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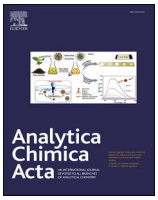

Determination of hexanal and heptanal in saliva samples by an adapted magnetic headspace adsorptive microextraction for diagnosis of lung cancer

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HIGHLIGHTS

- A modification of magnetic headspace adsorptive microextraction is presented.
- Magnetic nanoparticles–Strata™-X-RP composite is used as sorbent material.
- A simple and affordable method for bioanalysis of small sample volumes is proposed.
- Analysis of hexanal and heptanal in saliva as lung cancer biomarkers is performed.
- The presented method shows high potential for earlier and easier cancer diagnosis.

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Magnetic headspace adsorptive microextraction

ABSTRACT

In this work, an analytical method for the determination of two endogenous aldehydes (hexanal and heptanal) as lung cancer biomarkers in saliva samples is presented for the first time. The method is based on a modification of magnetic headspace adsorptive microextraction (M–HS–AME) followed by gas chromatography coupled to mass spectrometry (GC-MS). For this purpose, an external magnetic field generated by a neodymium magnet is used to hold the magnetic sorbent (*i.e.*, $CoFe_2O_4$ magnetic nanoparticles embedded into a reversed-phase polymer) in the headspace of a microtube to extract the volatilized aldehydes. Subsequently, the analytes are desorbed in the appropriate solvent and the extract is injected into the GC-MS system for separation and determination. Under the optimized conditions, the method was validated and showed good analytical features in terms of linearity (at least up to 50 ng mL^{-1}), limits of detection (0.22 and 0.26 ng mL^{-1} for hexanal and heptanal, respectively), and repeatability (RSD $\leq$12%). This new approach was successfully applied to saliva samples from healthy volunteers and those with lung cancer, obtaining notably differences between both groups. These results reveal the prospect of the method as potential diagnostic tool for lung cancer by saliva analysis. This work contributes to the Analytical Chemistry field presenting a double novelty: on the one hand, the use of M–HS–AME in bioanalysis is unprecedentedly proposed, thus expanding the analytical potential of this technique, and, on the other hand, the determination of hexanal and heptanal is carried out in saliva samples for the first time.

1. Introduction

Lung cancer is the leading cause of cancer-related death in adults [1] being tobacco its main risk factor, related since 1954 by Doll et al. [2]. Worldwide, it is estimated that there are 1.6 million deaths due to lung cancer annually [3]. Clinical outcome for non-small cell lung cancer is directly related to stage at the time of diagnosis. In addition, within early lung cancers (stage I), there is a relationship between tumour size and survival [4].

Many characteristics of lung cancer suggest that screening should be effective: high morbidity and mortality, significant prevalence (0.5–2.2%), identified risk factors allowing targeted screening for high-

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risk individuals, a lengthy preclinical phase for some types of lung cancer, and evidence that therapy is more effective in early-stage disease [5]. Prevention (by preventing smoking and promoting smoking cessation) will have far greater impact on reducing lung cancer mortality than screening. Nonetheless, lung cancer screening with low-dose computed tomography (LDCT) and treatment has been shown to significantly reduce the burden of lung cancer morbidity and mortality [6,7].

Therefore, it is crucial to identify, detect and quantify biomarkers that allow its early diagnosis. Endogenous aldehydes are by-products of cellular lipid oxidation caused by high levels of free radicals, which have been linked with some diseases, including cancer. Hence, aldehydes are considered potential biomarkers of oxidative activity [8,9]. To this regard, it has been reported that hexanal and heptanal are present in both breath and blood samples from cancer patients but not in control group [10,11], what makes them useful for the early diagnosis of lung cancer. Besides exhaled breath [12–14] and blood [11,15–23], they have been determined in other biological matrices such as urine [15,18,24–31], pleural effusion [32] and sweat [33].

Due to the correlation between numerous salivary and blood biomarkers and the non-invasive, painless, and straightforward nature of saliva collection, saliva has recently emerged as a potent and promising platform for bioanalysis [34]. However, to the best of our knowledge, there are no methods in the literature for the quantitative determination of hexanal and heptanal in saliva. Nonetheless, at this point, it should be mentioned the paper published by Shigeyama et al., who identified them as potential cancer biomarkers in an untargeted study carried out in saliva [35].

