Journal of Chromatography B 1133 (2019) 121824

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Multiple headspace sampling coupled to a programmed temperature vaporizer to improve sensitivity in headspace-gas chromatography. Determination of aldehydes

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ARTICLE INFO

Keywords:
Multiple headspace sampling
Programmed temperature vaporizer
Solvent vent mode
Increased sensitivity
Proof of concept
Aldehydes

ABSTRACT

The improvement of sensitivity in headspace (HS) sampling of not very volatile analytes constitutes a challenge that has usually been approached through coupling with additional techniques. Here we propose a new methodology for increasing sensitivity through a multistep approach. This proof of concept is based on direct coupling of a headspace sampler with a programmed temperature vaporizer (PTV) and a gas chromatograph (GC), with mass spectrometry (MS) detection. Analytes are extracted from the same vial in a stepwise procedure, splitting the headspace generation time of conventional HS into four periods and using the PTV to cryogenically trap the analytes during the successive HS samplings. Solvent vent mode is mandatory in order to retain the analytes, purging the gas solvent at an adequate initial low temperature and flash-heating the PTV liner in a quick ramp (720 °C/min), once the HS samplings are finished. Linear aldehydes, from pentanal to decanal, possible biomarkers of several diseases have been selected as model compounds. This multiple HS method has been compared with conventional HS, and it has been validated in terms of linearity, limits of detection, repeatability, reproducibility and accuracy. The limits of detection (LOD) ranged from 0.004 to 0.159 μg/L. Enrichment factors (EF) in relation to the conventional HS method ranged from 3.0 to 6.7, except for pentanal (EF: 0.8), which is too volatile and polar to be trapped in the PTV with the multiple HS methodology. Similar enrichment factors were obtained in a urine sample.

1. Introduction

Headspace sampling coupled to gas chromatography is known to solve many analytical problems by minimizing sample treatment, and eliminating interferences of major non volatile compounds [1]. However, in many cases the limited sensitivity and the discrimination towards the extraction of not very volatile compounds constitute a limitation and additional preconcentration steps are required [2]. Other techniques used for this purpose have been coupled with HS, such as headspace solid-phase microextraction (HS-SPME) [3,4], headspace single-drop microextraction (HS-SDME) [5], and headspace stir-bar sorptive extraction (HS-SBSE) [6].

The use of a programmed temperature vaporizer (PTV) inlet offers an alternative for increasing sensitivity. When using the solvent vent injection mode, analytes can be focused cryogenically in the liner of the injector, packed with different trapping materials, whilst major more volatile compounds, are eliminated. Later application of a rapid temperature ramp allows these analytes to be introduced into the GC column, with the advantage of a considerable narrowing of the chromatographic peaks [7,8].

In this work, a new use of the PTV coupled with HS, is proposed for enhanced analyte detection. It consists of performing multiple HS samplings from the same vial into the PTV cold inlet in solvent vent injection mode. A stepwise gas extraction is performed comparable to a repeated liquid extraction; the compounds are taken from the gas phase altering the equilibrium, which reestablishes again, generating a new amount of volatile compounds in each step. The liner of the PTV is used in this case as a cold trap, in a sort of dynamic extraction procedure and the split valve is open during given times after each sampling, in order to eliminate the gas solvent. Then the PTV inlet is ramped to a high final temperature to transfer the analytes into the GC column, and data collection is performed to obtain the chromatogram.

Concerning other multistep HS approaches, multiple headspace extraction (MHE) [9,10] was initially described as a procedure where

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https://doi.org/10.1016/j.jchromb.2019.121824
Received 30 July 2019; Received in revised form 23 September 2019; Accepted 3 October 2019
Available online 24 October 2019
1570-0232/

