Journal of Chromatography A 1634 (2020) 461657

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Analysis of non-derivatized 2-(4-R-2,5-dimethoxyphenyl)-N-[(2-hydroxyphenyl)methyl]ethanamine using short column gas chromatography – mass spectrometry

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ARTICLE INFO

Article history:
Received 15 June 2020
Revised 22 October 2020
Accepted 26 October 2020
Available online 29 October 2020

Keywords:
GC-MS
Short column
25R-NBOH
Thermal degradation

ABSTRACT

The 25R-NBOH family is a group of thermally labile compounds that are relevant for forensic sciences and traditionally analyzed by GC-MS after derivatization — a step that is time consuming in a routine work. In this paper, the use of short analytical columns (4 and 10 m) showed to decrease compound degradation in the GC oven during chromatographic separation and to allow the analysis of non-derivatized 25R-NBOH compounds by GC-MS. A shorter column demanded a higher gas flow rate, and both factors decreased residence time of the analytes in the column and their degradation. The inlet temperature (250° C or 280°C) did not impact the response of 25R-NBOH. A 25R-NBOH fragmentation pathway by electron ionization was also presented for the first time. The GC-MS method with a 4 m column was successfully applied to other compounds of forensic interest, and it can be tested in the analysis of biological samples in toxicological investigations.

1. Introduction

The family of 2-(4-R-2,5-dimethoxyphenyl)-N-[(2-hydroxyphenyl)methyl] ethanamine (25R-NBOH, with R being a halogen or an ethyl group) is emerging as LSD alternatives in the illicit drug market [1,2], associated with blotter paper seized in Brazil [3,4] and other countries [5]. They are potent serotonin receptor agonists [6], responsible for subjective and behavioral effects [7]. 25R-NBOH compounds are also metabolites of 25R-NBOMe [8,9,10], which have been reported to be involved in human intoxications [2,11].

Until now, the thermally labile compounds of the 25R-NBOH family were considered unsuitable for GC-MS analysis unless they went through a derivatization step [12,13,14]. Other authors confirmed the presence of 25R-NBOH in blotter papers through the detection of 2C-R, which is the degradation product on GC-MS [15,16]. LC-QTOF-MS [3] and LC-MS/MS [1,17] have also been used to analyze 25R-NBOH in blotter papers. However, these equipment are not available in most forensic laboratories, which rely on GC-MS analysis for routine work. Hence, a method that allows the analysis of 25R-NBOH and other thermally labile substances by this technique would be of great use not only in the forensic scenario but also in analytical chemistry laboratories in general.

There are many factors that can affect the thermal degradation of the analyte on the GC-MS system, such as injector and oven temperatures, liner type, and contact time with the analytical column [18]. To minimize the analyte breakdown, some authors have used deactivated liners, lowered the oven temperature, and shortened the column [19–21]. The use of short column has been successfully implemented for the analysis of the thermally labile pesticide aldicarb [21,22]; the anabolic steroid stanozolol, which exhibits poor gas chromatographic behavior [21,23]; and the synthetic cannabinoids, which require extended methods and high oven temperature [24,25].

The aim of this study was to investigate the use of short columns (4 m and 10 m) for 25R-NBOH GC-MS analysis, without derivatization. Aldicarb, stanozolol, the synthetic cannabinoids JWH-081 and JWH-210 were also analyzed and used as parameter of thermal degradation behavior, column reactivity, and reduction of elution temperature.

2. Materials and methods

2.1. Chemical and reagents

Methanol, n-octadecane and N-trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) were purchased from Sigma Aldrich (USA); acetonitrile (ACN) and chloroform LC–MS grade were purchased from Scharlau (Barcelona, Spain). Certified reference standards of

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https://doi.org/10.1016/j.chroma.2020.461657
0021-9673/

25C-NBOH, 25B-NBOH, 25E-NBOH and 25I-NBOH were purchased from Cayman Chemical (USA); 2C-B was donated by the United Nations Office on Drugs and Crime (UNODC); 2C-I, JWH-081, and JWH-210 were provided by the United States Drug Enforcement Administration (DEA). Aldicarb was purchased from AccuStandard (USA). Stanozolol standard solution was prepared in ACN (500 µg/mL) from material seized by the Civil Police of the Federal District, Brazil, after being characterized and having its purity determined by NMR analysis. Standard solutions of 25B-NBOH, 25C-NBOH, 25E-NBOH, 25I-NBOH (200 and 400 µg/mL each), aldicarb (200 µg/mL), 2C-B, and 2C-I (400 µg/mL each) were prepared using methanol. JWH-081 and JWH-210 were diluted in acetonitrile. All standards were analyzed in triplicate. Individual blotter papers (from 36 to 100 mm^2) seized by the Civil Police of the Federal District, Brazil, were put inside a vial containing 1 mL of methanol, sonicated for 5 min and the extract analyzed by GC-MS.

