

Analysis of Selected Carbonyl Compounds in Tobacco Samples by Using Pentafluorobenzylhydroxylamine Derivatization and Gas Chromatography-Mass Spectrometry *

by

Mingliang Bao, Peter J. Joza, Andrew Masters, and William S. Rickert
Labstat International ULC, 262 Manitou Drive, Kitchener, Ontario N2C1L3, Canada

SUMMARY

A simple, accurate, and reliable method for routine analysis of trace carbonyl compounds, including formaldehyde, acetaldehyde, acetone, propionaldehyde, methyl-ethyl ketone (MEK), butyraldehyde, and crotonaldehyde, in processed tobacco products was developed. One gram of tobacco sample was spiked with a mixture of isotope-labeled carbonyls as internal standards and extracted with water. A portion of aqueous extract was derivatized with *o*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride (PFBHA). The PFBHA derivatives of carbonyls were extracted with hexane and analysed by gas chromatography-mass spectrometry (GC-MS). The accuracy and precision of the method were evaluated with spiked Kentucky Reference Cigarette 3R4F and CORESTA smokeless reference products CRP1, CRP2, CRP3, and CRP4. For the investigated carbonyl compounds, excellent recoveries (95–107%) and precisions (5–10%) were achieved with different spiked tobacco products, with the exception of acrolein, which was found unstable in all tested tobacco products. The linear range of the developed method was from 0.07 to 36 µg/g with limits of quantification ranged from 0.10 to 0.15 µg/g. Using this method, formaldehyde (0.31–6.24 µg/g) and acetaldehyde (0.84–17.7 µg/g) were detected in all tested reference tobacco products. Acetone (0.55–2.12 µg/g) was found in 3R4F, CRP1, CRP2, and CRP3. Detectable levels of propionaldehyde were only found in CRP1 and CRP3. The

levels of MEK, butyraldehyde, and crotonaldehyde in all tested reference products were below the method quantification limits. It was found that the effects of storage conditions (storage time, container, and temperature) on yields of carbonyls detected in reference tobacco product samples (3R4F, CRP2, CRP3) were dependent on compound and sample matrix: the concentrations of formaldehyde in all tested reference products gradually increased as the storage time increased, while the concentrations of acetone in 3R4F samples dramatically decreased as the storage time increased. [Beitr. Tabakforsch. Int. 26 (2014) 86–97]

KEY WORDS: Carbonyl compounds, tobacco, derivatization, GC-MS analysis.

ZUSAMMENFASSUNG

Es wurde eine einfache, genaue und zuverlässige Methode für die Routineanalyse von Spuren von Carbonylverbindungen, einschließlich Formaldehyd, Acetaldehyd, Aceton, Propionaldehyd, Methyl-Ethyl-Keton (MEK), Butyraldehyd und Crotonaldehyd, in verarbeiteten Tabakerzeugnissen entwickelt. Ein Gramm einer Tabakprobe wurde mit einer Mischung aus isotopenmarkierten Carbonylen als internen Standards präpariert und mit Wasser extrahiert. Ein Teil des wässrigen Extrakts wurde mit *o*-(2,3,4,5,6-Pentafluoro-

benzyl)-hydroxylamin-Hydrochlorid (PFBHA) derivatisiert. Die PFBHA-Derivate der Carbonyle wurden mit Hexan extrahiert und mittels Gaschromatographie/Massenspektrometrie (GC/MS) analysiert. Die Genauigkeit und Präzision der Methode wurden mit einer präparierten Kentucky-Referenzzigarette 3R4F und den CORESTA rauchlosen Referenzprodukten CRP1, CRP2, CRP3 und CRP4 ermittelt. Für die untersuchten Carbonylverbindungen wurden mit den verschiedenen präparierten Tabakprodukten hervorragende Wiederfindungsraten (95–107 %) und Genauigkeiten (5–10 %) erreicht. Die Ausnahme bildete Acrolein, welches sich in allen getesteten Tabakprodukten instabil zeigte. Der lineare Bereich der entwickelten Methode erstreckte sich von 0,07 bis 36 µg/g mit Nachweisgrenzen im Bereich von 0,10 bis 0,15 µg/g. Formaldehyd (0,31–6,24 µg/g) und Acetaldehyd (0,84–17,7 µg/g) wurden in allen untersuchten Referenz-Tabakerzeugnissen mit dieser Methode nachgewiesen. Aceton (0,55–2,12 µg/g) wurde in 3R4F, CRP1, CRP2 und CRP3 gefunden. Nachweisbare Propionaldehydkonzentrationen wurden nur in CRP1 und CRP3 festgestellt. Die Mengen an MEK, Butyraldehyd und Crotonaldehyd lagen in allen getesteten Referenzprodukten unter den Nachweisgrenzen der Methode. Es wurde festgestellt, dass die Auswirkungen von Lagerungsbedingungen (Lagerdauer, Behälter und Temperatur) auf die in den Referenztabakerzeugnis-Proben (3R4F, CRP2, CRP3) nachgewiesenen Carbonylausbeuten von der Verbindung und der Probenmatrix abhängig waren: Die Formaldehydkonzentrationen stiegen in allen untersuchten Referenzprodukten mit zunehmender Lagerungsdauer allmählich an, während die Acetonkonzentrationen in 3R4F-Proben mit zunehmender Lagerzeit drastisch abnahmen. [Beitr. Tabakforsch. Int. 26 (2014) 86–97]

RESUME

Nous avons mis au point une méthode, à la fois fiable, précise et simple, d'analyse courante des composés carbonylés, en ce compris le formaldéhyde, l'acétaldéhyde, l'acétone, le propionaldéhyde, la méthyléthylcétone (MEC), le butyraldéhyde et le crotonaldéhyde, présents à l'état de traces dans les produits de tabac ouvré. Un échantillon d'un gramme de tabac, additionné d'un mélange de carbonyles marqués d'un isotope en guise de normes internes, a été extrait par l'eau. Une part de l'extrait aqueux a subi un processus de dérivation à l'aide de chlorhydrate d'*o*-(2,3,4,5,6-pentafluorobenzyle)-hydroxylamine (PFBHA). Les dérivés carbonylés obtenus à l'aide du PFBHA ont ensuite été extraits par l'hexane et analysés par chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC-MS). La précision et l'exactitude de la méthode ont été évaluées au regard d'une cigarette de référence Kentucky « additionnée » 3R4F et de produits de référence sans fumée CORESTA CRP1, CRP2, CRP3 et CRP4. Eu égard aux composés carbonylés analysés, d'excellents taux de précision (5–10%) et de récupération (95–107%) ont été observés avec divers produits du tabac « additionnés », à l'exception de l'acroléine, qui s'est avérée instable dans tous les produits du tabac testés. L'intervalle linéaire de la méthode mise au point s'est étendu de 0,07 à 36 µg/g avec des limites de quantification

variant de 0,10 à 0,15 µg/g. Grâce à la présente méthode, le formaldéhyde (0,31–6,24 µg/g) et l'acétaldéhyde (0,84–17,7 µg/g) ont été décelés dans tous les produits de référence testés. L'acétone (0,55–2,12 µg/g) a été trouvée dans les produits 3R4F, CRP1, CRP2 et CRP3. Des niveaux décelables de propionaldéhyde n'ont été observés que dans les produits CRP1 et CRP3. Les niveaux de MEC, butyraldéhyde et crotonaldéhyde décelés dans tous les produits de référence testés étaient inférieurs aux limites de quantification définies pour la présente méthode. Il s'est avéré que les effets des conditions de stockage (durée de stockage, conteneur et température) sur les quantités de carbonyles décelés dans les échantillons de référence (3R4F, CRP2, CRP3) varient selon la matrice de l'échantillon et du composé : Les concentrations de formaldéhyde dans tous les produits de référence testés augmentent graduellement avec la prolongation de la durée de stockage; tandis que les concentrations en acétone dans les échantillons 3R4F opèrent une chute spectaculaire lors de la prolongation de la durée de stockage. [Beitr. Tabakforsch. Int. 26 (2014) 86–97]