It should be noted that bioanalysis usually comprises the determination of compounds at very low concentrations in complex biological matrices. Hence, selective and sensitive methods are necessary, typically combining (micro)extraction techniques with highly sensitive detectors. In this sense, chromatographic systems (both, liquid (LC) and gas chromatography (GC)) coupled to mass spectrometry (MS) have been extensively used for the determination of hexanal and heptanal [11,13,14,16,21–24,27,28,30,32,33]. Among the vast selection of (micro) extraction techniques available in literature, headspace solid-phase microextraction (HS-SPME) has been widely employed, exploiting the volatility of the analytes [15,22,23,25,30,32].

As a consequence of the physical and chemical characteristics of aldehydes, such as lack of intrinsic chromophores, high volatility and low chemical stability, the methods for their determination usually include a derivatization step. 2,4-Dinitrophenylhydrazine (DNPH) and O-2,3,4,5,6-(pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) are mainly proposed as derivatization reagents for both LC and GC analysis [13,14,16–23,26,31]. However, some approaches without derivatization have also been proposed for GC [11,12,15,24,25,28,30,32,33].

As previously stated, HS-SPME has become a benchmark for the separation and preconcentration of volatile analytes from a wide variety of matrices [36]. HS-SPME has clear advantages such as its simplicity and reproducibility, requiring minimal sample pretreatment and low (if not completely avoided) consumption of organic solvents, in consonance with the principles of Green Sample Preparation [37]. However, the fibers used in the HS-SPME method are usually expensive and the commercially-available sorbents are limited, thus reducing the possibilities for analytical laboratories.

Recently, magnetic (nano)materials (*e.g.*, magnetic nanoparticles (MNPs) or composites made of MNPs and polymers) have been used to expand the possibilities of various sample preparation techniques including those based in headspace [38]. The main advantage of this approach is the simplicity of its implementation in the sorption process due to the magnetic properties of the material, which allow to keep it in the headspace using an external magnet. Furthermore, the nanometric size of MNPs and the high porosity of polymers, provide these materials with large surface areas, which facilitates efficient adsorption of analytes and their miniaturization.

To our knowledge, Nie et al. [39] were the first to use a magnetic material (*i.e.*, polystyrene-coated MNPs) in headspace extraction, specifically to extract volatile organic compounds (VOCs) from dried medicinal plants. Later, Vidal et al. [40] proposed a technique called magnetic headspace adsorptive extraction (Mag-HSAE) for environmental water analysis employing magnetic graphene oxide as extraction phase, and Timofeeva et al. [41] used a smaller version of this approach for food analysis, using Fe_3O_4@$Cr(OH)_3$ as sorbent material and renaming the technique as magnetic headspace adsorptive microextraction (M–HS–AME). However, in no case it was applied to the biological field.

In this work, a useful and reliable analytical method for the determination of hexanal and heptanal as lung cancer biomarkers in saliva samples is presented. For this purpose, the excellent performance that M–HS–AME presents has been exploited, thus expanding the possibilities of this technique. Nevertheless, it has been adapted by miniaturizing it and applied for the analysis of biological samples. A magnetic composite material made of cobalt ferrite ($CoFe_2O_4$) MNPs embedded into a commercial reversed-phase polymer (*i.e.*, Strata™-X-RP) was used for the first time as sorbent material in M–HS–AME [42]. The $CoFe_2O_4$ MNPs confer to this sorbent the magnetism needed and were preferred rather than usually-employed Fe_3O_4 MNPs due to their higher chemical stability [43].

The main parameters affecting the extraction and desorption stages were optimized by either a response surface methodology or a univariate approach. After the extraction, samples were injected into a GC-MS system for quantification. This is the first time, to the best of our knowledge, that the determination of hexanal and heptanal in saliva and the application of M–HS–AME in biological matrices are reported.

2. Experimental

2.1. Reagents

Hexanal (98%) and heptanal (97%) were purchased from Fisher Scientific (Loughborough, United Kingdom). 2,2-Dimethyl-4-pentenal (90%) (used as surrogate) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Chemical structures and some relevant information are given in Table S1.

For the synthesis of $CoFe_2O_4$-Strata™-X-RP composite, $CoCl_2{\cdot}6H_2O$ and $FeCl_3{\cdot}6H_2O$ were purchased from Acros Organics (New Jersey, USA), and NaOH (reagent grade) was purchased from Scharlau (Barcelona, Spain). Strata™-X-RP 33-μm polymeric reversed-phase was taken from SPE cartridges (500 mg/6 mL) from Phenomenex (Torrance, CA, USA). Octyl and octadecyl silica (C8 and C18) microparticles were obtained from Sigma-Aldrich.