2.2. Chromatographic conditions

GC–MS analyses were performed using an Agilent 7890A gas chromatograph coupled to a 5975C mass spectrometer and the system controlled by Agilent Chemstation Software version E 02.02.1431 (Agilent Technologies, Santa Clara, CA, USA). Results were also evaluated by the Agilent MassHunter Qualitative Analysis, version 10.0.

Instrumental parameters were adjusted as follows: Scan mode (40-500 m/z), 1µL injection volume, 20:1 split ratio, deactivated Ultra Inert liner without wool. Four injector port temperatures were evaluated: 150°C, 200°C, 230°C, 250°C, and 280°C. GC oven temperature programming was initiated at 50°C, followed by a ramp of 20°C/min up to 280°C, which was held for 7 min; total run time was 18.5 min and solvent delay was 2 min. MS conditions were ionization energy of 70 eV, ion source temperature at 280° C and interface heated to 280°C.

J&W Ultra Inert DB-1mscolumns (0.25 mm I.D., 0.25 μm film thickness; Agilent Technologies, # 122-0132) of 30, 10, and 4 m lengths were used. The helium gas flow was 1 mL/min for the 30 m column, 2 mL/min for the 10 m column and 4 mL/min for the 4 m column, to maintain a minimum positive pressure in the system. Thus, shortened columns have a lower pressure system compared to 30 m-column system.

2.3. Chromatographic performance of the qualitative GC-MS method for 25R-NBOH using a 4 m column

Selectivity of the method was assessed by analyzing a blank blotter paper (drug free) for any response at the 25R-NBOH retention times for possible interferences. The limit of detection (LOD) was set at a signal-to-noise ratio of 3:1 (n=3). The repeatability of the retention time was estimated by injecting each analyte 15 times (same day and different days) in the GC-MS and expressed as relative standard deviation (RSD, %). Peak width, number of theoretical plates per column, peak symmetry, tailing factor and resolution were estimated by the Agilent MassHunter Qualitative Analysis. Mass loading was assessed by determining the peak tailing [26,27] of two-fold serial dilution solutions (n=6) of 25E-NBOH (3880 to 243 µg/mL) and n-octadecane (5000 to 312 µg/mL), dissolved in methanol:chloroform (1:1).

2.4. Derivatization of 25R-NBOH

The 25R-NBOH analytical standards were derivatized with MSTFA as described previously for 25I-NBOH [13]. In summary, 100 µL of each standard (800 µg/mL) was added to a vial, the liquid evaporated under vacuum (Genevac EZ-2 series, United Kingdom) at room temperature, 150 µL of MSTFA was added, vortexed for 30s and kept at 70 °C for 1h in a dry oven. Each derivatized standard solution was directly injected into the GC-MS with a 4 m column.

3. Results

Table 1 shows the chemical structure, molecular formulas, molecular ions of the 25R-NBOH and 2C-R, in addition to their major fragments in the mass spectrometer. The fragmentation pattern of the 25R-NBOH family was described for the first time and will be discussed later in this paper.

3.1. Behavior of the analytes under different column lengths

First, the chromatographic behavior of the 25R-NBOH analytical standards (at 200 µg/mL) was assessed using a standard 30 m column. Only the degradation products (2C-R) were detected (data not shown), as it was previously reported for 25I-NBOH [3]. When the 10 m column was used, in addition to the degradants, the intact 25R-NBOH molecules were detected, but elevated baselines were also observed on the chromatograms (Fig. 1A, C, E, G). With a 4 m column, the peak area and shape of the 25R-NBOH compounds were dramatically improved, while the area of the 2C-R degradants was reduced and, in some cases, they were undetected, considering a signal-to-noise ratio of 3:1 as the LOD, even when higher 25R-NBOH concentration (400 µg/mL) was used (Fig. 1B, D, F, H).

The detectability of 2C-B and 2C-I (400 µg/mL) was confirmed using a 4 m column (Fig. 2A) and the four 25R-NBOH compounds had a satisfactory chromatographic separation in the short column (Fig. 2B). Fig. 2C illustrates the application of the method for the analysis of a seized blotter paper containing 25E-NBOH. Additionally, the proposed method is also suitable for a multi-compound forensic screening, performing well for 25R-NBOMes and was used to detect LSD in a blotter paper (Fig. S1, Supplementary Material).

Fig. 3 shows the GC-MS electron ionization mass spectra of the NBOH compounds and Fig. 4 the fragmentation pathway proposed for the drug family. Low abundance molecular ions (M^+) were observed for each compound (*m/z* 365/367, 321, 315, and 413). Five common fragmentation routes can be seen: loss of methoxy radical ($[M\text{-}OCH_3]^+$ at *m/z* 334, 290, 284, and 382 respectively), which was also observed for the 2C-R compounds (Fig. S2); cleavage of the C–N bond, leading both to the common *o*-cresol ion (*m/z* 107) and to the 2C-R fragments; cleavage of the benzylic C—C bond with hydrogen migration, leading to radical cation fragments (*m/z* 230/232, 186, 180, and 278); and the cleavage of the C–aromatic ring, producing a fragment at *m/z* 136, also a common ion to all NBOH.