INTRODUCTION

Over the past years, the yields in tobacco smoke of some carbonyl compounds, including formaldehyde, acetaldehyde, acetone, propionaldehyde, acrolein, methyl-ethyl ketone (MEK), butyraldehyde, and crotonaldehyde, have been requested by several regulatory authorities, including Canada, Brazil, Australia, UK (1, 2). In 2012 the U.S. Food and Drug Administration (U.S. FDA) established a list of harmful and potentially harmful constituents (HPHC) in tobacco products and tobacco smoke (3). Among the 93 compounds listed in U.S. FDA's HPHC list, six are carbonyl compounds including formaldehyde, acetaldehyde, acetone, acrolein, methyl-ethyl ketone, and crotonaldehyde. Currently, the U.S. FDA requires the determination of the yields of these carbonyl compounds in mainstream tobacco smoke, but it could be expected that for some carbonyl compounds such as formaldehyde, acetaldehyde, crotonaldehyde, their yields in processed tobacco products such as smokeless tobacco will also be required in the future.

For the determination of carbonyl compounds in tobacco smoke, several methods have been published (4–9). For example, carbonyl compounds in mainstream tobacco smoke can be determined by Health Canada Method T-104 (4) or CORESTA Recommended Method CRM74 (5) using derivatization with 2,4-dinitrophenylhydrazine (DNPH) followed by high-performance liquid chromatography (HPLC) with UV detection or by DNPH derivatization with gas chromatography-mass spectrometry (GC-MS) (6) or ultra-high pressure liquid chromatography coupled with mass spectrometry (UHPLC-MS) (7). Until now, however, there are no published methods available for the quantitative determination of carbonyl compounds in processed tobacco samples, other than the method described by STEPANOV and co-workers (10). The analysis of carbonyl compounds in processed tobacco samples is more of a challenge because of their extremely low concentrations expected and the high variety and chemical complexity of

processed tobacco products. For these reasons, those methods developed for the analysis of carbonyls in tobacco smoke may not be suitable for processed tobacco sample analysis. For example, DNPH/HPLC-UV methods are simple, less expensive but they lack selectivity, especially in complex matrices. The DNPH/GC-MS method published by DONG and MOLDOVEANU (6) is selective and sensitive but with limited linearity range, especially at low concentrations. The DNPH/UHPLC-MS method provides faster analysis with high selectivity and sensitivity but high cost to run due to high expensive instrument and internal standards.

The purpose of this study was to establish and validate a simple and reliable method suitable for routine analysis of selected carbonyl compounds in processed tobacco such as cigarette filler and smokeless tobacco. The carbonyl compounds included in this study are formaldehyde, acetaldehyde, acetone, propionaldehyde, MEK, butyraldehyde, and crotonaldehyde. Acrolein was included in the initial study but was found unstable in all tested tobacco samples, and was then excluded from further study. The method was based on derivatization with *o*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine (PFBHA) in the aqueous tobacco extracts (Figure 1) followed by extraction with hexane from liquid and GC-MS analysis. Even though there are no applications of PFBHA derivatization for the analysis of carbonyls in tobacco smoke or tobacco products, this method has been widely used for the determination of trace level carbonyls in environmental samples (11–13), food samples (14–16), and body fluids (17).

EXPERIMENTAL

Reagents and materials

The unlabeled and isotope-labeled carbonyl standards were obtained from Sigma-Aldrich (Milwaukee, WI, USA), except D₅-1,1,1,3,3-methyl-ethyl ketone (D₅-MEK), which

was obtained from C/D/N Isotopes INC. (Pointe-Claire, Quebec, Canada). All individual carbonyl standard stock solutions were prepared in methanol. The mixed carbonyl standard stock solutions and calibration standard solutions were prepared in Type I water. The derivatizing reagent, *o*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine (PFBHA), was purchased from Sigma-Aldrich and prepared as 20 mg/mL solution in Type I water. All reagent grade solvents were purchased from Fisher Scientific Co. (Norcross, GA, USA). The Kentucky Reference Cigarette 3R4F was purchased from University of Kentucky (Lexington, KY, USA). The CORESTA Reference Products CRP1, CRP2, CRP3, and CRP4 were obtained from Analytical Service Laboratory at the North Carolina State University. All reference tobacco samples were kept in a freezer (–20 °C) until analysis and moved from the freezer 2 h before use.

Tobacco sample extraction and derivatization

One gram of tobacco sample was placed in a 15 mL glass tube. A 100 µL of mixed deuterated carbonyl internal standards was added on the tobacco. After adding 10 mL of Type I water, the tube was capped and shaken on a wrist-shaker for 1 h. The sample was centrifuged at 3000 rpm for 5 min to separate the tobacco from the aqueous supernatant. After centrifuging, 5 mL of supernatant was transferred to a 10 mL glass tube and mixed with 100 µL of 20 mg/mL PFBHA aqueous solution. The glass tube was capped with a PTFE-lined septum and placed in the dark at room temperature for 2 h. After 2 h of derivatization with PFBHA, four drops of 18 N H₂SO₄ solution was added to stop the derivatization reaction. The PFBHA derivatives were extracted with 2 mL of hexane using a multi-tube vortex for 10 min. After vortex, the sample was centrifuged at 3000 rpm for 5 min to separate hexane layer from aqueous phase. The hexane extract was then transferred to an autosampler vial for GC-MS analysis.

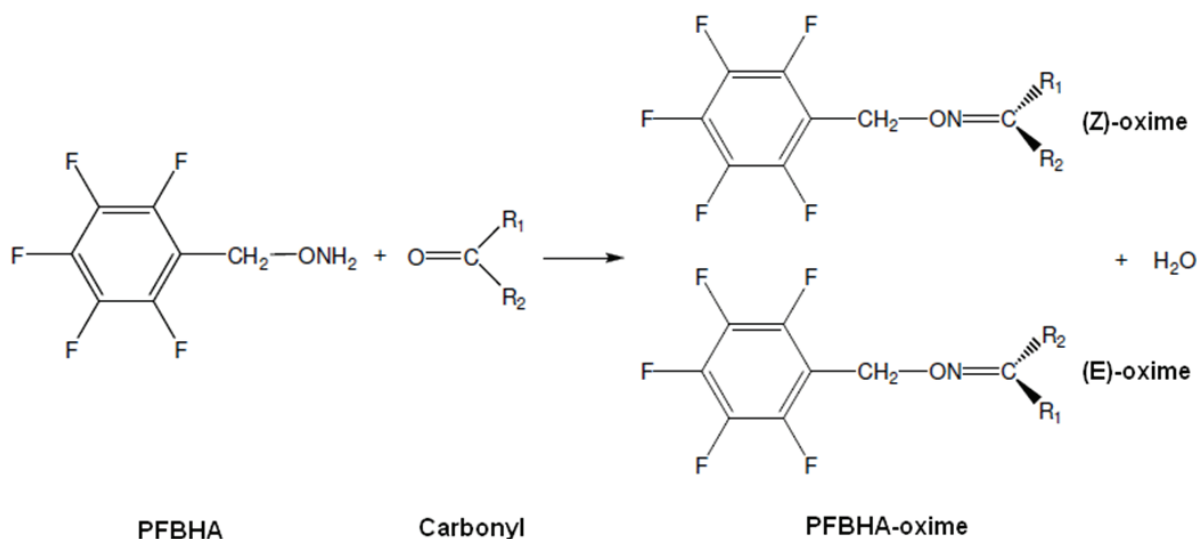


Figure 1. Derivatization reaction of carbonyls with PFBHA.