Ultrapure water was provided by a Connect water purification system provided by Adrona (Riga, Latvia). Ethanol absolute (99.9%) and LC-grade acetonitrile were obtained from Panreac (Barcelona, Spain). Anhydrous Na_2SO_4 was purchased from Scharlau.

For the preparation of synthetic saliva, NaCl from Fisher Scientific, KCl, $CaCl_2{\cdot}H_2O$, KSCN and urea from Panreac, and $NaH_2PO_4{\cdot}H_2O$ from Scharlau were used.

Extra pure helium (>99.999%), used as carrier gas, was obtained from Carburos Metálicos S.A. (Paterna, Spain).

2.2. Apparatus and materials

For the synthesis of the magnetic composite, a heating plate with magnetic stirrer from Stuart Scientific (Staffordshire, United Kingdom) and an oven from J.P. Selecta (Barcelona, Spain) were used.

For the extraction stage, 1.5-mL centrifuge microtubes from Fisher Scientific, neodymium magnetic cubes (5 mm, 42 MGO) from Supermagnete (Gottmadingen, Germany), and a 24-position thermo-shaker model TS-100 SC-24 N from BioSAN (Madrid, Spain) were used. For the dispersion of the magnetic composite in the desorption stage, a 10-

position multiple stirring plate model MS-M-S10 from Labbox (Barcelona, Spain) was used. An EBA 21 centrifuge from Hettich (Tuttlingen, Germany) was used to centrifuge the injection vials.

2.3. Sample collection

Saliva samples were collected in 15-mL centrifuge tubes from volunteers by the passive drooling method, to ensure the absence of interferences with the salivary analytes being measured [44]. Conversely, other collection methods like swab-based ones (*e.g.*, Salivette®, from Sarstedt, Germany) usually introduce variability and negatively affect results [45]. Following collection, samples were centrifuged for 10 min at 6000 rpm to separate saliva from other materials, such as phlegm or food leftovers, and the supernatant was then transferred to a microcentrifuge tube. To reduce the viscosity of saliva, samples were frozen at −20 °C to precipitate mucins [46], which was demonstrated to have negligible effects on the concentration of the analytes, as described in Supplementary Material. Just before the analysis, they were thawed and centrifuged again at 18000 rpm for 5 min.

The samples included in this study were managed and provided by Biobanco La Fe (PT17/0015/0043) with the approval of both ethics and scientific committees and have been processed following standardized procedures.

2.4. Synthesis of $CoFe_2O_4$-Strata™-X-RP magnetic composite

The $CoFe_2O_4$-Strata™-X-RP composite was synthesized in two steps: first, $CoFe_2O_4$ MNPs were synthesized using a modified methodology by wet chemical co-precipitation [43], and then MNPs were incrusted onto the pores of the polymer.

Briefly, 10.80 g of $FeCl_3$ and 4.76 g of $CoCl_2$ were dissolved in 200 mL of ultrapure water, and then 100 mL of a 3 M NaOH aqueous solution was added dropwise while being continuously stirred for an hour at 80 °C. After this, it was allowed to cool to ambient temperature and a magnet was used to decant the formed solid. The solid was then washed with water and ethanol, dried in an oven at 80 °C overnight and ground into a fine black powder.

Subsequently, 0.15 g of MNPs and 0.15 g of Strata™-X-RP were weighed for the preparation of the composite, and 50 mL of ethanol were also added. To make sure the MNPs were embedded on the polymer pores, the mixture was stirred for 72 h at room temperature. The residual non-magnetic polymer was then removed from the solid by magnetic decantation washed three times with ethanol. In order to remove the free MNPs and isolate the composite, it was then resuspended in ethanol and filtered under vacuum through a paper filter with a 10-μm pore size. It was dried at 80 °C in an oven overnight before being ground into a fine powder.

2.5. Preparation of standard solutions

A multicomponent standard solution of the analytes (hexanal and heptanal) containing 1000 μg mL^{-1} in methanol was prepared. From this solution, an intermediate solution of 10 μg mL^{-1} in methanol was prepared. Similarly, 1000-μg mL^{-1} and 10-μg mL^{-1} solutions of 2,2-dimethyl-4-pentenal (surrogate) in methanol were prepared. These solutions were kept at −20 °C and protected from light.

A multicomponent solution of hexanal and heptanal of 100 ng mL^{-1} and a solution of 2,2-dimethyl-4-pentenal of 1 μg mL^{-1} were prepared daily from these solutions, both in synthetic saliva (prepared as described in Supplementary Material).