As expected, synthetic cannabinoids JWH-210 and JWH-081, which are high boiling point compounds, were detected in a 30 m column using the GC conditions recommended by UNODC (240°C, for 1 min and 6°C/min to 310°C, for 8 min) [24] (Fig. S3). When the compounds were analyzed using a 10 m column and the GC-MS conditions optimized for the present study, which uses a milder oven temperature, the instrument responses were about 2 times higher, the elution times were drastically reduced, and the two JWH-081 isomers were separated (Fig. S3). The peak area and the shape of stanozolol, a synthetic steroid hormone, were drastically improved when using a 10 m chromatographic column (Fig. S4).

3.2. Effect of the inlet temperature

Lowering the inlet port temperature is one option to reduce thermal degradation of the analyte during the GC-MS analysis, but it should not compromise solvent evaporation in the inlet. For the 25R-NBOH, the inlet temperatures (250 °C or 280 °C) practically did not affect the instrument response of the parent compounds (Fig. 1B, D, F, H). Fig. 5 shows the impact of inlet tem-

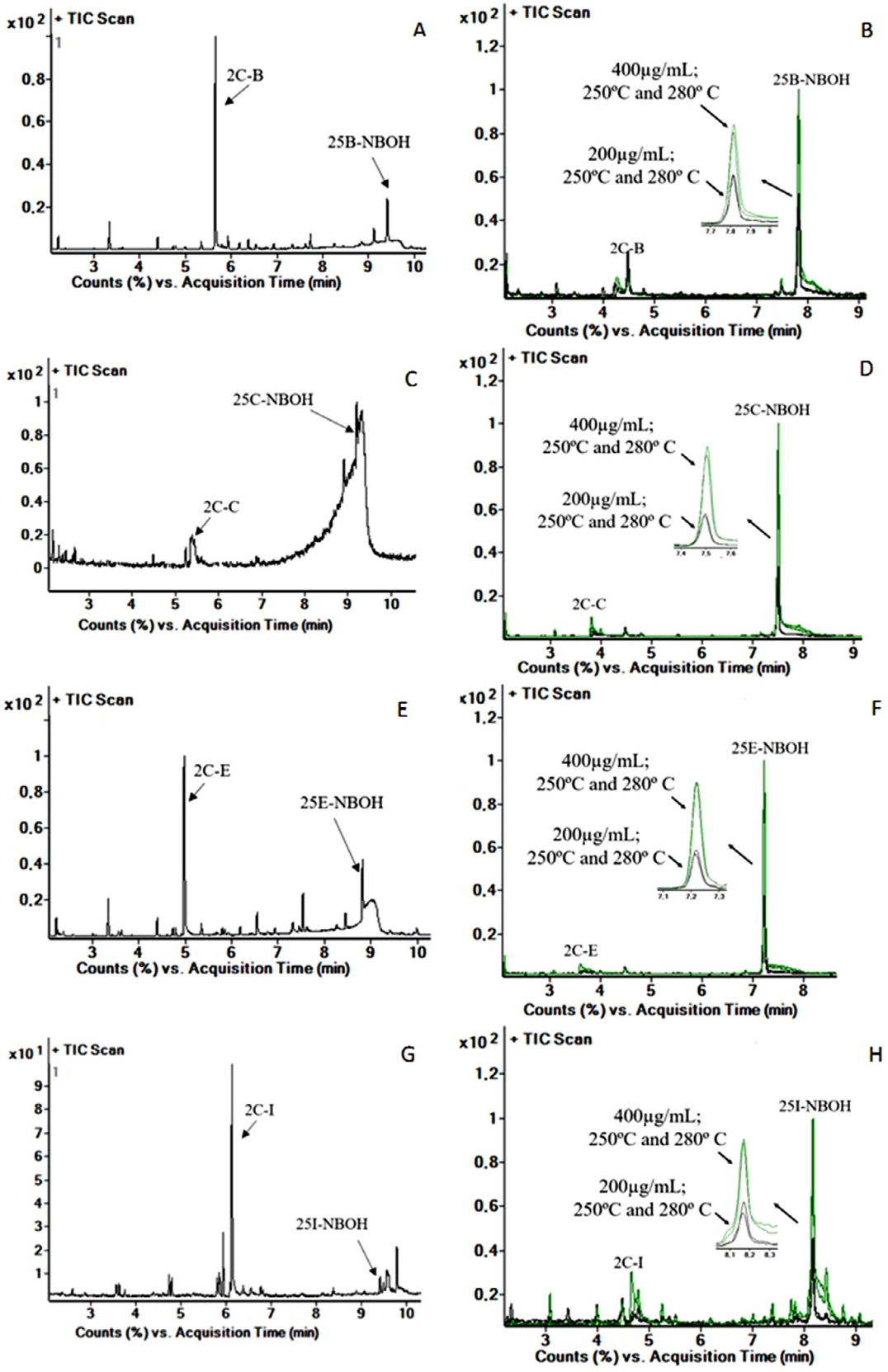
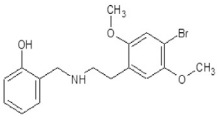
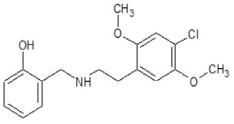
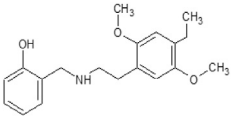
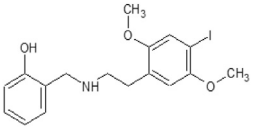
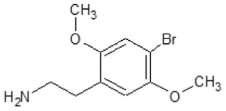
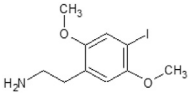
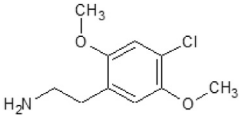
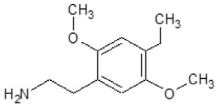