Calibration standard preparation

The calibration standards prepared in aqueous solutions with individual carbonyl standards are derivatized and extracted using exactly the same procedure as tobacco samples. This will compensate for any inefficiency in the processing procedure. The concentration ranges of the calibration standards were chosen to cover the concentrations possibly found in processed tobacco products.

GC/MS analysis

The PFBHA derivatives of carbonyl compounds were analyzed by using a Varian CP-3800 GC coupled to a Varian 320 TQ mass spectrometer (Varian, Palo Alto, CA, USA) and a CTC CombiPAL autosampler. The GC was equipped with a 30 m \times 0.25 mm I.D., 0.25- μ m film thickness, ZB-5ms capillary column (Phenomenex, Torrance, CA, USA). The GC oven temperature program was as follows: initial 50 $^{\circ}$ C, 5 $^{\circ}$ C/min to 180 $^{\circ}$ C, and then 25 $^{\circ}$ C/min to 280 $^{\circ}$ C, where it was held for 5 min. The carrier gas was helium at a flow rate of 1 mL/min. The GC injector was operated in the split mode with a 10:1 split ratio at 250 $^{\circ}$ C. The GC-MS transfer line was maintained at 280 $^{\circ}$ C. The temperature of the ion source was 250 $^{\circ}$ C and the manifold temperature was 41 $^{\circ}$ C. The mass spectrometer was operated under selected ion monitoring (SIM) with

the ion source configured for electron impact (IE) mode at 70 eV electron energy. For each studied carbonyl compound, at least two ions were selected for quantitation and confirmation purposes.

RESULTS AND DISCUSSION

Figure 2 shows typical GC-MS chromatograms of a calibration standard at concentration range of 0.2 to 0.6 μ g/mL obtained after PFBHA derivatization. The GC resolution, peak shape, and sensitivity are perfectly acceptable for this type of application. As shown in Figure 1, with the exception of formaldehyde and acetone, the PFBHA derivatization reaction of each studied carbonyl compound forms two PFBHA-oxime isomers, referred to as (*E*) and (*Z*). The peaks of the (*E*) form and the (*Z*) form can be separated by the column used for this study. The peak area ratio of the (*E*) form and the (*Z*) form is constant and the calibration and quantitation can be done by using either the sum of the isomer peak areas for each compound or the peak area of each individual isomer. For this method, the sum of the isomer peak areas for each compound was used for quantitation. Table 1 shows the retention times and ions used for quantification and confirmation for the studied carbonyl compounds.

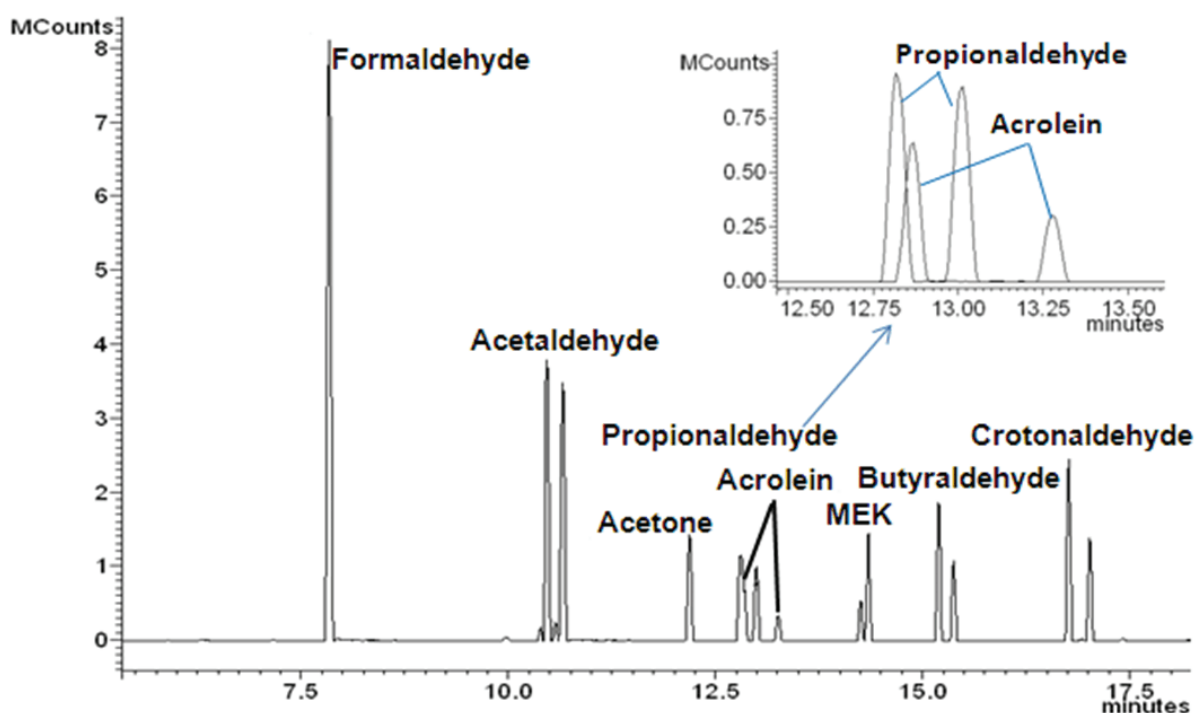


Figure 2. Typical SIM chromatograms of a calibration standard (0.2 to 0.6 μ g/mL) after PFBHA derivatization.

Table 1. Retention times and ions selected for the PFBHA oximes of studied carbonyl compounds.

| PFBHA Oxime | Retention time ^a (min) | Quantification ion (m/z) | Confirmation ion (m/z) |
|------------------------------|--------------------------------------|-----------------------------|---------------------------|
| Formaldehyde | 7.83 | 181 | 195 |
| D ₄ -acetaldehyde | 10.42, 10.61 | 213 | 181 |
| Acetaldehyde | 10.50, 10.68 | 209 | 181, 239 |
| D ₆ -acetone | 12.07 | 259 | 181 |
| Acetone | 12.19 | 253 | 181, 236 |
| Propionaldehyde | 12.84, 13.03 | 236 | 181, 253 |
| Acrolein | 12.88, 13.30 | 250 | 181 |
| D ₅ -MEK | 14.18, 14.28 | 255 | 181 |
| MEK PFBHA | 14.28, 14.38 | 250 | 181 |
| Butyraldehyde | 15.23, 15.41 | 239 | 181, 250 |
| Crotonaldehyde | 16.80, 17.06 | 250 | 181 |

^a Retention times of PFBHA-oxime isomers (E, Z), if applicable.