2.6. M–HS–AME procedure

The caps of the 1.5-mL centrifuge microtubes were previously separated from them. Inside the caps, 1 mg of magnetic composite was weighed, and a piece of double-sided adhesive tape was attached on the outside face to hold a cubic neodymium magnet, used to hold the magnetic composite when they are flipped, as it will be seen later.

The working standard solutions of hexanal and heptanal (1–10 ng mL^{-1}) were prepared directly in the cap-less centrifuge microtubes by adding the appropriate volume of the multicomponent solution of 100 ng mL^{-1} and diluting up to 1 mL with synthetic saliva. Subsequently, 50 μL of the 1-μg mL^{-1} surrogate solution were added.

The sample solutions were similarly prepared by adding 50 μL of the 1-μg mL^{-1} surrogate solution to 1 mL of saliva, obtained as described in Section 2.3. If sample volume was limited (*i.e.*, <1 mL), saliva samples were diluted up to 1 mL with synthetic saliva.

Caps containing the magnetic composite were turned upside down and attached to the tubes containing standard and sample solutions. Then, they were placed in the thermo-shaker at 30 °C, and shaked at 1400 rpm for 40 min. After this time, the caps of the microtubes were removed and turned again placing the composite upwards. Then, the external magnets were removed.

To perform the liquid desorption of the analytes, a tiny amount of anhydrous Na_2SO_4 to remove the possible condensed water during extraction and 50 μL of acetonitrile were added. An empty microtube was placed onto the cap to facilitate handling. Afterwards, they were placed in the multiple-position magnetic stirrer and stirred for 30 s. Once this time had elapsed, the assembly was placed on a neodymium magnet to deposit the magnetic composite, then the microtube was removed and the extract was transferred with the aid of a pipette from the cap to an injection vial provided with a conical insert. Finally, they were centrifuged at 6000 rpm for 5 min to deposit the possible collected magnetic composite or desiccant at the bottom and prevent their injection into the GC-MS system. Fig. 1 shows a schematic diagram of the proposed M–HS–AME procedure.

2.7. GC-MS analysis

An 8860 GC system coupled to a 5977B single quadrupole mass spectrometer, both from Agilent Technologies (Palo Alto, CA, USA), and provided with a PAL LSI 85 autosampler from CTC Analytics (Zwingen, Switzerland) was employed. Separations were carried out in a VF-WAXms capillary column (polyethylene glycol, 30 m, 0.25 mm i.d., 0.25 μm) from Agilent Technologies.

One microliter of each extract was injected in split mode (split ratio 5:1) into the chromatographic system. Helium as carrier gas was set at a flow rate of 1 mL min^{-1}. The oven temperature program was set as follow: from 50 °C (3 min) to 100 °C at 15 °C min^{-1}, then to 230 °C at 50 °C min^{-1}. Injector, transfer line and source temperatures were set at 230 °C.

The chromatograms were recorded in Selected Ion Monitoring (SIM) mode. The *m/z* values for quantification were 56, 70 and 55 for hexanal, heptanal and surrogate, respectively. For identification, they were 72 and 82 for hexanal, 55 and 86 for heptanal, and 41 and 43 for surrogate. A chromatogram of a standard solution is shown in Fig. S2 of Supplementary Material.

3. Results and discussion

3.1. Adaptation of the extraction device

As previously described, to the best of our knowledge, only three configurations have been described using magnetic (nano)materials as sorbent phase for headspace extraction.

In the first case [39] (Fig. 2a), the magnetic sorbent was placed in a 2-mL vial inside of a 10-mL vial containing the sample, both placed in an oven. The magnetism was only used to separate the sorbent from the extract after liquid desorption. Later, in the approach proposed by Vidal et al. [40] (Fig. 2b), the magnetic sorbent material was attached to the end of a small neodymium magnet, located in the headspace of a 25-mL extraction vial. To fix this magnet in the headspace, another neodymium