Fig. 1. GC-MS total ion chromatogram of 25R-NBOH with 10 m and 4 m columns. 25B-NBOH, A:10 m column and B: 4 m column; 25C-NBOH, C:10 m column and D: 4 m column; 25E-NBOH, E: 10 m column and F: 4 m column; 25I-NBOH, G:10 m column and H: 4 m column. Inlet temperatures 250° C and 280° C were evaluated (n=3, each) and no statistically significant differences were observed.

Table 1
Chemical structures, molecular formulas, molecular ions and the major fragments of the 25C-NBOMe, 25R-NBOH and 2C-R.

Substance	Chemical structure	Molecular formula	Molecular ion	Major fragments, m/z
25B-NBOH		$C_{17}H_{20}BrNO_3$	365	107, 136, 230-232
25C-NBOH		$C_{17}H_{20}ClNO_3$	321	107, 136, 186
25E-NBOH		$C_{19}H_{25}NO_3$	315	107, 136, 180, 165
25I-NBOH		$C_{17}H_{20}INO_3$	413	107, 136, 278
2C-B		$C_{10}H_{14}BrNO_2$	260	259-261, 230-232, 215-217
2C-I		$C_{10}H_{14}INO_2$	307	278, 262, 307
2C-C		$C_{10}H_{14}ClNO_2$	215	215, 186, 171
2C-E		$C_{12}H_{19}NO_2$	209	209, 180, 165

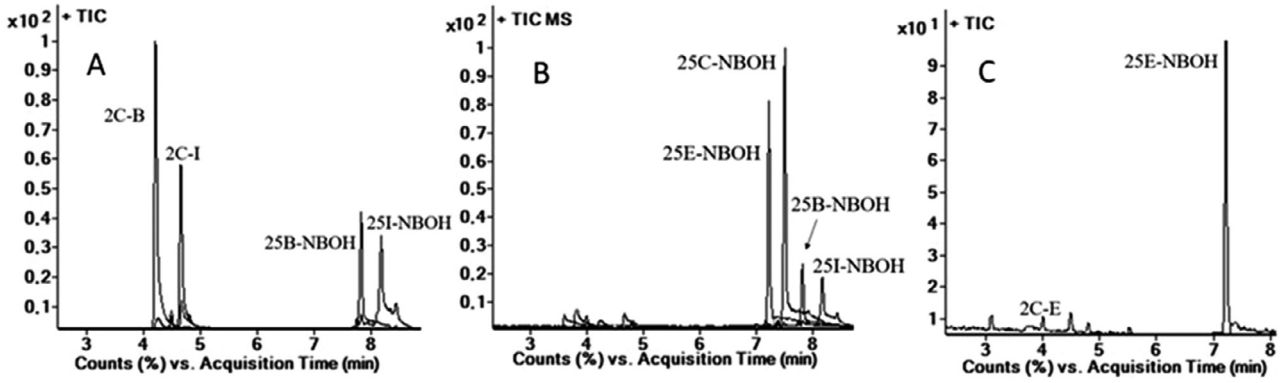


Fig. 2. GC-MS total ion chromatogram using a 4 m analytical column. A: 2C-B, 2C-I, 25B-NBOH and 25I-NBOH at 400µg/mL; B: 25R-NBOH. C: Blotter paper containing 25E-NBOH (signal-to-noise of 2C-E < 1:3).

perature (from 150 to 280 °C) on the response of 25C-NBOH, aldicarb and stanozolol, which are models for thermally labile and low volatile reactive compounds, respectively. Increasing the inlet temperature from 150 to 200 °C increased the response of all three compounds, but higher temperatures lead to the loss of aldicarb response, which disappear completely at 280 °C. While for stanozolol the response remains constant from 230 to 280 °C, the optimum inlet temperature for 25C-NBOH is reached at 250 °C (Fig. 5).