Table 2. Mean recoveries and relative standard deviations (RSD) of studied carbonyls from spiked 3R4F, CRP2, and CRP3 using 4-fluorobenzaldehyde as internal standard.

| Compound | Spike amount (µg/g) | 3R4F % Recovery (RSD) | CRP2 % Recovery (RSD) | CRP3 % Recovery (RSD) |
|-----------------|------------------------|--------------------------|--------------------------|--------------------------|
| Formaldehyde | 4.54 | 108 (7) | 149 (8) | 128 (13) |
| Acetaldehyde | 3.97 | 114 (9) | 151 (8) | 145 (16) |
| Acetone | 3.82 | 121 (7) | 101 (8) | 151 (14) |
| Propionaldehyde | 2.53 | 105 (3) | 164 (14) | 140 (13) |
| Acrolein | 2.37 | 9 (13) | 6 (17) | 4 (53) |
| MEK | 1.80 | 115 (7) | 89 (9) | 121 (14) |
| Butyraldehyde | 1.92 | 98 (7) | 160 (14) | 132 (14) |
| Crotonaldehyde | 2.09 | 99 (5) | 88 (5) | 100 (11) |

Selection of internal standard

As mentioned in the introduction section, one of the major challenges for the analysis of trace levels of carbonyl compounds in processed tobacco products is the high variety and complexity of tobacco matrices. To minimize the sample matrix effects on method performance, the use of appropriate internal standards (IS) is critical. The first IS chosen for testing was 4-fluorobenzaldehyde. Several published PFBHA methods used this compound as IS for aqueous sample analysis such as water (12) and beer (15). Table 2 shows the recoveries of studied carbonyl compounds from spiked reference tobacco samples 3R4F, CRP2, and CRP3, calculated by using 4-fluorobenzaldehyde as IS. The spiked amounts ranged from 1.8 to 4.54 µg/g. For the data shown in this table, the recoveries of acrolein from all tested reference tobacco samples are very low, below 10%. Recovery tests by using CRP1 and CRP4 get same low recovery results for this compound (not shown). It is unlikely that the extremely low recovery of acrolein from tobacco samples is caused by low extraction efficiency as acrolein is highly water soluble. A possible cause for acrolein's poor recovery is its instability in the tobacco matrix or in the aqueous tobacco extract. This was confirmed by another recovery test: one gram of CRP2 sample was extracted with 10 mL of Type I water; the aqueous extract was separated from the tobacco, spiked with a known amount of acrolein, and went through the

PFBHA derivatization and hexane extraction as described above. The recovery of acrolein from spiked CRP2 aqueous extract was still very low (< 36%). It is known that acrolein is very active and readily polymerizes to form acrolein dimer in aqueous solution in the presence of catalysts such as heat, oxidizing agents, acid (8). The instability of acrolein in aqueous tobacco extracts precluded it from this study. For other studied carbonyl compounds, matrix effects on their recoveries are sample matrix and compound dependent. For the data shown in Table 2, using 4-fluorobenzaldehyde as IS, recoveries for all compounds from 3R4F are acceptable but higher than 125% for formaldehyde, acetaldehyde, propionaldehyde, and butyraldehyde from CRP2 and CRP3. For acetone, recovery is good from CRP2 (101%) but poor from CRP3 (151%).

The second IS was chosen for testing was deuterated acetaldehyde, D₄-acetaldehyde. The recoveries of studied carbonyl compounds from 3R4F, CRP2, and CRP3 calculated with D₄-acetaldehyde as IS are shown in Table 3. Compared to the recovery data shown in Table 2, it is clear that using D₄-acetaldehyde as IS improves the recoveries for most of studied carbonyl compounds, but recoveries of acetone and MEK from CRP2 and crotonaldehyde from CRP2 and CRP3 are still poor.

As a single IS failed to provide satisfactory recovery for all studied carbonyl compounds from different sample matrices, multiple isotope labeled carbonyl compounds as IS

Table 3. Mean recoveries and relative standard deviations (RSD) of studied carbonyls from spiked 3R4F, CRP2, and CRP3 using D₄-acetaldehyde as internal standard.

| Compound | Spike amount (µg/g) | 3R4F % Recovery (RSD) | CRP2 % Recovery (RSD) | CRP3 % Recovery (RSD) |
|-----------------|---------------------|--------------------------|--------------------------|--------------------------|
| Formaldehyde | 4.54 | 102 (9) | 109 (5) | 104 (6) |
| Acetaldehyde | 3.97 | 107 (7) | 111 (2) | 112 (2) |
| Acetone | 3.82 | 109 (9) | 78 (4) | 120 (4) |
| Propionaldehyde | 2.53 | 92 (9) | 99 (11) | 106 (10) |
| MEK | 1.80 | 103 (14) | 58 (10) | 102 (11) |
| Butyraldehyde | 1.92 | 88 (3) | 100 (13) | 104 (7) |
| Crotonaldehyde | 2.09 | 90 (5) | 45 (29) | 78 (20) |

Table 4. Mean recoveries and relative standard deviations (RSD) of studied carbonyls from spiked 3R4F, CRP2, and CRP3 using multiple isotope labeled carbonyls as internal standards.

| Compound | Internal standard | 3R4F % Recovery (RSD) | CRP2 % Recovery (RSD) | CRP3 % Recovery (RSD) |
|-----------------|------------------------------|--------------------------|--------------------------|--------------------------|
| Formaldehyde | D ₄ -acetaldehyde | 100 (7) | 109 (5) | 106 (6) |
| Acetaldehyde | D ₄ -acetaldehyde | 105 (6) | 109 (4) | 111 (2) |
| Acetone | D ₆ -acetone | 97 (10) | 90 (3) | 99 (3) |
| Propionaldehyde | D ₄ -acetaldehyde | 97 (9) | 100 (10) | 107 (9) |
| MEK | D ₅ -MEK | 109 (2) | 102 (3) | 102 (5) |
| Butyraldehyde | D ₄ -acetaldehyde | 93 (8) | 103 (11) | 107 (7) |
| Crotonaldehyde | D ₅ -MEK | 95 (5) | 98 (6) | 86 (4) |

were then tested: D₄-acetaldehyde for formaldehyde, acetaldehyde, propionaldehyde, and butyraldehyde; D₆-acetone for acetone; D₅-MEK for MEK and crotonaldehyde. For the data shown in Table 4, using multiple isotope labeled carbonyls as IS, excellent recoveries were obtained for all studied carbonyl compounds from all tested reference tobacco samples: mean recovery is between 86 to 111% with relative standard deviation (RSD) ranging from 5 to 11%.

Stability of PFBHA-carbonyl derivatives in tobacco samples

PFBHA-carbonyl derivatives are usually stable once extracted into organic solvents such as hexane. Since this is the first application of the PFBHA derivatization approach for the analysis of carbonyls in tobacco samples, the stability of studied carbonyl compounds in tobacco samples after PFBHA derivatization was evaluated by re-analyzing laboratory fortified matrix (LFM) samples prepared with 3R4F and CRP2 on day 2, 5, and 8 after the original analysis. The LFM samples were kept in a freezer after the original analysis. As shown in Figure 3, the PFBHA derivatives of studied carbonyls are all stable in prepared reference tobacco samples 3R4F and CRP2 for at least 8 days if kept in freezer.