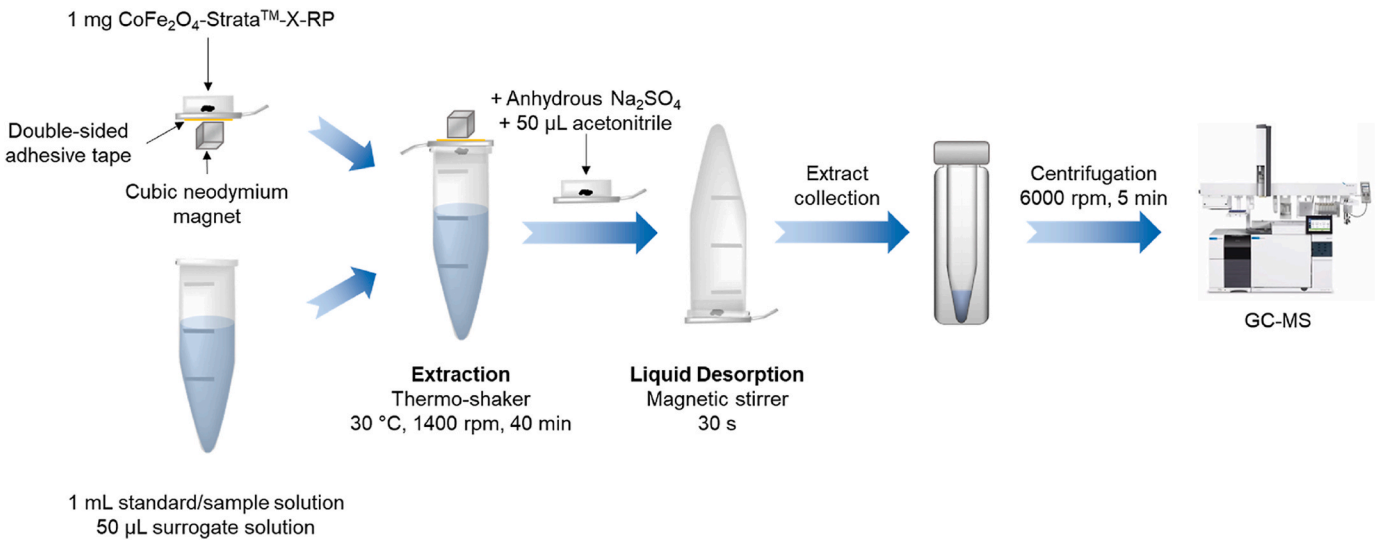

Fig. 1. Schematic diagram of the proposed M–HS–AME procedure.

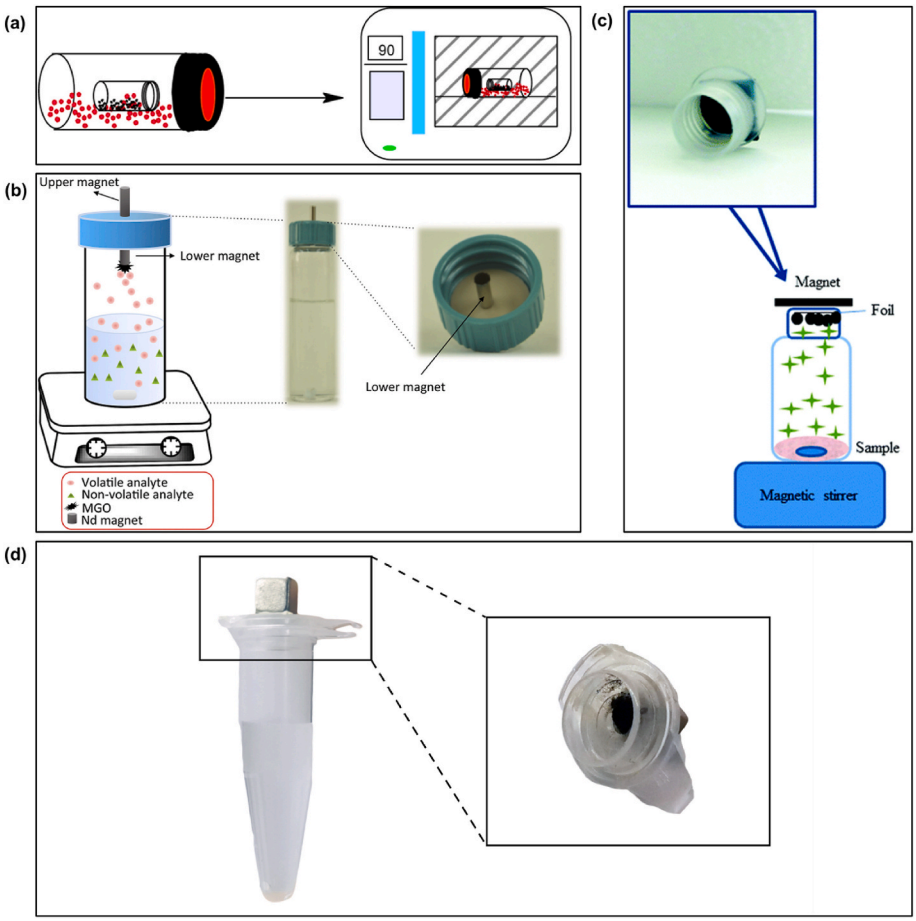

Fig. 2. Different configurations of the extraction device for M–HS–AME: (a), (b) and (c) are reproduced with permission from Refs. [39–41], respectively, and (d) is the proposed extraction device.

magnet was placed on the cap of the vial, without the need for any other device. The inner magnet was then eliminated by Timofeeva and co-workers [41] (Fig. 2c), placing the sorbent directly on the cap of the vial.

