, there is still a need for a fully validated method for quantitation of 2-nitropropane in cigarette smoke.

Herein we detail the development and complete validation of a selective and accurate method for the determination of 2-nitropropane in mainstream cigarette smoke. Method development was focused on overcoming the challenges associated with the capture and quantitation of this volatile small molecule. Mainstream cigarette vapor phase was collected in Tedlar gas sampling bags, and deuterated 2-nitropropane (2-nitropropane-d_6) was used as an internal standard to attenuate the possibility of matrix effects and any losses due to sample handling and aging (7, 18). A solid-phase extraction (SPE) step on bare silica cartridges was utilized for the dual purposes of sample cleanup and concentration. Gas

chromatography (GC) was selected as the means of sample separation due to the volatility of 2-nitropropane, and tandem mass spectrometry (MS/MS) was selected as the means of detection due to its inherent sensitivity and specificity.

EXPERIMENTAL

Chemicals, materials, and equipment

2-Nitropropane (2NP, CAS# 79-46-9, 97% purity) certified reference material (CRM) was obtained as a 1 mg/mL solution in acetone from SPEX Certiprep (Metuchen, NJ, USA) and stored at −70 °C until use. Deuterated internal standard 2-nitropropane-1,1,1,3,3,3-d_6 (2NPd6; ISTD, CAS # 52809-86-6, 99.5%) was obtained at a concentration of 1 mg/mL in methylene chloride from o2si smart solutions (Charleston, SC, USA) and stored at −70 °C until use. Methylene chloride (DCM, CAS # 75-09-2, HPLC grade) and hexanes (CAS # 92112-69-1, ≥60% n-hexane, ≤40% various methylpentanes, HPLC grade) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Tedlar PLV gas sampling bags with Thermogreen LB-2 septa (2 L) were purchased from Sigma Aldrich (St. Louis, MO, USA). Strata SI-1 silica solid phase extraction (SPE) cartridges (500 mg/3 mL) were obtained from Phenomenex (Torrance, CA, USA).

Fifteen different popular American cigarette products were purchased from The Lab Depot, Inc. (Dawsonville, GA, USA), representing four major domestic cigarette manufacturers: Philip Morris USA (five products), R.J. Reynolds (five products), Imperial Brands (four products), and Liggett Group (one product). CORESTA Monitor #6 (CM6) test pieces and University of Kentucky 3R4F reference cigarettes (Lexington, KY, USA) were used as quality control (QC) materials. These materials were selected because they represent the American and Virginia tobacco blends found in many popular U.S. cigarette products. These test articles have been widely studied and used as quality control materials in the past (19-23). Unfortunately, there are no reference cigarettes with certified published yields for 2NP; accordingly, the best option for quality control in sampling was the independent characterization and application of these industry-standard research materials.

Cigarette products were labeled and stored in a −20 °C freezer (maintained at or below −16 °C) within 10 days of receipt in their original packaging in accordance with International Organization for Standardization (ISO, Geneva, Switzerland) guidance document ISO 3402:1999 (24). Opened cigarette packs were returned to a −20 °C freezer in sealed bags within 10 days of opening. Prior to smoking, cigarette samples and Cambridge filter pads were placed in the temperature- and humidity-controlled smoking chamber and conditioned at 22 ± 1 °C and 60 ± 3% relative humidity for at least 48 h and no more than 10 days.

Positive-displacement repeating pipettes and adjustable volumetric pipettes were purchased from Eppendorf Corporation (Hauppauge, NY, USA). An eVol digital positive displacement system and accessory syringes (100 mL, 500 mL, and 1000 mL volumes) were obtained from Trajan (Ringwood, Victoria, Australia). Bag shaking was carried out with the help of an Eberbach 6010 fixed speed, reciprocal shaker (Eberbach Corporation, Ann Arbor, MI, USA).

Cigarette smoking

Cigarettes were smoked using Cerulean SM450 20-port smoking machines (Cerulean, Richmond, VA, USA) located and operated inside a temperature- and humidity-controlled smoking chamber (Parameter Generation & Control Inc., Black Mountain, NC, USA) under ISO conditions (22 ± 1 °C and 60 ± 3% relative humidity) (17). Cigarette filter holders purchased from Cerulean (44 mm; Molins PLC, Milton Keynes, UK) were fitted with Cambridge filter pads (44 mm; Borgwaldt, Hamburg, Germany). Smoking machine puff volume was verified using a soap bubble meter (Borgwaldt, Hamburg, Germany). Cigarette smoke vapor phase was collected using 2 L Tedlar collection bags connected to puff engine exhaust ports using PVC tubing. A simplified scheme of this sample collection approach is illustrated in Figure 1. Each sample comprised smoke from three cigarettes, collected according to the Health Canada Intense (HCI) regimen; specifically, cigarettes were smoked with 100% filter ventilation blockage, a 55 mL puff volume, a 2 s puff duration and a 30 s puff interval (25). One cigarette clearing puff was collected after each cigarette was smoked, and one run clearing puff was collected at the end of each smoking run. Quality control materials (CM6 and 3R4F) were smoked in tandem with commercial cigarette products during each smoking run. Quality control materials were assessed with respect to a modified set of Westgard rules (26, 27).

Standard and extraction solution preparation

Extraction solution was prepared by diluting labeled internal standard to a concentration of 70 ng/mL with hexanes in a volumetric flask. After thorough mixing, extraction solution was portioned into 60-mL amber autosampler vials with PTFE-faced screw caps and sealed with Parafilm. Aliquots of extraction solutions were stored at −70 °C if not intended for same-day use and allowed to equilibrate to room temperature prior to use.

Standards were prepared from CRMs and covered the full range of 2NP concentrations observed during preliminary investigations of a variety of different cigarette products. The preparation process was designed to approximate the treatment of cigarette samples as closely as possible and thus reduce the likelihood of errors due to differences in preparation and handling.