3.3. Performance of the 4 m column GC-MS qualitative method

The method was shown to be selective as no peaks were found near the 25R-NBOH eluting times. The LOD was found at 5 or 10 µg/mL, repeatability of the retention times was lower than 0.2%,

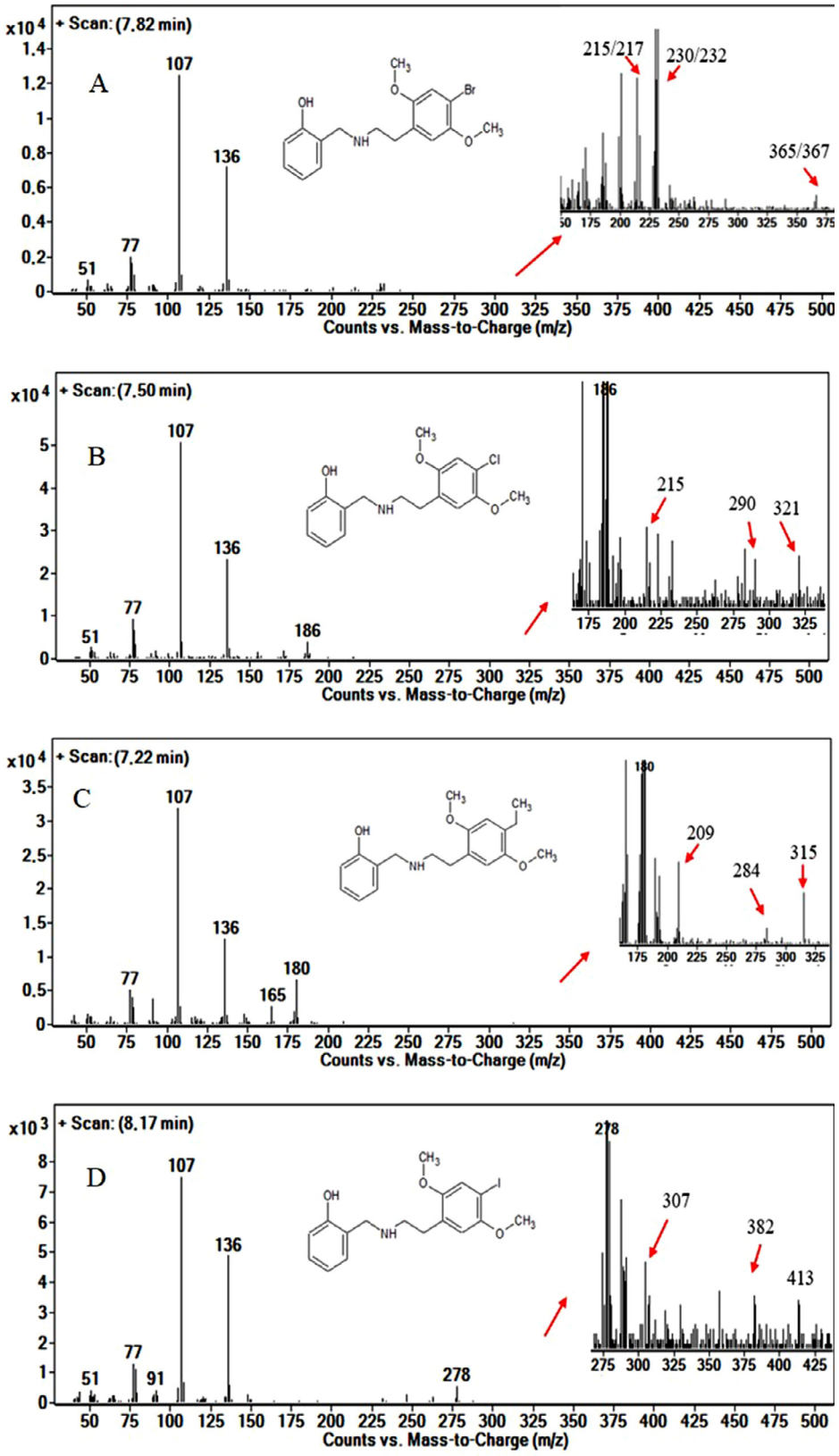


Fig. 3. Fragmentation of 25R-NBOH using a 4 m column. A: 25B-NBOH; B:25C-NBOH; C: 25E-NBOH and D: 25I-NBOH.