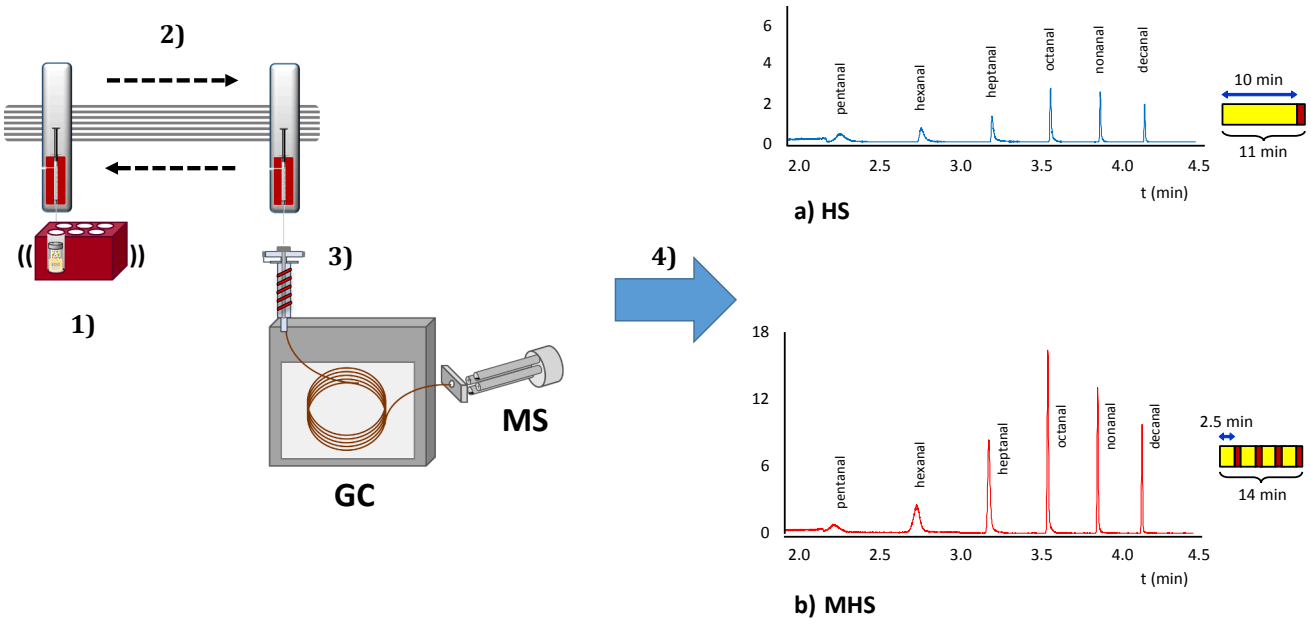

Fig. 1. Experimental setup of the proceeding used in multiple HS and conventional HS approaches. (**1**) HS generation time: (a) 10 min for conventional HS; (b) 4 × 2.5 min for optimum MHS (this work); (**2**) N samplings of headspace: (a) N = 1, 1 sampling without return to HS oven for conventional HS; (b) N = 4, 4 successive samplings with return to HS oven for optimum MHS (this work); (**3**) Solvent vent injection into PTV: (a) 1 injection; (b) 4 injections; (**4**) Transfer of analytes and separation in the GC column. HS generation time; transfer of gas sample and (a) injection in the PTV for conventional HS; (b) injection in the PTV plus return of syringe to the HS oven to withdraw a new sample from the same vial in MHS.

successive HS aliquots are equally sampled from the same vial. However, the purpose of MHE is to avoid the matrix effects by exhaustively extracting all volatiles from the sample. In this procedure several chromatograms are obtained, one per HS extraction; analyte concentration decays exponentially, and the total analyte peak area can be calculated as the sum of the areas of each individual extraction. In practice, a reduced number of extraction steps allows quantification, since extrapolation models can be applied. As well as headspace sampling, MHE has also been combined with SPME [11] and SDME [12], in order to broaden the applicability of these microextraction techniques to quantitative determination of analytes in complex liquid and solid matrices [13].

As a proof of concept, in this work a multistep HS sampling, in combination with PTV trapping through multiple injections per run is used to gain sensitivity. The possibilities of the proposed enrichment methodology have been checked using a family of volatile organic compounds (VOCs) as model analytes, specifically linear C5-C10 aldehydes, *i.e.* from pentanal to decanal. These analytes are of interest because they are lipid-peroxidation products, and have been included in different studies as possible biomarkers of several diseases, such as cancer [14–16]. Most of the current methods used for determination of biomarker aldehydes in aqueous solutions require derivatization reactions [17,18], which can be a source of additional errors and require an additional time to perform the analyses. A conventional HS-PTV-GC-MS method developed at our laboratory for determination of these aldehydes [19] was used as starting point to study this new approach.

2. Experimental

2.1. Reagents and standards

Pentanal, hexanal, heptanal, octanal, nonanal, and decanal (approx. 99% purity) were supplied by Sigma-Aldrich (Steinheim, Germany). Methanol of HPLC grade was purchased from Merck (Darmstadt, Germany) and sodium chloride was from Scharlau (Barcelona, Spain). Stock solutions (2500 mg/L in methanol) of each aldehyde were prepared and stored at 8 °C. These solutions were used to spike the samples at the different concentrations analysed. Optimization of the method was performed with ultra-high quality water (UHQ), obtained with a Wasserlab Ultramatic water purification system (Noain, Spain).