For standard preparation, an ampoule of 2NP CRM was brought to room temperature, vortexed, then opened; without delay, ampoule contents were diluted to 2 μg/mL in hexanes using a volumetric flask and an adjustable-volume pipette to prepare a secondary stock solution. Partially inflated Tedlar bags were then spiked through their septa with appropriate volumes of the 2 μg/mL 2NP solution using an eVol digital positive displacement system fitted with appropriately-sized syringes. Spiked standards in Tedlar bags were extracted with 10 mL of extraction solution containing 70 ng/mL internal standard, which was introduced into Tedlar bags via the push-lock valve. After the addition of extraction solution, bags were shaken briefly by hand to distribute the solvent and internal standard, then bags were shaken on a reciprocal shaker for a further 10 min at 180 rpm to finalize sample extraction. Standards were then subjected to SPE (detailed below) prior to analysis by GC-MS/MS. Following SPE, each standard sample was eluted with 1.00 mL DCM. The fully-prepared

standards contained ISTD concentrations of 700 ng/mL and 2NP concentrations between 150–1400 ng/mL.

Sample preparation

The cigarette vapor phase was collected in Tedlar bags for extraction and analysis; Cambridge filter pads were discarded after smoking as the analyte was not detected in this fraction of the smoke. Samples were extracted with 10 mL of extraction solution containing 70 ng/mL internal standard, which was introduced into Tedlar bags via the push-lock valve. Extraction solution was introduced 10 min after termination of the smoking run to rapidly homogenize the internal standard and reduce the likelihood of sample loss in the polymeric Tedlar matrix (7, 28). After the addition of extraction solution, bags were shaken briefly by hand to distribute the solvent and internal standard, then bags were shaken on a reciprocal shaker for a further 15 min at 180 rpm to finalize sample extraction. Samples were then concentrated and cleaned up using SPE (detailed below) prior to analysis by GC-MS/MS.

Solid phase extraction cleanup and concentration steps

Smoke samples, quality control samples, blanks, and standards were subjected to SPE on normal phase (500 mg/3 mL) silica cartridges using a 20-port manifold outfitted with disposable liners. Cartridges were washed once with 100% DCM and once with 100% hexanes; cartridges were fully filled (3 mL) during each wash step and contents were fully evacuated under vacuum following each of the two washing steps. Cartridges were then conditioned by refilling with 3 mL 100% hexanes and allowing the liquid to run to the top of the SPE stationary phase. The entire contents of each Tedlar bag (smoke sample or standard) were loaded onto respective conditioned cartridges. Loaded samples were washed with 1 mL of 10% DCM in hexanes, evacuated to dryness, eluted with 1 mL 100% DCM, and transferred without delay into PTFE-capped autosampler vials fitted with glass inserts to minimize headspace losses. Eluates were analyzed by GC-MS/MS. If not intended for same-day use, aliquots of standards were stored at −20 °C and allowed to equilibrate to room temperature prior to use.

Screening for 2NP in mainstream smoke vapor phase and particulate matter collected on filter pads and cigarette filters

Relative recoveries of 2NP were assessed separately in the mainstream smoke vapor phase, particulate matter, and cigarette filters for three commercial cigarette products, one monitor test piece product obtained from CORESTA, and one research cigarette product obtained from the University of Kentucky. The particulate matter smoke fraction generated from three cigarettes was collected on a Cambridge filter pad placed inside a filter holder into which the filter end of the cigarette was inserted, and the corresponding vapor fraction of the cigarette smoke was collected using a 2-L Tedlar bag. The three smoked cigarette filters corresponding to each smoke sample were combined in a vial as they were left after smoking (with intact tipping paper and rod wrapper paper); following completion of the smoking run, pads were removed from their holders, folded and placed inside separate vials. To each vial, 10 mL of extraction solution containing 70 ng/mL internal standard was added, and vials were closed and placed on a Barnstead Lab-line E-class orbital shaker at 180 rpm for 10 min. Extraction solution (10 mL) was added to bags via the push-lock valve as per the

usual method. The bags were then placed on an Eberbach reciprocal shaker at 180 rpm for 10 min. Extracts from bags, pads, and filters were subjected to the SPE procedure described above. Eluates were analyzed by GC-MS/MS to determine the relative contents of 2NP in each smoke fraction.

Instrumentation and chromatographic method

Sample analysis employed an Agilent 7890B gas chromatography system interfaced to an Agilent 7000C tandem mass spectrometer (GC-MS/MS; Agilent Technologies, Santa Clara, CA, USA) and a Gerstel MPS autosampler rail (GERSTEL GmbH & Co. KG, Mülheim an der Ruhr, Germany). The GC inlet was maintained at 230 °C and outfitted with an Agilent ultra-inert universal gooseneck inlet liner with glass wool. Samples were introduced using a 10:1 split ratio with a 30 psi injection in constant flow mode. Research-grade helium (Airgas Inc., Radnor, PA, USA) was employed as mobile phase. The column was a 40 m Agilent J&W DB-VRX capillary column with a 180 μm I.D. and a 1.0 μm film thickness. During chromatography, the initial oven temperature was 30 °C with no preliminary hold time, and the temperature was ramped to 61 °C at a rate of 5 °C/min, held at 61 °C for 7 min, then ramped to 230 °C at a rate of 100 °C/min and held at this temperature for 3 min. Mass spectrometry was carried out using electron ionization and multiple reaction monitoring (MRM), with the source heated to 230 °C and both the first and second mass analyzers heated to 150 °C. Ultra-high purity grade nitrogen (Airgas) was used as the collision cell gas. Three MRM transitions were selected based on abundance, with the primary (quantitation) transition selected as the highest abundance transition and the two secondary (confirmation) transitions selected as the second and third most abundant transitions. A fourth MRM transition was monitored for the internal standard. For all transitions, the MS1 and MS2 resolution was set to “wide” and a dwell time of 20 ms was employed. The transitions and collision energies used in the data collection are summarized in Table 1. Data acquisition and analysis were carried out using Agilent MassHunter Workstation software. Concentrations of 2NP were determined using the ratio of the analyte peak area to the ISTD peak area.