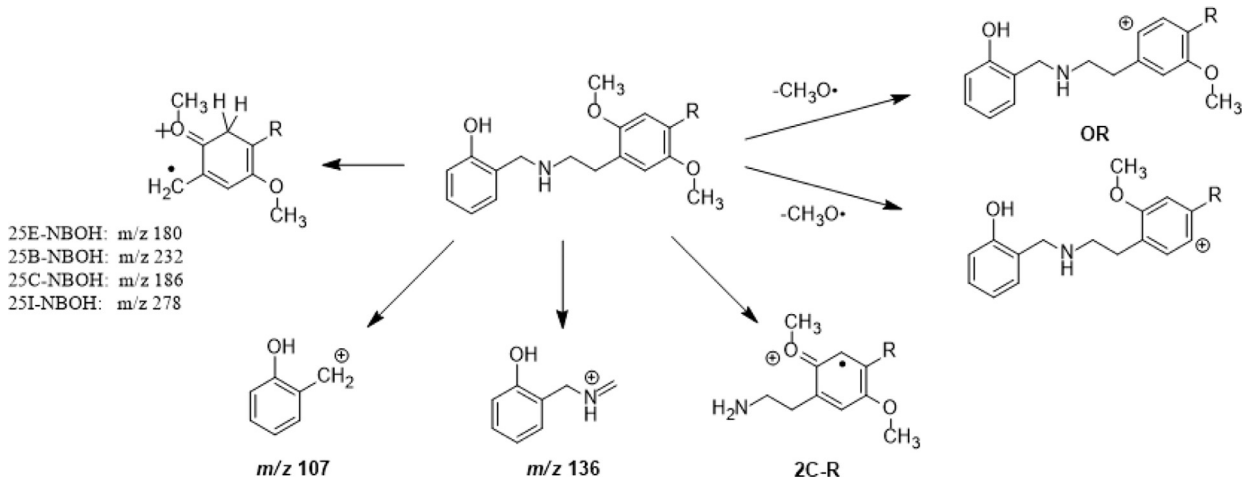


Fig. 4. Proposed fragmentation pathway for 25R-NBOH compounds using electron ionization. R = 25B-NBOH: Br; 25C-NBOH: Cl; 25E-NBOH: C_2H_5; 25I-NBOH: I. 2C-R = 2C-B (m/z 260); 2C-C (m/z 215); 2C-E (m/z 209); 2C-I (m/z 307).

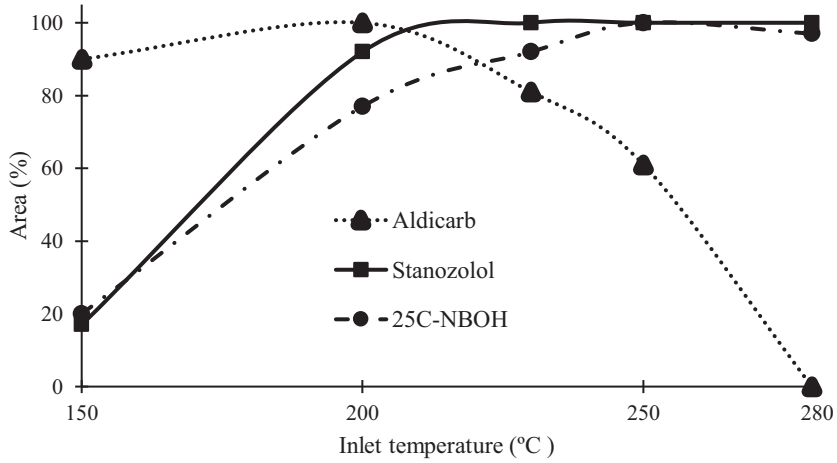


Fig. 5. Peak area percentage of aldicarb, stanozolol and 25C-NBOH using different inlet temperatures (150, 200, 230, 250 and 280 °C) in the CG-MS method (4 m column).

Table 2
Chromatographic parameters of 25R-NBOH at 200 µg/mL.

Substance	LOD (µg/mL)	RT repeatability (RSD, %)	Peak width (W, min.)	Theoretical plate (N)	Symmetry	Tailing factor
25B-NBOH	5	0.13	0.203	212372	0.96	1.7
25C-NBOH	5	0.09	0.197	228727	0.94	1.1
25E-NBOH	5	0.07	0.145	195329	0.83	1.35
25I-NBOH	10	0.06	0.227	185936	0.99	1.1

LOD: Limit of detection; RT = retention time; RSD= relative standard deviation.

peak width was below 0.3, peak symmetry higher than 0.8 and maximum tailing factor was 1.7 (Table 2). Resolution was higher than 1.5 in all cases (data not shown). Even at high mass loading of the analyte (25E-NBOH), the peak tailing was within the expected range, and similar to that of n-octadecane (Fig. S5; Supplementary Material).

3.4. Analysis of derivatized 25R-NBOH

The method performance of MSTFA derivatized 25R-NBOH was also evaluated using the 4 m column, and is illustrated in Fig. 6 for 25C-NBOH. The 2C-R degradant was not detected and the silylation generated two derivatives, mono-TMS, with m/z 73, 179 e 208 as most abundant fragments, and di-TMS, with m/z 73, 179, 280. The mono-TMS and di-TMS fragmentation pattern was quite similar for all the 25R-NBOH tested (Fig. S6, S7 and S8).

4. Discussion

Thermolabile and reactive substances exhibit poor gas chromatographic behavior and are a challenge for the analyst [18,19]. Although derivatization of the analyte before GC-MS analysis can overcome most of the problems [12], there is a burden of increasing complexity, analysis time, and cost.