Method validation

Method validation parameters including linearity ranges, calibration slopes and correlation coefficients (R^2), limits of quantification (LOQs), method accuracy and precision were determined and listed in Table 5. As shown in Table 5, all studied carbonyl compounds showed linearity

over two orders of magnitude with correlation coefficients better than 0.999. It should be noted that trace levels of acetone, formaldehyde, and acetaldehyde could be present in water used for the preparation of calibration standards and for extracting tobacco samples. It may be necessary to subtract these values from the measurements, depending on the concentration levels found in the tested samples. The method LOQ was determined by analyzing the lowest level of standard ten times over several days. The LOQ was reported as ten times the standard deviation of the ten measurements, respectively. The method LOQs obtained ranged from 0.102 to 0.153 µg/g with the exception for acetone, which showed relatively higher LOQ (0.274 µg/g) mainly due to background contribution.

The accuracy and precision of the method were determined with reference tobacco samples of 3R4F, CRP1, CRP2, CRP3, CRP4 spiked with known amounts (1.80 to 4.54 µg/g) of studied carbonyl compounds. A minimum of 3 replicates for each tested reference tobacco products were prepared and analyzed on different days. The total replicates, mean recovery, and RSD obtained for each compound are listed in Table 5. For the data shown in Table 5, the mean recoveries of all studied carbonyl compounds from different fortified reference tobacco samples were between 95 to 107% with RSD ranging from 5 to 10%.

Application of the method

The yields of the studied carbonyl compounds in reference tobacco samples of 3R4F, CRP1, CRP2, CRP3, and CRP4 were determined using the above described method. Figure 4 shows the typical SIM chromatograms of reference tobacco product 3R4F (top) and CRP3 (bottom).

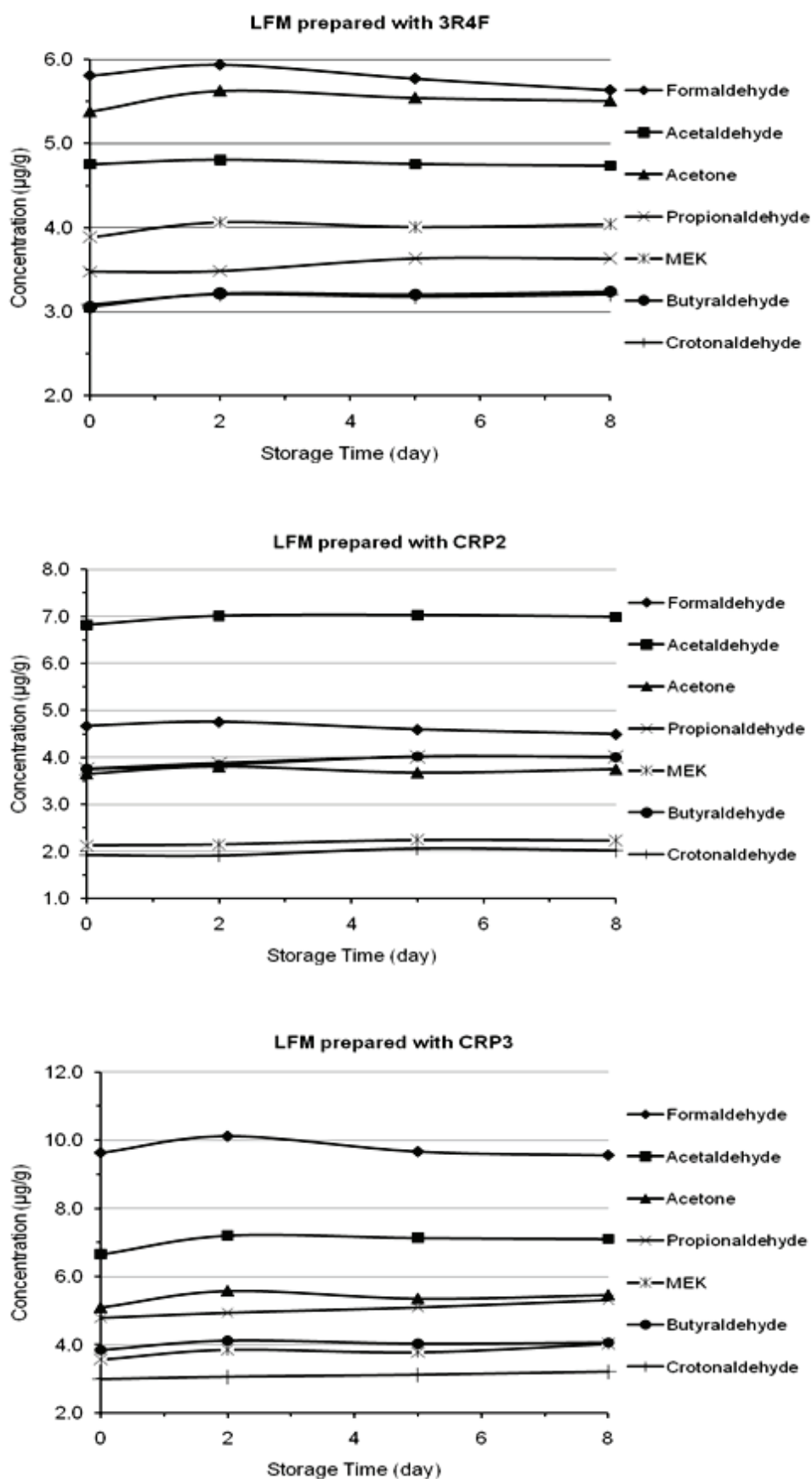


Figure 3. Stability of PFBHA derivatives of carbonyl compounds in laboratory fortified matrix (LFM) samples prepared with 3R4F, CRP2, and CRP3. Spiked amounts: 1.8 to 4.54 µg/g.

Table 5. Method characteristics of the PFBHA derivatization and GC-MS method for the analysis of studied carbonyl compounds in tobacco samples.

| Compound | Linearity range (µg/g) | Calibration curve | | LOQ (µg/g) | Replicate number | Recovery (%) | RSD (%) |
|-----------------|------------------------|-------------------|----------------|------------|------------------|--------------|---------|
| | | Slope | R ² | | | | |
| Formaldehyde | 0.181 – 36.3 | 13.66 | 0.9996 | 0.145 | 33 | 106 | 6 |
| Acetaldehyde | 0.159 – 31.7 | 1.100 | 0.9997 | 0.102 | 33 | 107 | 6 |
| Acetone | 0.153 – 30.6 | 1.126 | 0.9997 | 0.274 | 15 | 98 | 10 |
| Propionaldehyde | 0.095 – 19.0 | 0.723 | 0.9991 | 0.135 | 33 | 102 | 9 |
| MEK | 0.084 – 16.7 | 1.053 | 0.9995 | 0.153 | 15 | 105 | 5 |
| Butyraldehyde | 0.072 – 14.4 | 0.938 | 0.9992 | 0.107 | 33 | 102 | 9 |
| Crotonaldehyde | 0.077 – 15.3 | 1.784 | 0.9996 | 0.120 | 15 | 95 | 6 |