RESULTS AND DISCUSSION

SPE method development and optimization

In many volatile organic compound panels, 2NP cannot be quantified because of chromatographic interferents and low signal. Solid-phase extraction was selected for sample cleanup as it was found to be capable of addressing both of these concerns. Normal phase silica sorbent SPE cartridges were selected for their excellent retention of nitro compounds. A “classic” normal phase separation was carried out on these cartridges using hexanes and DCM as the respective weak and strong solvents; hexanes were determined to be a suitable extraction solvent for 2NP and DCM was found to provide complete sample elution from the cartridge. The SPE method was optimized by considering the relative percentages and volumes of DCM necessary to promote adequate sample cleanup and elution. Eluates were analyzed separately following sample loading and addition of aliquots of elution solution containing increasing percentages of DCM (in 10% intervals). No analyte “breakthrough” or loss was observed following loading of the full extract on the SPE cartridge, nor was

any loss observed following the addition of 1 mL 10% DCM in hexanes. However, upon treatment with 1 mL aliquots of hexanes solutions containing higher percentages of DCM, sample breakthrough was observed, with complete sample elution occurring when the percentage of DCM was increased to 100%. Thus, 1 mL of 10% DCM in hexanes was chosen as the wash solvent and 1 mL of 100% DCM was chosen as the elution solvent. Chromatograms obtained using this approach and the previously detailed chromatographic method displayed significantly fewer interferents despite the small *m/z* values of the MRM transitions and showed baseline resolution of the analyte peak from those of the interferents that remained. This SPE process effectively reduces the sample volume from approximately 10 mL to 1 mL without significant sample loss, thereby increasing the signal intensity nearly tenfold compared with samples obtained without SPE cleanup. To ensure that no errors were introduced that might bias 2NP quantitation, both standards and smoke samples were prepared in Tedlar bags and subjected to the same SPE process prior to analysis.

Assessment of 2NP content in cigarette filters and the vapor and particulate phases of mainstream smoke

All of the measurable 2NP content in cigarette smoke was found in the vapor phase; any quantities present in the particulate phase collected in the cigarette filter or the inline filter pad applied during smoking were below the limits of detection (S/N < 3). The chromatographic results of a comparison of bags, pads, and filters for a representative cigarette product are shown in Figure 2. As the contribution of the particulate matter (in both cigarette filters and Cambridge filter pads) is negligible to the total delivery of 2NP, further particulate matter analysis is unnecessary to the quantification of this analyte and was not considered further.

Method validation

The developed method was validated to ensure fitness for purpose. Validation parameters included the assessment of accuracy, precision, linearity, limit of detection, matrix effects, stability and ruggedness.

Accuracy

Accuracy was evaluated by determining the percent recoveries for spiked smoke vapor samples. Smoke vapor from a reference cigarette was collected in Tedlar bags and spiked with standard 2NP solution in triplicate at low, medium, and high concentrations, using an eVol syringe prior to extraction; “blank” reference bags containing only cigarette smoke vapor were also collected for calculation of recoveries. Average calculated percent recoveries for the low, medium, and high spikes were 110%, 102%, and 102%, respectively.

Precision

Intermediate precision was calculated by analyzing samples of QC materials (CM6 and 3R4F) collected over the course of 20 separate smoke collection runs (n = 20 per product), each conducted on a separate day. Analysis of intermediate precision yielded relative standard deviations of 14.9% for CM6 and 9.49% for 3R4F. Within-product variability was estimated from an analysis of smoke samples obtained from 15 different cigarette products

(n = 7 for each product), resulting in relative standard deviations ranging from 5.4 to 15.7%. Variability in the measured values may be ascribed to the pyrosynthetic origin of 2NP and the intrinsic heterogeneity of both the product itself and the smoke matrix it generates.

Limit of detection and linearity

Limit of detection was estimated by calculating the signal-to-noise (S/N) ratio for the lowest calibration standard (150 ng/mL) during 10 separate instrumental runs, carried out over the course of a three-month period (n = 10). The limit of detection was extrapolated for a S/N ratio of 3, based on the mean S/N value of the 10 measurements (29, 30). The limit of detection calculated in this manner was 56 ng/mL.

A calibration range was chosen such that the calibrator concentrations were higher than the limit of detection, fell within the linear range, and bracketed the range of 2NP concentrations measured for a number of representative domestic products. Analysis of the calibration plot indicated that a linear regression with no weighting resulted in an optimal distribution of residuals, and calibration plots displayed a coefficient of determination (R^2) higher than 99.

Matrix effects

The measuring of matrix effects was necessary because no matched matrix (i.e., cigarette smoke vapor phase) free of 2NP was available. Matrix effects between smoke vapor phase collected in Tedlar bags and smoke-free “blank” Tedlar bags were assessed by comparing the slopes of two sets of calibrators prepared in non-matrix (hexanes) solution and hexanes-based smoke vapor extract (matrix) solution. Ten-point curves spanning the range of calibrator concentrations were prepared in hexanes and in smoke matrices generated by extraction with hexanes. These were equivalent to respective preparations of calibrators and smoke samples. Each sample was subjected to the SPE process detailed above. Sample sets were prepared in triplicate. Least squares slopes were calculated for sets of calibration curves prepared in smoke matrix and “blank” hexanes matrix in triplicate, averaged separately for the matrix-based and non-matrix-based samples, and the percent error in averaged slopes was computed. A plot of average responses for calibrators prepared in smoke matrix and blank matrix as a function of relative added 2NP concentrations is provided in Figure 3. Acceptable linearity ($R^2 \geq 0.99$) was exhibited by all curves, and no matrix effects were observed; the difference between the average slopes for matrix-based and non-matrix based calibrators was 0% (31-33).