2.2. Sample preparation

In a 10 mL headspace vial, 2.4 g of NaCl were added, followed by 4.0 mL of aqueous or urine sample [19]. The vial was hermetically closed and placed in the autosampler tray. Automatic processing was then operated.

2.3. Headspace

HS sampling was performed with a MPS2 Multi-Purpose Sampler (Gerstel, Mülheim an der Ruhr, Germany). Operation mode included the transport of the vial into the heated six position incubator (set at 84 °C).

2.3.1. Conventional HS

The vial was shaken in the incubator at 750 rpm for 10 min to allow headspace conditioning; then the HS sample (2.5 mL) was withdrawn with a 2.5 mL gas-tight syringe (heated at 120 °C), transferred to the PTV injector and injected into the GC column. Fill speed and injection speed were fixed at 100 μL/s and 250 μL/s, respectively. After injection, the hot syringe was automatically cleaned purging with He (99.999%, Air Liquid) for 2 min.

2.3.2. Multiple HS

Temperature, volume and syringe conditions were the same as those used in conventional HS. The MPS2 sampler was operated in multiple injections mode, which is capable to perform successive transferences (up to 100) from the HS to the PTV, without injection into the GC column until the end of the whole process.

The vial was shaken in the incubator for 2.5 min to allow headspace conditioning; then the HS sample was withdrawn and it was transferred to the PTV injector, which acted as a cold trap. The process of 2.5 min headspace generation, withdrawing and transference to the PTV was

repeated three more times before the chromatographic run began. Fig. 1 shows a schematic setup of the procedure used in multiple HS and conventional HS approaches.

2.4. PTV-GC-MS conditions

Analyses of the aldehydes were performed on a GC–MS instrument (Agilent Technologies, Santa Clara, CA, USA) consisting of an Agilent 7890A series gas chromatograph equipped with a 6890 Agilent Technologies PTV injector and an Agilent 5975C inert XL MSD.

The method was developed from a previous one, optimized in our laboratory [19]. The PTV was operated in solvent vent mode. A liner packed with Tenax TA® (71 mm × 2 mm I.D., Gerstel CIS-4) was selected in the optimized methods. Cooling was accomplished with liquid CO_2. One transference (in conventional HS, after 10 min conditioning) or four consecutive samplings (in multiple HS, after each successive 2.5 min conditioning time) were made. During each transference to the PTV the split valve was opened, and temperature was set at 50 °C for 0.5 min (purge time, at a vent flow of 20 mL/min and vent pressure of 5.00 psi). Then the split valve was closed until the next injection into the PTV, repeating the previous process. Once the purge time after the last injection had finished, the split valve was closed and the liner of the PTV flash-heated at 720 °C/min up to 300 °C. The analytes were then splitless injected into the capillary column (1 min). After that, the split valve was opened again and the liner temperature was held at 300 °C during 4 min (cleaning step).

Aldehydes were separated on a HP-5MS UI (30 m × 0.25 mm, 0.25 μm) (J&W Scientific, Folsom, CA, USA). The carrier gas was He (99.999% pure; Air Liquide). The column oven temperature program began at 45 °C, held for 2.00 min; then it was increased at 60 °C/min until 175 °C and finally a 45 °C/min increase was applied until 240 °C, holding this temperature for 0.5 min. Once the chromatographic ramp had finished, the column was heated up to 250 °C and maintained at this temperature during 3 min (post-run time). The total chromatographic run time was 6.11 min.

The detector used was a quadrupole mass spectrometer, operated in electron ionization mode using an ionization voltage of 70 eV. The ion source temperature was 230 °C, and the quadrupole was set at 150 °C. The analyses were performed in synchronous scan/SIM mode which allowed collection of both full scan data and SIM in a single run. Full scan (25–160 *m/z*) was used for identification and SIM for quantification, selecting as quantitation ions (with a dwell time of 10 ms) the base peaks in the mass spectrum of each aldehyde, except for pentanal and hexanal, for which the second most intense peaks were selected given that *m/z* 44 also corresponds to the base peak of CO_2 (Table 1). A solvent delay of 2.0 min was established, during which the filament was turned off. Data acquisition was performed with an MSD ChemStation, Ver. E.02.00.493 software from Agilent Technologies. Compounds were identified using the NIST_98 database (NIST/EPA/NIH Mass Spectral Library, version 2.0).