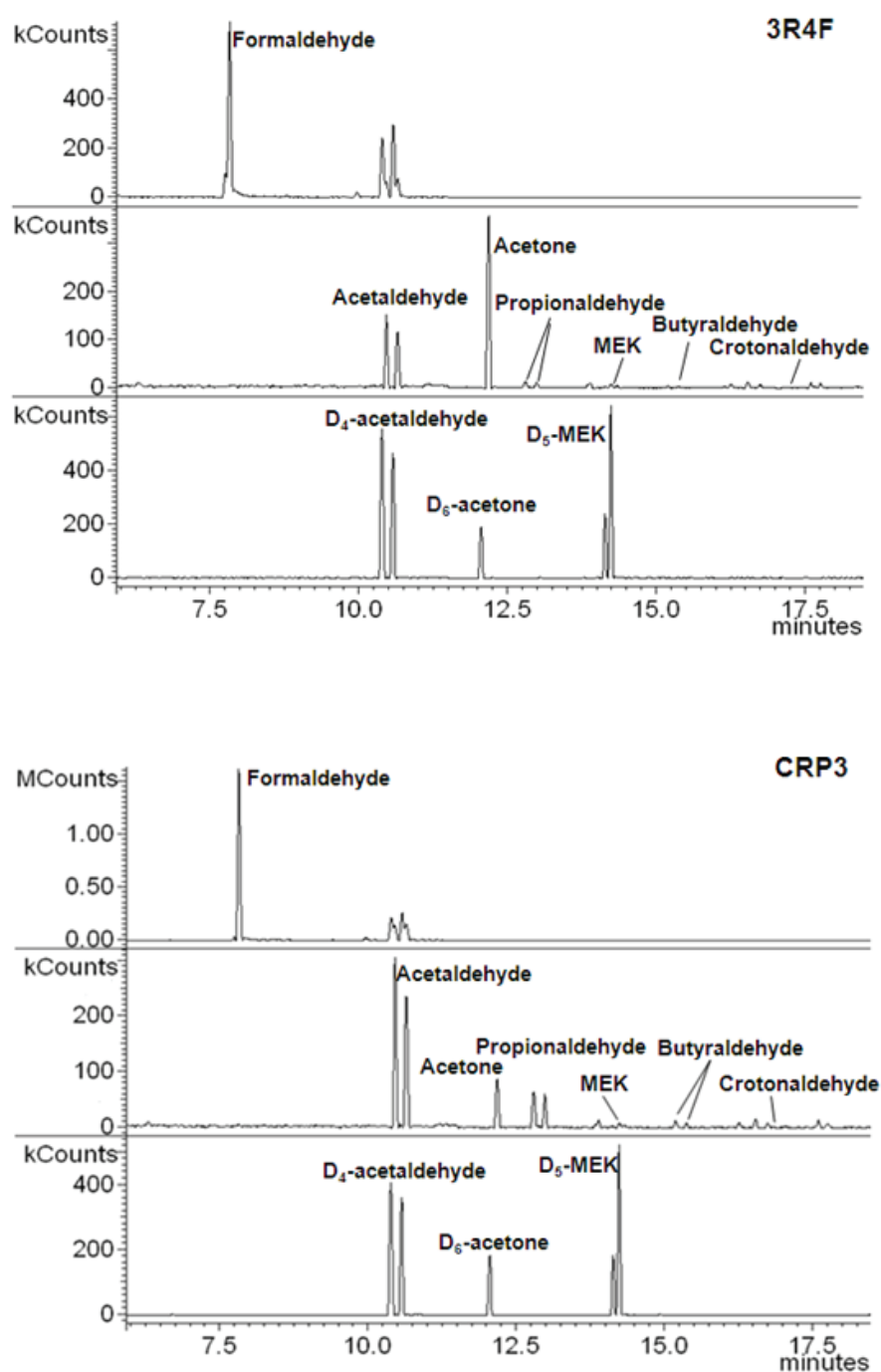


Figure 4. Typical SIM chromatograms of 3R4F and CRP3 samples obtained by using PFBHA derivatization and GC-MS method.

The chromatograms were clean with good peak shape and separation for all detected carbonyl compounds and internal standards. Table 6 gives the means and standard deviations (SD) calculated from at least 9 replicates analyzed on different days for those carbonyl compounds with detectable levels found. For the data shown in Table 6, formaldehyde and acetaldehyde were detected in all tested reference tobacco products with concentrations ranged from 0.31 to 6.24 µg/g for formaldehyde and 0.84 to 17.7 µg/g for acetaldehyde. Acetone (0.55–2.12 µg/g) was detected from 3R4F, CRP1, CRP2, and CRP3 but not from CRP4. Propionaldehyde was only found in CRP1 (0.76 µg/g) and CRP3 (1.13 µg/g). The levels of MEK, butyraldehyde, and crotonaldehyde in all tested reference tobacco samples were all below or close to the method detection limits. The levels of carbonyl compounds in cigarette reference products 3R4F and smokeless tobacco reference products CRP1, CRP2, CRP3, and CRP4 have never been reported prior to the present study. There have only been two studies, to date, that have evaluated the contents of carbonyl compounds in commercial smokeless tobacco products on the US market (10, 18). The results from those two studies are shown in Table 7 with the data from this study. As seen from Table 7, the levels of formaldehyde and acetaldehyde found in those commercial smokeless tobacco products by HOFFMANN *et al.* (18) and STEPANOV *et al.* (10) are generally in the same order of magnitude as those found in CRP products by the present study. Crotonaldehyde was

not detected in all reference tobacco samples tested in this study but was found in all those commercial smokeless tobacco products at concentrations from 0.2 to 19.4 µg/g. Detectable levels of acrolein were also found in most of commercial smokeless tobacco products tested by HOFFMANN *et al.* (18) and STEPANOV *et al.* (10). As mentioned before, the present method was not able to detect acrolein because of its instability in tobacco aqueous extract.

It has always been a challenge to accurately measure the carbonyl compounds selected for this study due to their volatility and instability. Information on properly handling the test samples before and during sample analysis is critical to achieve accurate measurement. Using this new developed method, the effects of tobacco sample storage conditions such as storage container (jar or plastic bag), storage temperature (refrigerator or ambient temperature) and storage time (0 to 7 days) on the yields of studied carbonyl compounds in reference tobacco products were evaluated. It was found that the storage container showed no significant effects on yields of carbonyls detected in CRP2 and CRP3, while the effects of storage time and temperature were dependent on compound and sample matrix. For example, when 3R4F samples were stored in a refrigerator, the concentrations of formaldehyde and acetaldehyde detected gradually increased as the storage time increased, while the concentrations of acetone were found dramatically decreased from day 0 to day 7 storage (Figure 5).