Stability

Stability of the 2NP spiking solution was assessed by comparing 2NP recoveries obtained from mid-range calibrators prepared from both a fresh spiking solution and an aged (71-day-old) spiking solution that had been stored at −70 °C. Calibrators used to calculate recovery were prepared in triplicate from a fresh spiking solution. Calibrator concentrations were then calculated against a separate calibration curve constructed using the fresh spiking solution. After 71 days at −70 °C, no significant change to the concentration of the spiking solution was observed. Recoveries of midrange calibrators prepared from fresh and aged calibrators demonstrated percent differences of 2.5% and 2.9% from the nominal values, respectively.

Ruggedness testing

Method ruggedness was assessed by changing five separate method parameters: (1) number of cigarettes smoked per sample, (2) number of run-clearing puffs, (3) time elapsed before adding extraction solution into the Tedlar bags, (4) total sample shaking time, and (5) volume of extraction solution. Recoveries of 2NP were compared when different numbers of cigarettes were smoked per sample in order to determine whether stability issues or leakage were present; no significant differences in recovery were observed on a per-cigarette basis when 1, 2, or 3 cigarettes were smoked per sample. Therefore a sample size of 3 cigarettes was chosen in order to maximize the 2NP signal. Changes in the number of run-clearing puffs following cigarette smoking were also assessed in order to ensure all of the analyte was collected during the smoking run. When no run-clearing puffs were carried out, 2NP recoveries were significantly lower; however, when either 1 or 2 run clearing puffs were carried out, 2NP recoveries were not significantly different; accordingly, the standard single run clearing puff was selected for the smoking method. Changes to the time elapsing before the addition of extraction solution were evaluated by waiting 5, 10, or 15 min after smoking to introduce extraction solution into the Tedlar bags; no significant change in recovery was observed and a 10-min period of time before bag extraction was chosen to allow adequate time for sample handling and transfer. Assessment of total sample shaking (extraction) time also indicated that as shaking time increased from 15–45 min, there was no change in 2NP recovery; because complete extraction could be attained in 15 min. This duration was chosen in order to reduce the sample preparation downtime. Changes to extraction volume indicated a slightly less efficient extraction using 5 mL of extraction solution, but equivalent extraction efficiencies for 7.5 mL, 8.5 mL, 10 mL, and 11.5 mL; thus a 10 mL extraction volume was selected.

Commercial cigarette analysis

Levels of 2NP were analyzed in one monitor test piece product obtained from CORESTA (n = 20), one research cigarette product obtained from the University of Kentucky (n = 20), and fifteen commercial cigarette products representing four major cigarette manufacturers (n = 7).

Variable levels of 2-nitropropane among different products may have resulted from differences in tobacco blending ratios and/or additives. As shown in Table 2, average 2NP levels ranged from 100 to 360 ng/cig. Most of the cigarette products analyzed were composed of American tobacco blends, which exhibited 2NP concentrations ranging between 210 and 360 ng/cig. For the two Virginia blend cigarette products (CM6 and product 1), levels were significantly lower (100 ng/cig). This correlation between nitro-compound content and tobacco blend type has been observed previously and attributed to variations in nitrate content between tobacco types: American blends contain a mixture of high-nitrate Burley tobacco and low-nitrate Virginia and Oriental tobaccos, while Virginia blends contain only low-nitrate Virginia tobacco (9, 34-38). Hoffmann and Rathkamp's studies on the effects of adding nitrates to tobacco prior to smoking substantiated the correlation between nitro-compound content and nitrates in tobacco. Their results indicate that as nitrate content increases, there is a concomitant increase in the nitrobenzene and nitroalkane compounds recovered from smoke, as well as a decrease in polycyclic aromatic

hydrocarbons (attributed to competition for starting materials in pyrosynthetic reactions) (9, 36). Within-product variability may be illustrated by the calculated percent relative standard deviations (% RSD), which ranged from 5.2% to 16% for the products analyzed. The observed range in variability may be attributable to the inherent heterogeneity of natural tobacco and the volatility and pyrosynthetic origins of 2NP.

Previously published 2NP yields from mainstream smoke range from 13.7 ng/cig to 1.5 μg/cig for commercial and reference cigarette products (14, 15, 17, 39). With the exception of reference products, product identities are not specified in published papers. Hoffmann *et al.* reported average 2NP yields of 1.08 μg/cig (n = 200 cig/sample) and 1.21 μg/cig (n = 20 cig/sample) for an 85 mm unfiltered U.S. blended cigarette product; Wang *et al.* reported a 2NP yield of 0.66 μg/cig for the 3R4F reference cigarette and 2NP yields ranging from 0.18–1.5 μg/cig for 10 different Chinese commercial brands. Both Coffa and Gaworski reported significantly lower 2NP yields (13, 14): Coffa reported 13.7 ng 2NP/cig in the 2R4F reference cigarette and Gaworski reported 19.1 ng 2NP/cig in a laboratory-constructed "control" cigarette. The similarity in their values may be attributed to their use of the same method, for which no published validation parameters could be located.

The published ranges of yields vary significantly across different manuscripts and are mutually exclusive, except in cases where the same analysis method was applied (14, 15). Furthermore, it would be expected that 2NP yields would vary in response to applied smoking protocols due to differences in vent blocking, puff volume, and puff frequency. Sample collection in the previously referenced publications was carried out in accordance with the ISO 3308 smoking regimen (40) and included 20–200 cigarettes per sample, whereas our method made use of the HCI regimen (25) and 3 cigarettes per sample. Under the ISO 3308 regimen, 2NP yields from 3 cigarettes were found to be below our method's limit of quantitation, and the number of cigarettes per sample could not be increased due to the limited volume capacity of the Tedlar bags.