2.5. Method validation

Limit of detection (LOD) and limit of quantification (LOQ) were calculated, as recommended by ISO 11843-1 [20] and other authors [21] for chromatographic analysis, by using the signal-to-noise ratio (S/N) criterion calculated by the expression:

$$\frac{S}{N} = 2\frac{H}{h}$$

where H is the height of the neat peak corresponding to the considered analyte and h is the range of the blank noise measured around the place where the analyte elutes. Values of H were calculated for a S/N = 3 (for LOD) and S/N = 10 (for LOQ) and the concentration was then calculated from each slope of the calibration curves.

Repeatability and reproducibility were determined as the relative standard deviations (RSDs) obtained at a 5 μg/L analyte concentration level. Conventional HS and multiple HS were performed ten times on the same day (repeatability) and three times per day on six different days (reproducibility). The validity of the linear regression models was checked by ANOVA.

Table 1
Aldehyde quantitation ions (in bold), qualifier ions, retention times, boiling points and octanol-water partition coefficients (log Kow).

Aldehyde	SIM ions (*m/z*)	t_R (min)	boiling point (°C) [22]	log Kow
Pentanal	44, **58**, 29	2.253	103	1.29 [23]
Hexanal	44, **56**, 41	2.784	129	1.80 [23]
Heptanal	**70**, 41, 44	3.212	153	2.32 [23]
Octanal	**43**, 44, 41	3.641	173	2.86 [23]
Nonanal	**57**, 41, 44	3.886	194	3.36 [23]
Decanal	**43**, 41, 44	4.212	213	3.71 [24]

3. Results and discussion

3.1. Multiple headspace enrichment approaches

Two multiple HS approaches were assayed, using the optimized conventional HS-PTV-GC-MS experimental conditions as a starting point [19].

In approach 1, an initial 10 min HS generation time was selected. In order to check the analyte enrichment, conventional HS sampling was compared with a stepwise gas transference (2, 4 and 8 steps, respectively) after the first one. No pause time for re-equilibration after the successive HS samplings was used, apart from the hardware cycle time (the time required for transferring each gas sample plus the return time of the syringe to the HS oven, to withdraw a new sample from the same vial). Fig. 2 plots the signals obtained, together with a diagram of the total times required. The relevance of analyte volatility and polarity (see Table 1) is evident; the higher values of these properties for pentanal determined that its signal was not improved, because it is the most volatile and polar of the studied analytes, which makes its trapping in the liner more difficult when the time associated with the process increases. The hexanal signal was initially improved, but it diminished when increasing the number of transferences. The rest of the aldehydes, less volatile and polar, were adequately retained in the PTV liner and their signals accordingly increased in all the experiments. Given the trends obtained adding more transference steps is not worthwhile.

In approach 2, the 10 min HS generation time was split into different periods: one (equivalent to conventional HS), two (5 min each), and four (2.5 min each) were assayed. HS gas phase was transferred to the PTV after each HS generation time. Fig. 3 compares the three chromatograms obtained, and the total times required.

In both approaches, after the syringe performs each HS extraction, a renewing of the partition gradient is generated in the headspace of the vial which contributes positively to the generation of a bigger amount of volatiles in the successive extractions. Somehow, this behaves as a not continuous, dynamic headspace generation in steps.

The best results (considering heptanal to decanal) were obtained with the maximum number of transferences (8, in approach 1) or maximum splitting of the HS generation time (four 2.5 min periods, in approach 2). Similar maximum enrichment results were obtained with approach 1 (for 10 min HS generation time and 8 HS transferences) and approach 2 (10 min HS generation time split in four 2.5 min periods). Repeatability of retention time values was similar for conventional HS and for both MHS approaches, with RSD values ranging from 0.01 to 0.14% (n = 10).

Regarding the required run times, the first approach at its optimum required 18 min (Fig. 2), versus the 14 min needed with the second approach (Fig. 3). Conventional HS demands 11 min. Therefore, both approaches can be used, but higher sample throughput will be obtained