Among the parameters related to sample degradation during the GC–MS analysis, those related to the residence time in the inlet, chromatographic column and oven temperature are the most important. Since the separation in the GC column is driven mainly by the analyte boiling points, temperature is normally the first parameter thought as being related to compound degradation [19]. Furthermore, the time the compound spends in the GC oven depends on the length of the column and/or the carrier gas flow rate,

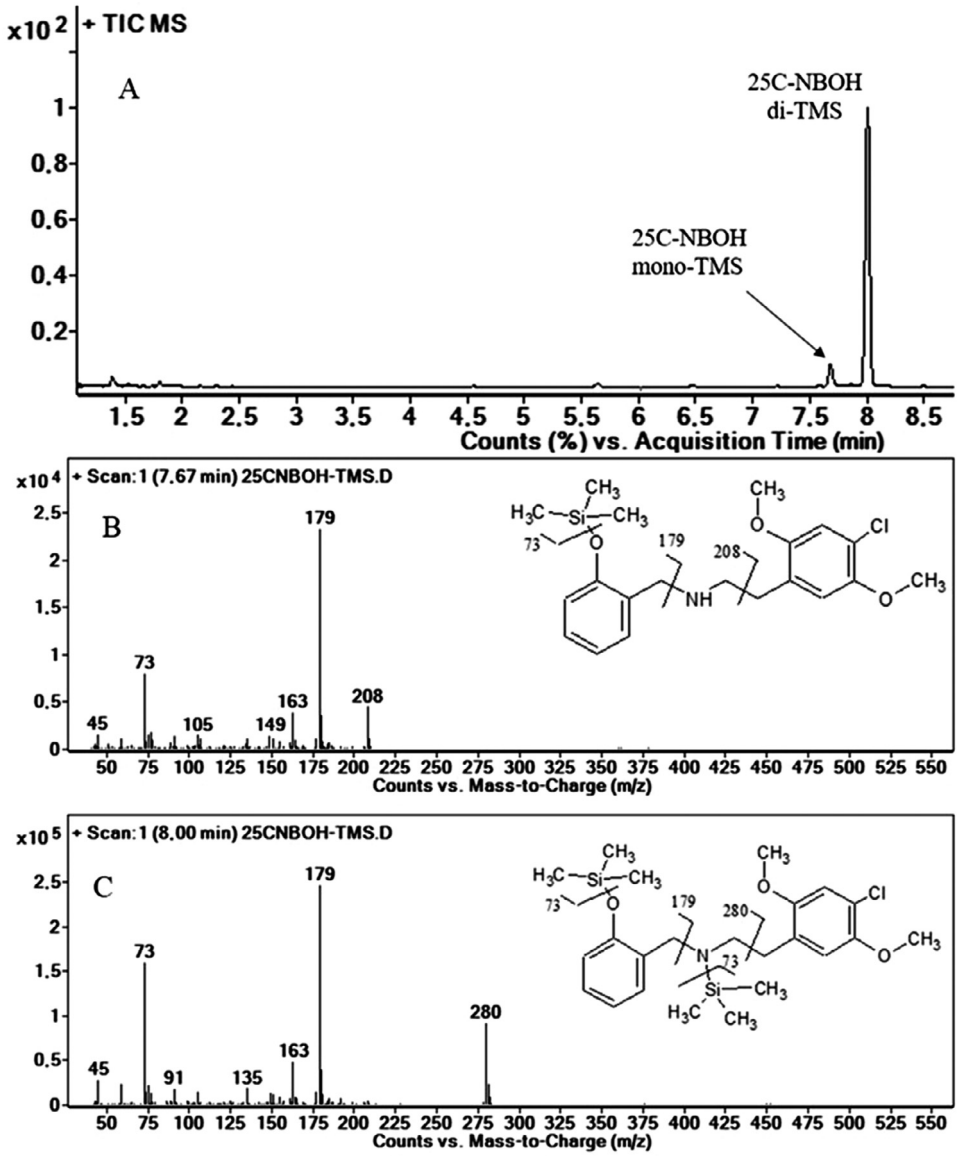


Fig. 6. A: GC-MS total ion chromatogram of 25C-NBOH derivatized with MSTFA, using a 4 m column; B: fragmentation and molecule structure of 25C-NBOH mono-TMS and C: 25C-NBOH di-TMS.

which will affect the degradation and the chromatographic performance of the analyte [22].

Thermal stability of the 25I-NBOH has been evaluated by thermogravimetric analysis and differential scanning calorimetry [13]. The authors concluded that the small temperature window between fusion and decomposition (203° C and 265° C, respectively) had a direct effect on its thermal stability. Previous work conducted by our research group has indeed showed that 25I-NBOH is not detected by GC-MS using a standard 30 m column, and the identification of this compound in blotter papers relied on the detection of its degradation derivative (2C-I) [15]. In the present study, the use of short columns to reduce the 25R-NBOH degradation during the GC separation phase was explored. Short column length reduces the elution temperature of the substances, which is important for thermolabile compound analysis [18]. With the 10 m column, the 25R-NBOH molecules were detected, but the chromatographic performance was poor, with the presence of an elevated baseline. When the NBOH compounds were analyzed with a 4 m column, the chromatographic performance improved considerably, showing a strong positive correlation between column length and analyte degradation and a negative correlation between column length and peak shape. With short columns, the gas flows were higher, which reduces the elution time and temperature of the analyte into the column [18,20].