The 2NP yields determined by our group are intermediate to those determined by others. Discrepancies in yield relative to the Hoffmann and Wang manuscripts may be attributable to additional factors outside of smoking protocols (9,17). For example, both the Hoffmann and Wang methods made use of extended sampling steps involving the collection of smoke from many cigarettes in 1–2 impingers, followed by manual combination of impinger contents prior to addition of a structurally distinct internal standard; thus, changes due to handling and sample instability prior to internal standard addition cannot be ruled out. Moreover, neither manuscript appraised the matrix effects that commonly occur in complex media such as cigarette smoke. Finally, the method reported by Hoffmann and Rathkamp may be subject to additional discrepancies due to the extensive sample preparation steps and the possibility of co-eluting interferents due to the use of less selective detectors.

CONCLUSIONS

The validated method described herein provides a robust, selective means for the determination of 2-nitropropane (2NP) levels in mainstream cigarette smoke without measurable matrix interference. The use of tandem mass spectrometry and solid phase

extraction reduces chromatographic interferents and provides for significant signal enhancement. The method is fully validated, and its experimentally determined precision and accuracy are fit for the purpose of quantifying 2NP in mainstream tobacco smoke. Although machine-based smoking cannot replicate human smoking behaviors, this method provides a reliable and reproducible way to compare 2NP yields in cigarette products. The cigarette products analyzed came from a onetime purchase of a single product lot and do not necessarily reflect the lot-to-lot variability of 2-nitropropane concentrations in these products over time.

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REFERENCES

1. Department of Health and Human Services (DHHS), Food and Drug Administration (FDA): Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke – Established List; Federal Register Vol. 77, No. 64 (3 April 2012) 20034–20037.
2. Pazo DY, Moliere F, Sampson MM, Reese CM, Agnew-Heard KA, Walters MJ, Holman MR, Blount BC, Watson CH, andChambers DM: Mainstream Smoke Levels of Volatile Organic Compounds in 50 U.S. Domestic Cigarette Brands Smoked With the ISO and Canadian Intense Protocols; Nicotine Tob. Res 18 (2016) 1886–1894. DOI: 10.1093/ntr/ntw118 [PubMed: 27113015]
3. Jenkins RA andGill BE: Determination of Oxides of Nitrogen (NO_x) in Cigarette Smoke by Chemiluminescent Analysis; Anal. Chem 52 (1980) 925–928. DOI: 10.1021/ac50056a035
4. Watson CV, Feng J, Valentin-Blasini L, Stanelle R, and Watson CH: Method for the Determination of Ammonia in Mainstream Cigarette Smoke Using Ion Chromatography; PLOS ONE 11 (2016) e0159126. DOI: 10.1371/journal.pone.0159126 [PubMed: 27415766]
5. Green CR and Rodgman A: The Tobacco Chemists' Research Conference: A Half Century Forum for Advances in Analytical Methodology of Tobacco and its Products; Fifty Years of Tobacco Science and Technology; Rec. Adv. Tob. Sci 22 (1996) 131–304.
6. Thielen A, Klus H, and Müller L: Tobacco Smoke: Unraveling a Controversial Subject; Exp. Toxicol. Pathol 60 (2008) 141–156. DOI: 10.1016/j.etp.2008.01.014 [PubMed: 18485684]
7. Sampson MM, Chambers DM, Pazo DY, Moliere F, Blount BC, and Watson CH: Simultaneous Analysis of 22 Volatile Organic Compounds in Cigarette Smoke Using Gas Sampling Bags for High-Throughput Solid-Phase Microextraction; Anal. Chem 86 (2014) 7088–7095. DOI: 10.1021/ac5015518 [PubMed: 24933649]
8. Oldham MJ, DeSoi DJ, Rimmer LT, Wagner KA, and Morton MJ: Insights from Analysis for Harmful and Potentially Harmful Constituents (HPHCs) in Tobacco Products; Regul. Toxicol. Pharmacol 70 (2014) 138–148. DOI: 10.1016/j.yrtph.2014.06.017 [PubMed: 24973503]
9. Hoffmann D and Rathkamp G: Chemical Studies on Tobacco Smoke. III. Primary and Secondary Nitroalkanes in Cigarette Smoke; Beitr. Tabakforsch 4 (1968) 124–134. DOI: 10.2478/cttr-2013-0175
10. Rodgman A and Perfetti T: Chemical Components of Tobacco and Tobacco Smoke; CRC Press, Boca Raton, FL, USA, 2009.
11. Hoffmann D, Hoffmann I, and El-Bayoumy K: The Less Harmful Cigarette: A Controversial Issue. A Tribute to Ernst L. Wynder; Chem. Res. Toxicol 14 (2001) 767–790. DOI: 10.1021/tx000260u [PubMed: 11453723]