In this work, we proposed an adaptation of the latter employing 1.5-mL centrifuge microtubes, whose caps are separated from the body. The magnetic material is placed in the inner part of the cap and an external cubic magnet is used to keep the sorbent in place. A double-sided adhesive tape is used to easily attach and detach the external magnet to the cap (Fig. 2d).

An important advantage of this new approach is that a large number of samples can be treated simultaneously in a semi-automated way by

using a thermo-shaker adapted for centrifuge microtubes. Additionally, the microtube cap can be easily turned upside down after the extraction, and liquid desorption can be performed in the same cap, avoiding the transfer of the magnetic composite. Moreover, the intrinsic magnetism of the composite is enough to be stirred by the magnet of the multi-position stirring plate during the desorption step, without the need to incorporate a stirring magnet, achieving an easy and effective dispersion of the sorbent within the desorption solvent.

3.2. Selection of the composite

Hexanal and heptanal possess a hydrophobic carbon chain and a carbonyl group. Hence, different sorbent materials based in MNPs embedded into commercial hydrophobic sorbents were tested. MNPs were embedded into C8 and C18-coated silica, following the same procedure described in Section 2.5, and a commercial pyrrolidone-modified styrene-divinylbenzene copolymer (*i.e.*, Strata™-X-RP).

The extraction performance of these magnetic composites and bare MNPs was evaluated and compared. As can be seen in Fig. 3, the magnetic composite based in Strata™-X-RP showed greater performance for the extraction of hexanal and heptanal due to its hydrophobic and dipole-dipole interactions with the analytes, unlike the other two materials that only offered hydrophobic interactions. Therefore, the former was selected for the subsequent work. It was also proved that the polymer, but not the MNPs, was the responsible for the extraction of analytes.

The characterization of this material, including synthesis repeatability, can be revisited in a previous work of our group [42]. Magnetization, particle size distribution, morphology, specific surface area and pore size were established. In addition, energy dispersive X-ray spectroscopy (EDS) was performed for elemental analysis.

3.3. Optimization of the M–HS–AME variables

3.3.1. Optimization of the desorption

In order to ensure maximum preconcentration of analytes, the minimum solvent volume (i.e., 50 μL) was used for the desorption step in subsequent experiments. Lower volumes were not feasible because its retrieval from the microtube cap containing the sorbent and the desiccant was highly complicated from the practical point of view. Both solvent and desorption time were optimized to maximize the recovery of analytes from sorbent material after the extraction. In this regard, aliquots of 1 mL of standard solution containing 50 ng mL^{-1} of the analytes were extracted under the following extraction conditions: 1 mg of sorbent material, 10 min, 40 °C, 1400 rpm.

For the optimization of the desorption solvent, three GC-compatible solvents were selected (*i.e.*, methanol (X_1), acetonitrile (X_2) and acetone (X_3)). A Simplex-Centroid mixture design [47], where the vertices of the triangle represent the three pure solvents and the distance from a vertex is a measure of their relative proportion, was used. The selected analytical responses were the peak area of the analytes. The description of the statistics of the Simplex-Centroid design are included in Supplementary Material. A total of 7 experiments, where analytes were desorbed for 1 min in 50 μL of solvent, were performed. StatGraphics Centurion XVI (Stat Point Inc. Herndon, VA, USA) software was employed for the statistical analysis. Fig. 4a shows the triangular surface constructed with the proposed Simplex-Centroid design and a quadratic function. On the triangular surface obtained with adjusted determination coefficients (61–97%) it can be observed that the desorption was more efficient when acetonitrile was used. This is probably due to the better dispersion of the sorbent material in this solvent. Therefore, acetonitrile was selected as desorption solvent.