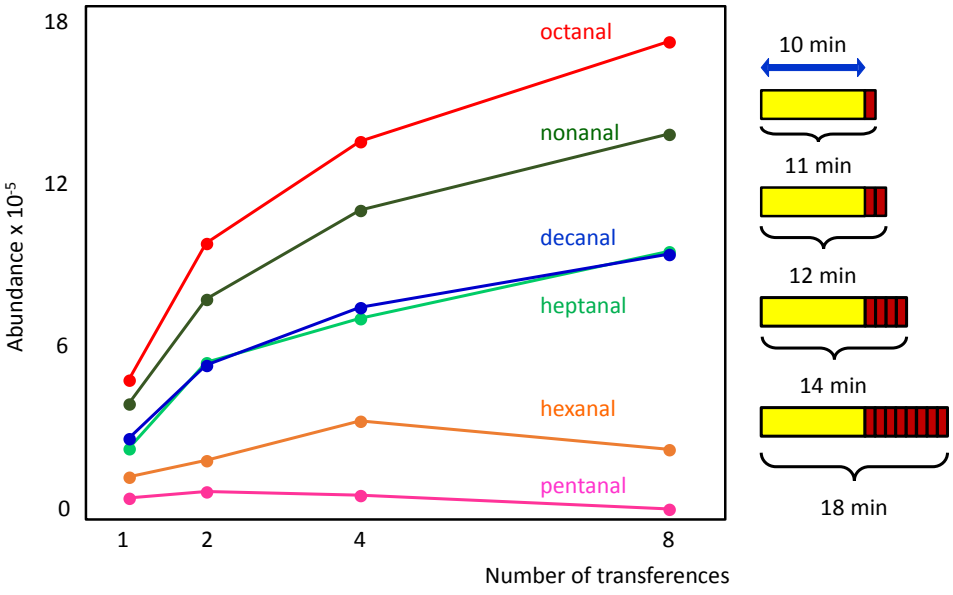

Fig. 2. Signals obtained with approach 1, selecting an initial 10 min HS generation time and 1 (conventional HS), 2, 4 and 8 gas transference steps. ▭ HS generation time; ▭ transfer of gas sample plus return of syringe to the HS oven to withdraw a new sample from the same vial. (1) pentanal, (2) hexanal, (3) heptanal, (4) octanal, (5) nonanal, (6) decanal.