12. International Agency for Research on Cancer (IARC): 2-Nitropropane; *in*: Re-Evaluation of Some Organic Chemicals, Hydrazine, and Hydrogen Peroxide, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Vol. 71, IARC, Lyon, France, 1999, pp. 1079–1094.
13. Gaworski CL, Oldham MJ, Wagner KA, Coggins CRE, and Patskan GJ: An Evaluation of the Toxicity of 95 Ingredients Added Individually to Experimental Cigarettes: Approach and Methods; Inhal. Toxicol 23S1 (2011) 1–12. DOI: 10.3109/08958378.2010.543187
14. Gaworski CL, Lemus-Olalde R, andCarmines EL: Toxicological Evaluation of Potassium Sorbate Added to Cigarette Tobacco; Food Chem. Toxicol 46 (2008) 339–351. DOI: 10.1016/j.fct.2007.08.012 [PubMed: 17905505]
15. Coffa BG, Coggins CRE, Werley MS, Oldham MJ, and Fariss MW: Chemical, Physical, and *in Vitro* Characterization of Research Cigarettes Containing Denicotinized Tobacco; Regul. Toxicol. Pharmacol 79 (2016) 64–73. DOI: 10.1016/j.yrtph.2016.05.016 [PubMed: 27181452]
16. Rustemeier K, Stabbert R, Haussmann H-J, Roemer E, and Carmines EL: Evaluation of the Potential Effects of Ingredients Added to Cigarettes. Part 2: Chemical Composition of Mainstream Smoke; Food Chem. Toxicol 40 (2002) 93–104. DOI: 10.1016/S0278-6915(01)00085-0 [PubMed: 11731039]
17. Wang X, Guo J, Shang J, Ding L, Zhao G, Xie F, Jia Y, Qin Y, Yu Y, Chen L, and Zhang S: Determination of Nitroalkanes in Mainstream Cigarette Smoke by Heart-Cutting Multidimensional Gas Chromatography System Coupled with Mass Spectrometry Detection; J. Chromatogr A 1424 (2015) 118–126. DOI: 10.1016/j.chroma.2015.10.078
18. Lanckmans K, Sarre S, Smolders I, and Michotte Y: Use of a Structural Analogue Versus a Stable Isotope Labeled Internal Standard for the Quantification of Angiotensin IV in Rat Brain Dialysates Using Nano-Liquid Chromatography/Tandem Mass Spectrometry; Rapid Commun. Mass Spectrom 21 (2007) 1187–1195. DOI: 10.1002/rcm.2950 [PubMed: 17328092]
19. Ding YS, Yan X, Wong J, Chan M, and Watson CH: *In Situ* Derivatization and Quantification of Seven Carbonyls in Cigarette Mainstream Smoke; Chem. Res. Toxicol 29 (2016) 125–131. DOI: 10.1021/acs.chemrestox.5b00474 [PubMed: 26700249]
20. Lisko JG, Stanfill SB, Duncan BW, and Watson CH: Application of GC-MS/MS for the Analysis of Tobacco Alkaloids in Cigarette Filler and Various Tobacco Species; Anal. Chem 85 (2013) 3380–3384. DOI: 10.1021/ac400077e [PubMed: 23394466]
21. Pappas RS, Fresquez MR, Martone N, and Watson CH: Toxic Metal Concentrations in Mainstream Smoke from Cigarettes Available in the USA; J. Anal. Toxicol 38 (2014) 204–211. DOI: 10.1093/jat/bku013 [PubMed: 24535337]
22. Ticha J and Wright C: Rapid Detection of Toxic Compounds in Tobacco Smoke Condensates Using High-Resolution ^{1}H-Nuclear Magnetic Resonance Spectroscopy; Anal. Methods 8 (2016) 6388–6397. DOI: 10.1039/C6AY00849F
23. Intorp M, Purkis SW, and Wagstaff W: Determination of Selected Volatiles in Cigarette Mainstream Smoke. The CORESTA 2009 Collaborative Study and Recommended Method; Beitr. Tabakforsch. Int 24 (2011) 243–251. DOI: 10.2478/cttr-2013-0904
24. International Organization for Standardization (ISO): ISO 3402:1999: Tobacco and Tobacco Products - Atmosphere for Conditioning and Testing; ISO, Geneva, Switzerland, 1999.
25. International Organization for Standardization (ISO): ISO 20778:2018: Cigarettes - Routine Analytical Cigarette Smoking Machine - Definitions and Standard Conditions with an Intense Smoking Regime; ISO, Geneva, Switzerland, 2018.
26. Caudill SP, Schleicher RL, and Pirkle JL: Multirule Quality Control for the Age-Related Eye Disease Study; Statist. Med 27 (2008) 4094–4106. DOI: 10.1002/sim.3222
27. Westgard JO, Barry PL, Hunt MR, and Groth T: A Multi-Rule Shewhart Chart for Quality Control in Clinical Chemistry. Clin. Chem 27 (1981) 493–501. [PubMed: 7471403]
28. Blount BC, McElprang DO, Chambers DM, Waterhouse MG, Squibb KS, and Lakind JS: Methodology for Collecting, Storing, and Analyzing Human Milk for Volatile Organic Compounds; J. Environ. Monit 12 (2010) 1265–1273. DOI: 10.1039/B927022A. [PubMed: 20358052]
29. 2.2.46 Chromatographic Separation Techniques; *in*: European Pharmacopoeia, 5th Edition, Council of Europe, Strasbourg, France, 2005.

30. International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH): Validation of Analytical Procedures: Text and Methodology Q2(R1); ICH, Geneva, Switzerland, 1996.

31. Thompson M: Standard Additions: Myth and Reality; AMCTB 37 (2009) 1–2.

32. Ellison SLR and Thompson M: Standard Additions: Myth and Reality; Analyst 133 (2008) 992–997. DOI: 10.1039/B717660K [PubMed: 18645637]

33. Guidi LR, Tette PAS, Evangelista WP, Fernandes C, and Glória MBA: Matrix Effect on the Analysis of Amphenicols in Fish by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS); J. Phys. Conf. Ser 575 (2015) 012036. DOI: 10.1088/1742-6596/575/1/012036

34. Fischer S, Spiegelhalder B, and Preussmann R: Preformed Tobacco-Specific Nitrosamines in Tobacco – Role of Nitrate and Influence of Tobacco Type; Carcinogenesis 10 (1989) 1511–1517. DOI: 10.1093/carcin/10.8.1511 [PubMed: 2752525]

35. Chapman GM, Bravo R, Stanelle RD, Watson CH, and Valentín-Blasini L: Sensitive and Selective Gas Chromatography-Tandem Mass Spectrometry Method for the Detection of Nitrobenzene in Tobacco Smoke; J. Chromatogr. A 1565 (2018) 124–129. DOI: 10.1016/j.chroma.2018.06.028 [PubMed: 29945789]

36. Rathkamp G and Hoffmann D: Chemical Studies on Tobacco Smoke. XIII. Inhibition of the Pyrosyntheses of Several Selective Smoke Constituents; Beitr. Tabakforsch 5 (1970) 302–306. DOI: 10.2478/cttr-2013-0255

37. Kallianos AG, Means RE, and Mold JD: Effect of Nitrates in Tobacco on the Catechol Yield in Cigarette Smoke; presented at the 20th TCRC, Winston-Salem, NC, USA, 1966; Tob. Sci 12 (1968) 125–129.