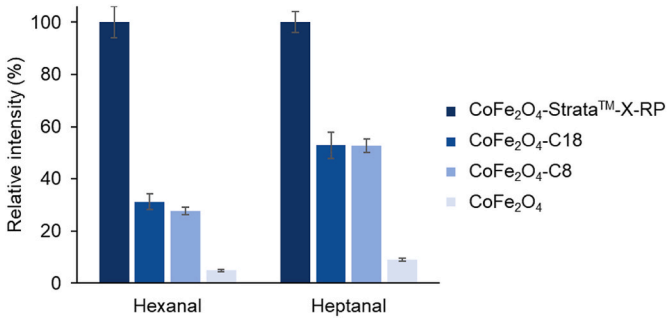

Fig. 3. Comparison of extraction performance between different magnetic composites and bare MNPs.

Then, the desorption time was tested by a univariate approach. The composite recovered from the extraction was stirred in 50 μL of acetonitrile for 0 (*i.e.*, placing the solvent on the microtube cap and retrieving it immediately without stirring), 0.5, 1, 3 and 5 min. Fig. 4b shows that 0.5 min provided the best results. Higher desorption times might lead to analyte loss by volatilization, so 0.5 min was selected as desorption time.

3.3.2. Optimization of the extraction

A response surface methodology (RSM) [48] based on a four-factor three-level Box-Behnken design was performed to stablish the optimal values of some variables involved in the extraction procedure. The studied variables and ranges were: (i) sorbent amount (X_1, 0.5–2 mg), (ii) extraction time (X_2, 5–60 min), (iii) temperature (X_3, 30–60 °C) and (iv) shaking rate (X_4, 600–1400 rpm). The volume of the donor solution was set to 1 mL trying to increase the ratio between donor an acceptor phases but as a compromise between the capacity of the microtube (<1.5 mL) where perform the microextraction and the restrictions encountered for sample availability. On the other hand, since the analytes do not present ionizable moieties in their chemical structure, their partition coefficients are not affected by the pH, and thus this variable was not considered relevant and as consequence it was not considered in the optimization.

Since human saliva is mainly composed of water (*ca.* 99%) [49], aliquots of 1 mL of standard solution containing 50 ng mL^{-1} of the analytes prepared in synthetic saliva were used to perform the experiments. After the extraction, the analytes were desorbed in 50 μL of acetonitrile for 0.5 min, as previously stablished. The selected analytical responses were the peak area of the analytes. The description of the statistics of the Box-Behnken design are included in Supplementary Material. A total of 27 experiments were performed including 3 replicates of the central point. StatGraphics Centurion XVI software was also employed for the statistical analysis.

Fig. 5 shows response surfaces of desirability function. As shown in Fig. 5a, the best responses were obtained when the mass of the sorbent was between *ca.* 0.8 and 1.1 mg. The mass transfer of analytes from the donor phase to the sorbent depends on the ratio of the sorbent area to the volume of the aqueous solution. Therefore, extraction performance increased from 0.5 to 1.1 mg of composite. However, for higher amounts, this value decreased because more sorbent within the same volume of elution solvent hindered its correct dispersion during the desorption step. Hence, 1 mg of sorbent material was used for extraction. Regarding the extraction time (Fig. 5a), it must be sufficient to reach the state of equilibrium. However, at very long times, an important amount of water condensed on the surface of the sorbent, hence, hindering the desorption of analytes and also broadening the chromatographic peaks. As a result, 40 min were selected as extraction time. Despite this prolonged time, it should be noted that up to 24 samples can be extracted simultaneously using the thermo-shaker.

Temperature had an inverse effect on the extraction performance, as shown in Fig. 5b and Fig. S3. Even though higher temperatures favour the volatilization of analytes, adsorption is an exothermic process [50], so a trade-off between both effects was necessary. Moreover, similarly to long extraction times, at high temperatures water condensed on the surface of the sorbent, with the drawbacks already mentioned.

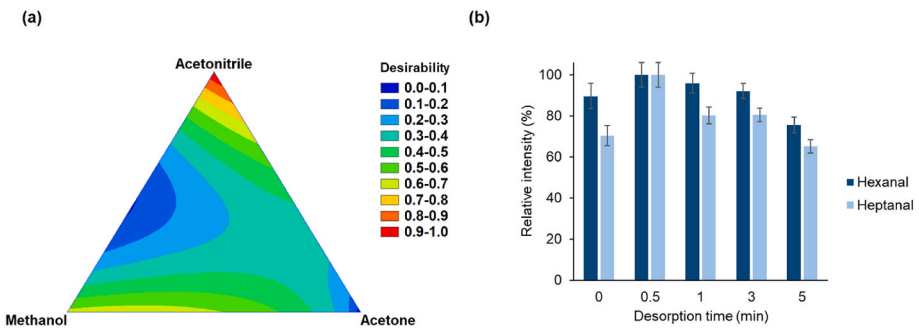

Fig. 4. (a) Triangular surface obtained through simplex-centroid design for the optimization of the desorption solvent. Extraction conditions: sample volume, 1 mL; sorbent amount, 1 mg; extraction time, 10 min; 40 °C; 1400 rpm. Desorption conditions: solvent volume, 50 μL; desorption time, 1 min. (b) Optimization of the desorption time. Extraction conditions: sample volume, 1 mL; sorbent amount, 1 mg; extraction time, 10 min; 40 °C; 1400 rpm. Desorption conditions: 50 μL acetonitrile.