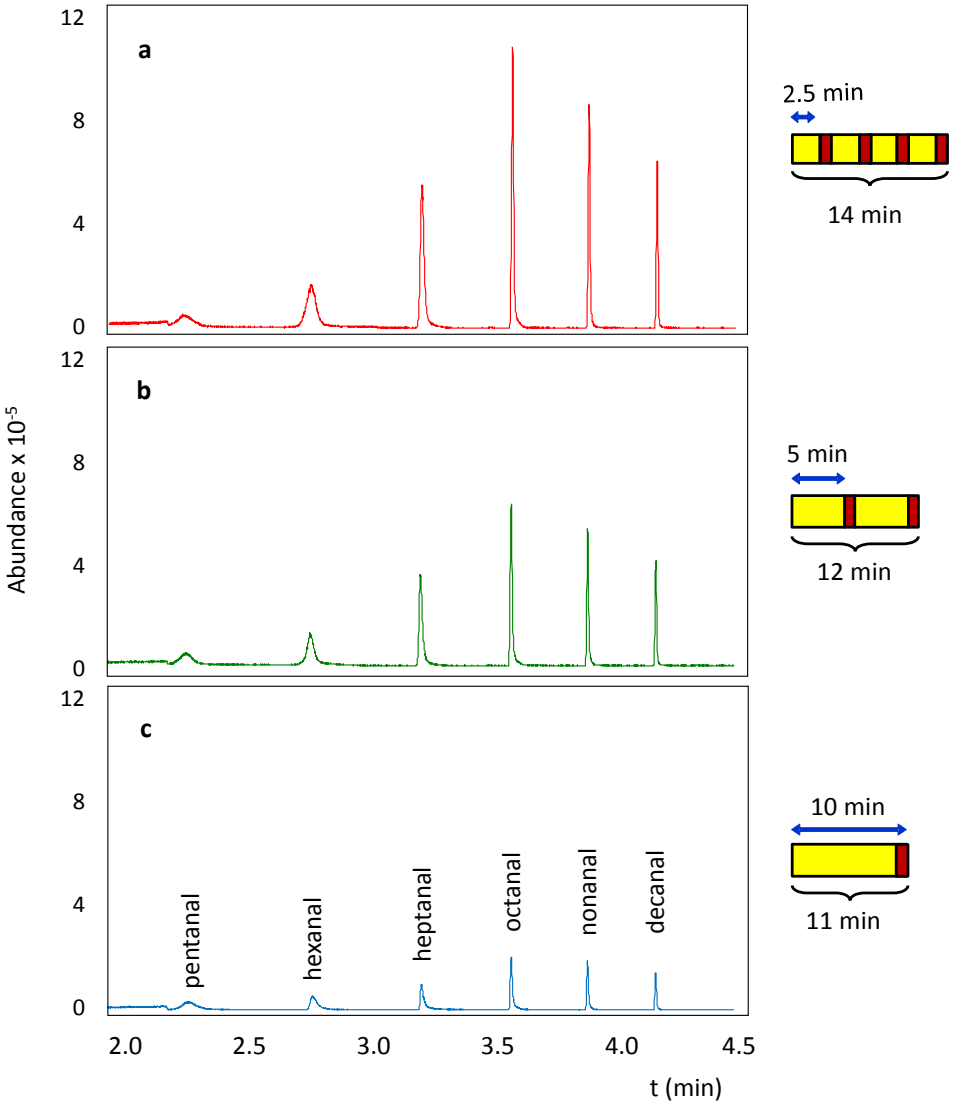

Fig. 3. Chromatograms obtained with approach 2. (a) HS generation time split in four, (b) HS generation time split in two, (c) conventional HS. ▭ HS generation time; ▭ transfer of gas sample and injection in the PTV plus return of syringe to the HS oven to withdraw a new sample from the same vial. (1) pentanal, (2) hexanal, (3) heptanal, (4) octanal, (5) nonanal, (6) decanal.

by splitting the HS generation time. This was the option selected in the following studies.

3.2. Influence of PTV temperatures and type of liner

Trapping of the analytes in the PTV liner is of paramount importance in order to achieve analyte enrichment. Initially, a liner packed with Tenax TA® was used in the PTV inlet. Tenax is a porous polymer, with low affinity for water, widely used as an adsorbent in PTV injection combined with solvent venting for high moisture content samples [25]. Given that pentanal and, to a lesser extent, hexanal could not be adequately retained with the multistep HS sampling, PTV initial temperatures lower than 50 °C were tested (35 °C and 20 °C, respectively) with the purpose of cryogenically trapping these more volatile analytes. However, the lower temperatures were not sufficient to adequately trap them, whilst the reproducibility in the retention times worsened. Taking also into account that lower PTV temperatures required more CO_2 to be used and longer time to reach initial conditions, 50 °C was kept as PTV initial temperature.

A different liner packing was then tested: Carbotrap B. This material, consisting of graphitized carbon black, is also recommended for trapping of C5-C12 organic compounds, and in combination with large volume injections [25]. Similar results were observed for pentanal and hexanal, maintaining the same PTV conditions; on the contrary, for more apolar compounds, a dramatic decrease in the signals was observed, probably due to their higher retention in the Carbotrap liner, leading to an inefficient desorption. With a view to enhancing analyte desorption prior to injection into the chromatographic column, the highest final temperature recommended when working with this type of liner (350 °C) was assayed. More efficient desorption was achieved, but still better results were obtained with Tenax TA®. Fig. 4 compares the chromatograms obtained with both types of liner at their maximum recommended final temperatures, 300 °C and 350 °C for Tenax TA® and Carbotrap B, respectively. As shown, the most polar compounds provide similar signals in both cases, but Tenax TA® is a better option for apolar compounds. This is quite evident comparing the signals of decanal in both chromatograms. In light of these results we decided to work with the liner packed with Tenax TA®.

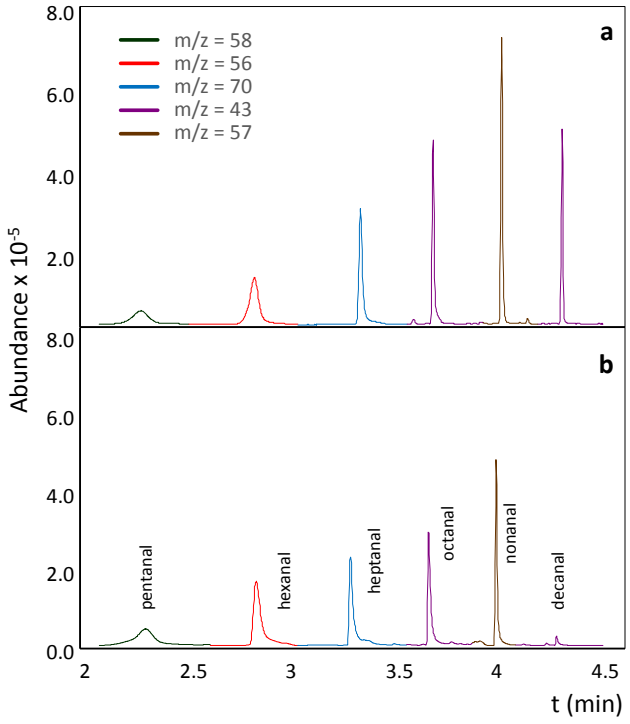

Fig. 4. Comparison of chromatograms recorded with (a) a TenaxTA® liner (final PTV temperature: 300 °C), (b) a Carbotrap B liner (final PTV temperature: 350 °C). For the rest of PTV conditions see Experimental section. (1) pentanal, (2) hexanal, (3) heptanal, (4) octanal, (5) nonanal, (6) decanal.