The respective degradation products (2C-R) were drastically reduced at both 25R-NBOH concentrations tested (200 and 400 µg/mL). The detection of 2C-R compounds as 25R-NBOH degradant has been also demonstrated in “cold chromatographic” analysis. Arantes et al. [3] analyzed 25I-NBOH by LC–QTOF-MS using different fragmentor voltages and demonstrated that the fragmentation could be induced in the region between the end of the transfer capillary and the first skimmer cone, producing 2C-I on the chromatogram.

Previous studies have not only successfully analyzed synthetic cannabinoids [25], stanozolol [21,23], and aldicarb [21,22] using short columns, but also demonstrated that the use of short columns improved the peak shape and the detectability of those analytes. The combination of short column and higher gas flow

provides high signal-to-noise ratio, lower elution temperature [18], improvements that were confirmed in the present study. The use of a 10 m column and low oven temperatures did increase the instrument response for JWH-081 and JWH-210 compared to the recommended method [24], while drastically reduced the elution temperatures and retention times of these compounds.

Increasing the inlet temperature from 250 to 280 °C did not impact the 25R-NBOH response in the GC-MS, although some studies have hypothesized that this may occur [13,15]. Increasing the inlet temperature also did not impact the stanozolol response, but had a significant impact on aldicarb, which is highly thermolabile [21].

The chromatographic GC-MS parameters evaluated showed that the 4 m column method fits the purpose for a qualitative method. The method was selective, and all the substances tested were unequivocally identified. LODs of 5 µg/mL (25B-NBOH, 25C-NBOH and 25E-NBOH) or 10 µg/mL (25I-NBOH) were considered acceptable to analyze the real samples using only one blotter paper [28]. Repeatability of the retention times, tailing factor, theorical plate number, resolution and mass loading were considered suitable for a qualitative analysis [26,27,28].

The fragmentation pathway for derivatized 25R-NBOH (mono-TMS and di-TMS) was shown to be similar for all four compounds of the family and was previously reported for 25I-NBOH [13]. Although the retention time of the derivatized compounds are different, the poor structure information of the derivatized compound can be a limitation for the detection of new compounds from the same class that shows up in the market in the future. The proposed 4 m column GC-MS method was also able to detect and discriminate other compounds commonly present in blotter papers, such as LSD and the 25R-NBOMe family.

To the best of our knowledge, this is the first time that intact 25R-NBOH molecules with good chromatographic peak shapes were obtained by GC-MS analysis without the need of a derivatization step. A fragmentation pattern that is common to all compounds within the family was also proposed, and included the loss of methoxy radical and the hydroxyl of the phenol group, and cleavage of the C–N bond to form the 2C-R degradants. The fragmentation pattern is similar to that for 25C-NBOMe proposed by Zuba et al. [29], which shows the fragments m/z 121 formed by the cleavage of the C-N bond yielding 2-methoxybenzyl cation and of m/z 150 corresponding to iminium cation formed by the dissociation of the α- and β-carbon atoms. For the 25C-NBOH, these pathways lead to the m/z 107 and m/z 136 respectively, which are characteristic fragments of the family. Complementary to the m/z 107, the 2C-R compound molecular ions were detected. A fragmentation pattern of 25R-NBOH molecules was proposed by Machado et al. [16] based on the work conducted by Coelho Neto et al. [15] and Zuba et al. [29], although the intact compounds were not detected in the GC-MS system. In their proposal, the fragmentation follows the thermal degradation of 25R-NBOH into 2C-R and o-cresol, therefore different from the present proposal, which is based on the mass spectra of the intact 25R-NBOH compounds and their prominent ion fragments.

6. Conclusion

A 4 m column GC-MS method for the detection of intact non-derivatized 25B-NBOH, 25C-NBOH, 25E-NBOH, and 25I-NBOH in seized materials was developed for the first time, and their fragmentation pathways using electron ionization were proposed for the first time. The method uses regular GC-MS setup, can be easily implemented in other laboratories and can be a way to overcome the poor detectability of thermolabile, high boiling point and reactive compounds in the forensic scenario. This method can also be further applied to biological matrices in cases of toxicological investigation, after appropriate matrix preparation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ettore Ferrari Júnior: Conceptualization, Methodology, Writing - original draft. **Luciano Chaves Arantes:** Methodology, Writing - review & editing. **Lívia Barros Salum:** Investigation. **Eloisa Dutra Caldas:** Supervision, Data curation, Writing - review & editing.

Acknowledgments

The authors thank A.D. Jones for helping to elucidate the fragmentation pathway of 25R-NBOH, and B.H.M. Leite for helping with the analysis of blotter papers.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461657.

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