38. Klus H and Kuhn H: Die Bestimmung von Nitro-phenolen im Tabakrauchkondensat (vorläufige Ergebnisse) [Determination of Nitrophenols in Tobacco Smoke Condensate (Preliminary Results)]; Fachl. Mitt. Austria Tabakwerke 15 (1974) 275–288.

39. Hoffmann D and Hoffmann I: Chemistry and Toxicology; *in*: Smoking and Tobacco Control Monograph No. 9, edited by Burns D, Cummings KM and Hoffmann D, U.S. Department of Health and Human Services, National Institutes of Health, National Cancer Institute, Bethesda, MD, USA, 1998.

40. International Organization for Standardization (ISO): ISO 3308:2012: Routine Analytical Cigarette-Smoking Machine – Definitions and Standard Conditions; ISO, Geneva, Switzerland, 2012.

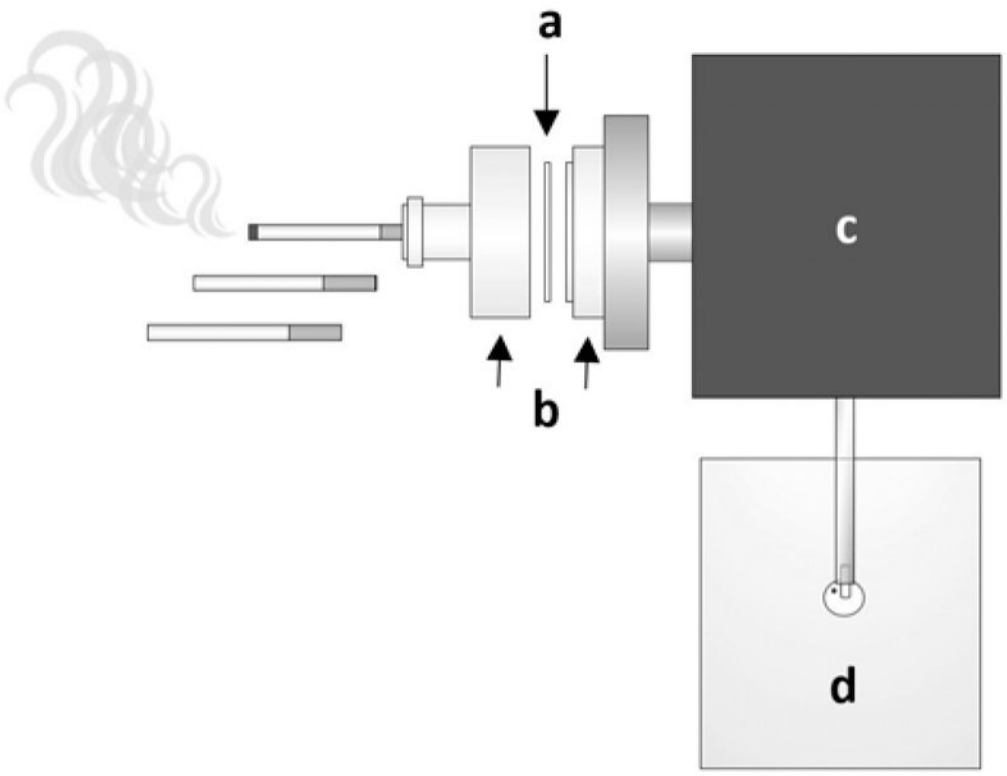

Figure 1. Smoke sample collection scheme.

(a) Cambridge filter pad, (b) Cambridge filter holder, (c) smoking machine puff engine, and (d) Tedlar bag connection to puff engine.