3.3. Evaluation of the method

Due to the difficulties in identifying the start and the end of a peak at low signal-to-noise ratio (S/N), peak height was used to obtain the analytical characteristics of both methodologies, conventional and multiple HS (Table 2). Linear range was found for all data (LOQs-20 μg/L) with three replicate measurements at each calibration level (five levels for analyte). The ANOVA lack-of-fit test was used to confirm that the linear models fitted the data. Enrichment factors (EF) for each analyte were calculated as the ratio of slopes obtained with multiple HS and conventional HS. In all cases, except for pentanal (EF = 0.8), favorable EF were obtained, from 3.0 for hexanal to 6.7 for octanal.

The differences in these values can be explained in terms of volatility and polarity of the analytes. For more volatile compounds retention in the PTV liner is less favourable; additionally, as the polarity decreases, analyte desorption is less favourable.

The relative standard deviations (RSDs) for repeatability and reproducibility were in most cases higher in multiple HS, but still with acceptable values, less than or equal to 10.7%, except for pentanal. Again, pentanal in multiple HS is the analyte presenting a higher value −18.3%– due to its physico-chemical characteristics.

Table 2 also shows the LODs and LOQs of the conventional and multiple HS methods. The LOD values (expressed in μg/L) are in the range of 0.017–0.094 for HS and 0.004–0.159 for multiple HS. Better LODs were achieved with multiple HS, except for pentanal, whose volatility makes it unadvisable to apply multiple HS for its determination. For the rest of the analytes, the improvement achieved in the LOD and the slope ratio (enrichment factor) differed slightly because the background noise range and standard deviation increased slightly in the multiple HS procedure.

Finally, apparent recoveries were calculated for the results obtained for four different spiked water samples, in order to evaluate the accuracy of the method. The mean values obtained as the ratio (in percentages) of the measured concentration to the spiked concentration, were between 82 and 100% for HS and 86 and 104% for multiple HS; both methodologies showed good recovery values for analysis of aldehydes in water samples.

To check the applicability of this proof of concept, the analysis of a urine sample was performed using multiple HS (right side of Fig. 5) in comparison with conventional HS (left side of Fig. 5). Among the studied aldehydes, only pentanal and hexanal were detected, so the sample was spiked with all the analytes (10 μg/L, except for pentanal-30 μg/L-), in order to calculate the enrichment factors. Similar values to those obtained in water were measured, showing the potential of the methodology in complex biological samples.

For the urine sample analyzed, determination of pentanal suffered an interference caused by 2-pentanone, an analyte naturally present in urine. However, as shown in the zoomed area in Fig. 5d, the overlapping of 2-pentanone and pentanal would not prevent individual chromatographic determination of pentanal, using the chromatogram extracted from the $m/z = 57$ ion, instead of $m/z = 58$, which is common to both compounds. For different samples, a specific study of interferences should be done.

4. Conclusions

A simple and effective methodology has been applied for the first time to gain sensitivity in HS sample treatment. Through repeated gas extraction from the same vial, coupled with a PTV inlet used as a cold trap (in solvent vent injection mode), the extraction efficiency of static HS sampling could be improved. Accordingly, the obtained signals were

Table 2
Comparison of analytical characteristics of the conventional HS and multiple HS methodologies.

Compound	Slope		Slope ratio	Repeatability (%)		Reproducibility (%)		LOD (μg/L)		LOQ (μg/L)		Apparent recoveries (%)*	
	HS	MHS	MHS/HS	HS	MHS	HS	MHS	HS	MHS	HS	MHS	HS	MHS
Pentanal	(274 ± 7) 10	(212 ± 2) 10	0.8	3.0	10.0	5.9	18.3	0.094	0.159	0.310	0.525	96 ± 8	92 ± 16
Hexanal	(61 ± 3) 10^2	(180 ± 6) 10^2	3.0	5.4	8.0	10.1	10.3	0.086	0.037	0.284	0.122	82 ± 14	94 ± 12
Heptanal	(65 ± 4) 10^2	(40 ± 3) 10^3	6.1	6.9	8.2	8.4	10.5	0.080	0.017	0.264	0.056	84 ± 12	86 ± 14
Octanal	(144 ± 6) 10^2	(96 ± 4) 10^3	6.7	5.5	10.3	9.6	6.4	0.037	0.007	0.122	0.023	100 ± 8	86 ± 12
Nonanal	(30 ± 1) 10^3	(154 ± 6) 10^3	5.1	5.5	7.2	9.3	7.9	0.017	0.004	0.056	0.013	94 ± 8	92 ± 10
Decanal	(188 ± 8) 10^2	(876 ± 2) 10^2	4.7	7.4	10.7	13.9	7.5	0.028	0.008	0.092	0.026	88 ± 10	104 ± 8

*Four samples spiked at 5 μg/L, confidence intervals 95% probability; each sample was measured in triplicate.