Table 6. Yields of studied carbonyl compounds found in reference tobacco products by using PFBHA derivatization and GC-MS method ^a.

| Compound | 3R4F (n = 13) (µg/g) | CRP1 (n = 9) (µg/g) | CRP2 (n = 24) (µg/g) | CRP3 (n = 27) (µg/g) | CRP4 (n = 9) (µg/g) |
|-----------------|-------------------------|------------------------|-------------------------|-------------------------|------------------------|
| Formaldehyde | 2.20 ± 0.29 | 2.11 ± 0.29 | 1.46 ± 0.23 | 6.24 ± 0.57 | 0.31 ± 0.04 |
| Acetaldehyde | 1.15 ± 0.11 | 17.7 ± 1.3 | 3.37 ± 0.53 | 2.88 ± 0.39 | 0.84 ± 0.10 |
| Acetone | 2.12 ± 0.34 | 0.74 ± 0.15 | 0.89 ± 0.15 | 0.55 ± 0.12 | LOQ |
| Propionaldehyde | 0.14 ± 0.02 | 0.76 ± 0.09 | LOQ | 1.13 ± 0.17 | LOQ |
| MEK | LOQ | LOQ | LOQ | LOQ | LOQ |
| Butyraldehyde | LOQ | LOQ | LOQ | 0.11 ± 0.02 | LOQ |
| Crotonaldehyde | LOQ | LOQ | LOQ | LOQ | LOQ |

^a LOQ: limit of quantification.

Table 7. Comparison of the results for the levels of carbonyl compounds in reference smokeless tobacco products obtained from this study and in the commercial smokeless tobacco products reported in the literature ^a.

| References | Sample category | Formaldehyde (µg/g) | Acetaldehyde (µg/g) | Acrolein (µg/g) | Crotonaldehyde (µg/g) |
|-----------------------------|--------------------|------------------------|------------------------|--------------------|--------------------------|
| HOFFMANN <i>et al.</i> (18) | Moist snuff | 0.3 – 6.8 | 2.4 – 7.4 | 0.3 – 0.6 | 1.0 – 2.4 |
| HOFFMANN <i>et al.</i> (18) | Dry snuff | 1.6 – 7.4 | 1.4 – 3.9 | 0.07 – 0.3 | 0.2 – 0.6 |
| STEPANOV <i>et al.</i> (10) | Moist snuff | 6.58 – 10.6 | 17.1 – 72.3 | 2.58 – 7.85 | 0.98 – 6.35 |
| STEPANOV <i>et al.</i> (10) | Snus | 0.21 – 7.04 | 0.97 – 16.4 | BDL – 4.42 | 0.55 – 19.4 |
| This study | Snus (CRP1) | 4.39 | 36.6 | ND | LOQ |
| This study | Moist snuff (CRP2) | 3.20 | 7.39 | ND | LOQ |
| This study | Dry snuff (CRP3) | 6.85 | 3.16 | ND | LOQ |

^a All data are based on dry weight. ND: not detectable, LOQ: limit of quantification.

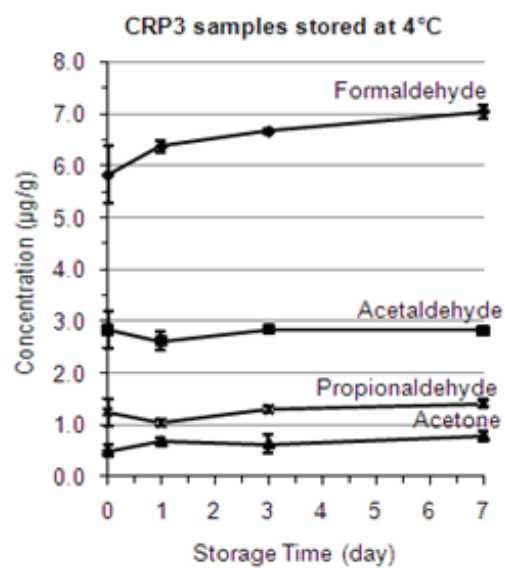
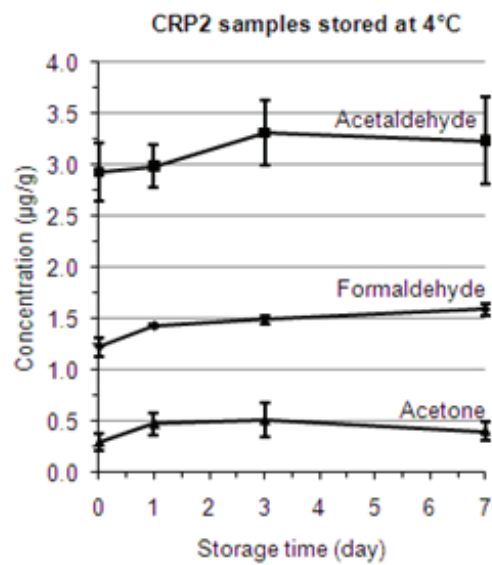
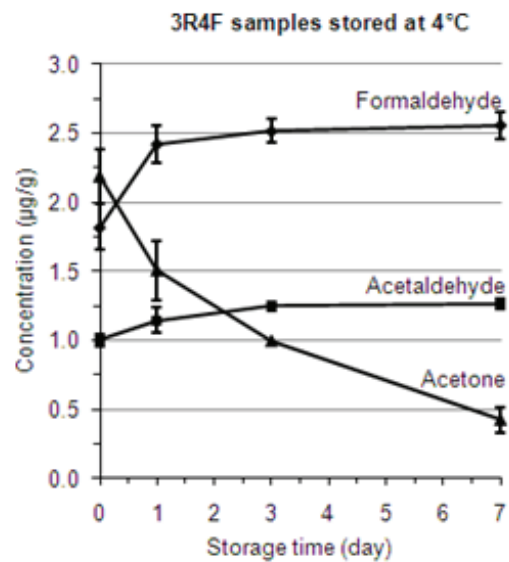


Figure 5. Effects of storage time at 4°C on the yields of carbonyl compounds in tobacco samples.

For CRP2 and CRP3 samples stored in a refrigerator, the concentrations of formaldehyde gradually increased during 7 days storage period. For other detected carbonyls, acetaldehyde, acetone and propionaldehyde, no significant changes on yields were determined during 7 days storage period (Figure 5). When CRP2 and CRP3 were stored at ambient temperature (Figure 6), the concentrations of formaldehyde determined in both tobacco samples on day 7 were significantly higher than that detected on day 0. For CRP2, the concentrations of acetaldehyde found on day 7 were significantly lower as compared to day 0 results. For CRP3, yields of acetaldehyde determined on day 0 and day 7 showed no significant difference.

CONCLUSIONS

The results of this study demonstrate that PFBHA derivatization followed by GC-MS analysis shows excellent performance: with a simple sample preparation procedure, clean chromatograms with no interference, good linearity, good accuracy and precision. By using multiple isotope labeled carbonyl compounds as internal standards, this method was validated with different reference tobacco products and proved to be suitable for the analysis of 7 carbonyl compounds in cigarette filler and smokeless tobacco products. Stability tests demonstrate the need for proper sample handling before and during sample analysis,