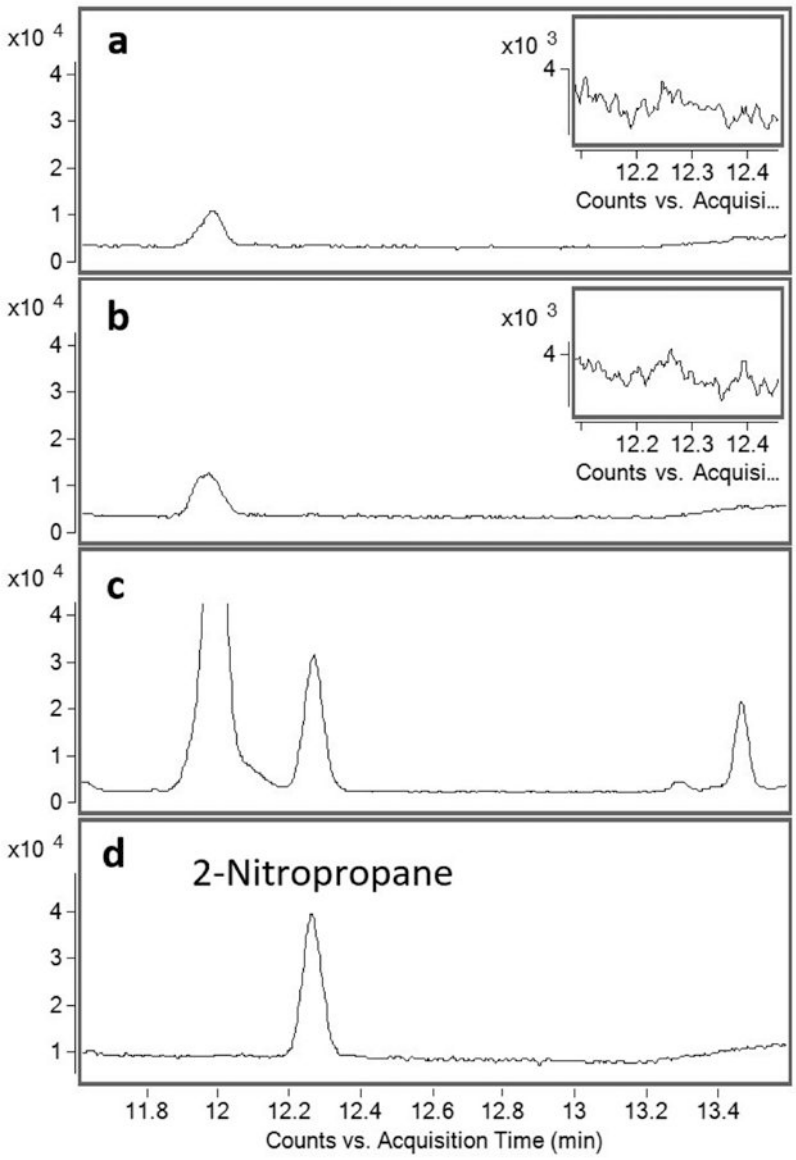

Figure 2. Representative chromatograms (quantitation transition, 43.2 ➔ 41.2) illustrating relative 2-nitropropane recoveries

from (a) cigarette filter particulate matter, (b) Cambridge filter pad particulate matter, and (c) Tedlar bag vapor phase from a representative cigarette product; representative chromatogram for a calibration standard (d) included for reference. Insets: magnified regions at 2-nitropropane retention time for both particulate matter samples; no detectable signal is observed.

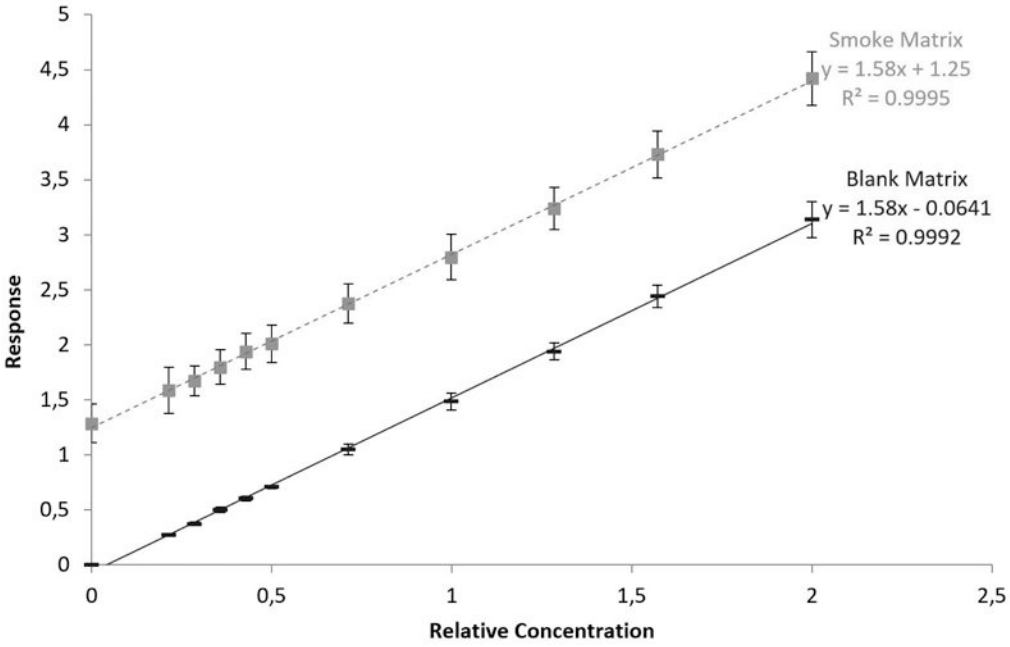

Figure 3. Matrix effects.

Average responses (n = 3) of calibrators prepared in smoke matrix (■) and those prepared in blank matrix (–) plotted as a function of relative concentration, with included standard deviations (error bars). Responses were calculated using the ratio of 2-nitropropane (2NP) peak area to internal standard (ISTD) peak area, and relative concentrations were calculated using the ratio of added 2NP to ISTD.

Table 1.

Ion transitions and collision energies for 2-nitropropane (2NP) and 2-nitropropane-1,1,1,3,3,3-d_6 internal standard (ISTD).

Compound	Transition type	Transition ion masses (*m* / *z*)	Collision energy (V)
2NP	Quantitation	43.2 ➔ 41.2	6
	Confirmation 1	43.2 ➔ 38.9	18
	Confirmation 2	43.2 ➔ 27.2	7
ISTD	Quantitation	49.2 ➔ 45.1	8

Table 2.

2-Nitropropane yields (ng/cig) from commercial cigarette products (n = 7).

Product	Average (ng/cig)	Standard deviation (ng/cig)	RSD (%)
1	100	14	14
2	280	29	10
3	260	41	16
4	300	44	15
5	280	27	9.6
6	240	13	5.4
7	300	25	8.3
8	260	22	8.5
9	360	20	5.6
10	250	13	5.2
11	310	31	10
12	230	14	6.1
13	280	21	7.5
14	220	22	10
15	210	11	5.2
3R4F	240	22	9.2
CM6	100	14	14