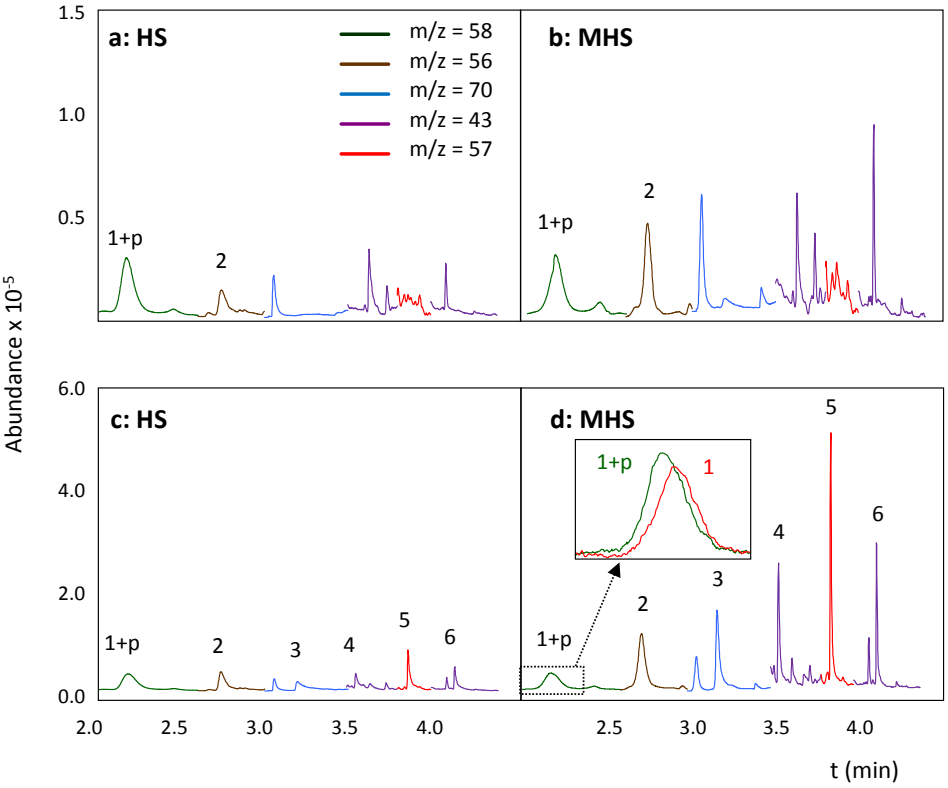

Fig. 5. Comparison of chromatograms obtained in the analysis of a urine sample. (a) Conventional HS, non-unspiked sample; (b) multiple HS, non-unspiked sample; (c) conventional HS, spiked sample; (d) multiple HS, spiked sample. Concentrations reported in the text (1) pentanal, (p) 2-pentanone, (2) hexanal, (3) heptanal, (4) octanal, (5) nonanal, (6) decanal.

improved up to six times for the less volatile aldehydes used as model compounds. The most volatile compound tested –pentanal- could not be adequately trapped in the PTV inlet with the multistep approach, but it could serve as a general strategy for enhancing HS sampling sensitivity for compounds with suitable volatility and polarity.

It has to be noted that no additional instrumentation to that required for conventional HS sampling is needed, in comparison with other methodologies, such as HS-SPME or HS-SDME procedures.

Analytical performance of the multistep HS approach has been proved, and compared with conventional HS. With a minimum increase in time (from 11 to 14 min) a significant increase in sensitivity can be achieved (more than 600% depending on the physico-chemical characteristics of the analytes). Good linearity, repeatability and reproducibility, as well as good accuracy (expressed as recovery for analysis of aldehydes in water samples) were observed.

Future work will be developed to address more extensive validation of this methodology, in order to extend it to further organic compounds in biological matrices.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitiveness (Project CTQ2017-87886-P), the Junta de Castilla y León (Project SA055P17) and the Samuel Solórzano Foundation (FS/30-2017). Javier Peña is also thankful to Junta de Castilla y León and European Regional Development Fund.

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