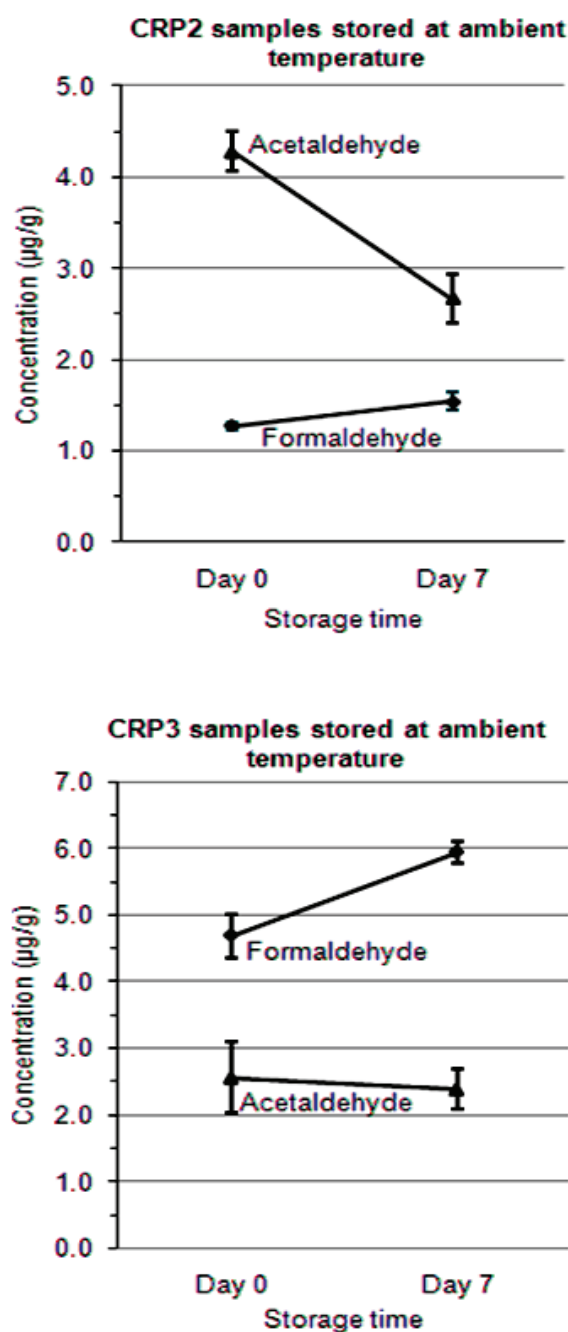


Figure 6. Effects of storage time at ambient temperature on the yields of carbonyl compounds in tobacco samples.

and also the need for timely sample analysis. Given the relative instability of the studied carbonyl compounds in the tobacco samples even stored in refrigerator, the tobacco samples should be kept in a freezer (-20°C) until analysis and once moved out from the freezer, all samples should be extracted and derivatized as soon as possible.

REFERENCES

1. Health Canada: Tobacco Reporting Regulations; The Canada Gazette Part II, June 29, 2005, available at: <http://www.hc-sc.gc.ca/hc-ps/tobac-tabac/legislation/reg/indust/index-eng.php> (accessed August 2014).
2. Liu, C., K.G. McAdam, and T.A. Perfetti: Some Recent Topics in Cigarette Smoke Science; *Mini Rev. Org. Chem.* 8 (2011) 349–359.
3. Food and Drug Administration: Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke; Established List, Federal Register Vol. 77, No. 64/Tuesday, April 3, 2012/Notices.
4. Health Canada: Official Method T-104 Determination of Selected Carbonyls in Mainstream Tobacco Smoke, made by the Department of Health, dated December 31, 1999.
5. CORESTA Recommended Method No. 74: Determination of Selected Carbonyls in Mainstream Cigarette by HPLC (second edition); Cooperation Centre for Scientific Research Relative to Tobacco, 2013, available at: [http://www.coresta.org/Recommended_Methods/CRM_74-update\(March13\).pdf](http://www.coresta.org/Recommended_Methods/CRM_74-update(March13).pdf) (accessed August 2014).
6. Dong, J.Z. and S.C. Moldoveanu: Gas Chromatography-Mass Spectrometry of Carbonyl Compounds in Cigarette Mainstream Smoke After Derivatization with 2,4-Dinitrophenylhydrazine; *J. Chromatogr. A* 1027 (2004) 25–35.
7. John, H.M. IV, P.G. William, and R.G. Richardo: UHPLC Separation with MS Analysis for Eight Carbonyl Compounds in Mainstream Tobacco Smoke; *J. Chromatogr. Sci.* 48 (2010) 12–17.
8. Risner, C.H. and P. Martin: Quantitation of Formaldehyde, Acetaldehyde, and Acetone in Sidestream Cigarette Smoke by High-Performance Liquid Chromatography; *J. Chromatogr. Sci.* 32 (1994) 76–82.
9. Fujioka, K. and T. Shibamoto: Determination of Toxic Carbonyl Compounds in Cigarette Smoke; *Environ. Toxicol.* 21 (2006) 47–54.
10. Stepanov, I., J. Jensen, D. Hatsukami, and S.S. Hecht: New and Traditional Smokeless Tobacco: Comparison of Toxicant and Carcinogen Levels; *Nicotine Tob. Res.* 10 (2008) 1773–1782.
11. US Environmental Protection Agency: Method 556.1 Determination of Carbonyl Compounds in Drinking Water by Pentafluorobenzylhydroxylamine Derivatization and Capillary Gas Chromatography with Electron Capture Detection; US EPA, Cincinnati, OH, USA, 1998, available at: <http://www.epa.gov/safewater/methods/pdfs/methods/met556.pdf> (accessed September 2014)
12. Bao, M.L., F. Pantani, O. Griffini, D. Burrini, D. Santianni, and K. Barbieri: Determination of Carbonyl Compounds in Water by Derivatization-Solid-Phase Microextraction and Gas Chromatographic Analysis; *J. Chromatogr. A* 809 (1998) 75–87.
13. Yu, J., H.E. Jeffries, and R.M. Le: Identifying Airborne Carbonyl Compounds in Isoprene Atmospheric Photooxidation Products by their PFBHA Oximes Using Gas Chromatography/Ion Trap Mass Spectrometry; *Environ. Sci. Technol.* 29 (1995) 1923–1932.
14. Deng, C., N. Li, W. Zhu, J. Qian, X. Yang, and X. Zhang: Rapid Determination of C6-Aldehydes in Tomato Plant Emission by Gas Chromatography-Mass Spectrometry and Solid-Phase Microextraction with On-Fiber Derivatization; *J. Sep. Sci.* 28 (2005) 172–176.
15. Saison, D., D.P. De Schutter, F. Delvaux, and F.R. Delvaux: Determination of Carbonyl Compounds in Beer by Derivatization and Headspace Solid-Phase Microextraction in Combination with Gas Chromatography and Mass Spectrometry; *J. Chromatogr. A* 1216 (2009) 5061–5068.
16. Iglesias, J., J.M. Gallardo, and I. Medina: Determination of Carbonyl Compounds in Fish Species Samples with Solid-Phase Microextraction with On-Fibre Derivatization; *Food Chem.* 123 (2010) 771–778.
17. Deng, C. and X.M. Zhang: A Simple, Rapid and Sensitive Method for Determination of Aldehydes in Human Blood by Gas Chromatography/Mass Spectrometry and Solid-Phase Microextraction with On-Fiber Derivatization; *Rapid Commun. Mass Spectrom.* 18 (2004) 1715–1720.
18. Hoffmann, D., J.D. Adams, D. Lisk, I. Fisenne, and K.D. Brunnemann: Toxic and Carcinogenic Agents in Dry and Moist Snuff; *J. Natl. Cancer Inst.* 79 (1987) 1281–1286.

Corresponding author:

*William S. Rickert
Labstat International ULC
262 Manitou Dr.
Kitchener, ON N2C1L3, Canada
E-mail: wrickert@labstat.com*