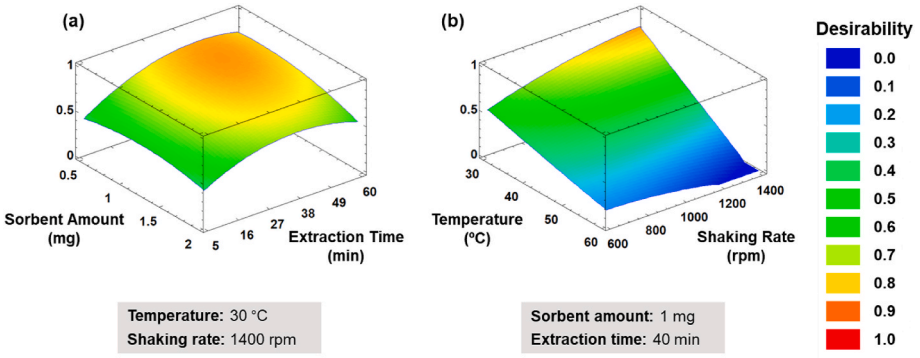

Fig. 5. Response surface of the desirability function representing the relation between the studied variables: (a) sorbent amount vs extraction time, and (b) temperature vs shaking rate.

Therefore, the selected working temperature was 30 °C.

Regarding the shaking rate (Fig. 5b), an improvement was observed at higher rates because the movement of the solution improved the transfer of the analytes to the headspace. Therefore, the selected value was the maximum value allowed by the thermo-shaker, *i.e.*, 1400 rpm.

Saline content of synthetic saliva was measured by conductimetry being equivalent to 0.25% (w/v) NaCl. This value was then considered as average saline content of real saliva samples, which is consistent with reported values [42]. To this regard, saline content of donor phase was studied employing 10-ng mL^{-1} standard solutions prepared with different saline contents from 0 to 5% (w/v) NaCl (Fig. 6). Extraction performance increased with the slightest presence of NaCl due to the salting-out effect (*i.e.*, 0.25% (w/v) NaCl was enough to improve sensitivity of the method). Signals slightly improved at higher salt concentrations, but worse precision was obtained, probably due to the higher viscosity of donor phase, what hindered reproducible extraction.

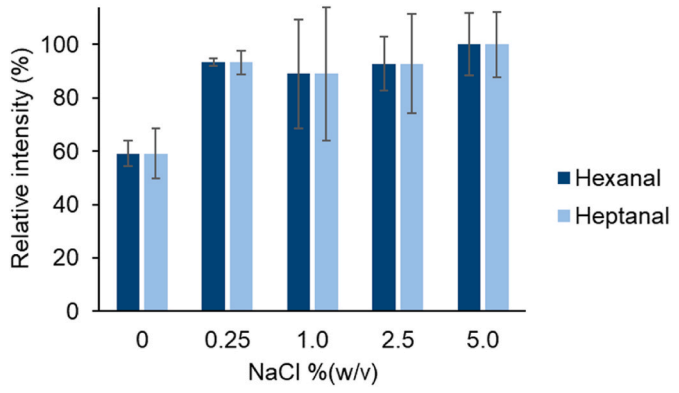

Fig. 6. Optimization of saline content of donor phase. Extraction conditions: sample volume, 1 mL; sorbent amount, 1 mg; extraction time, 40 min; 30 °C; 1400 rpm. Desorption conditions: 50 μL acetonitrile, 30 s.

Therefore, synthetic saliva was considered an appropriate solvent for standard solutions and saliva samples did not need any saline adjustment.

3.4. Analytical performance of the proposed method

Under the optimized conditions the analytical quality parameters of the proposed method, including linearity, enrichment factors (EF), extraction efficiency (EE), limits of detection (LOD) and quantification (LOQ), and intra- and inter-day precision, were evaluated. The figures of merit about the analytical performance of the proposed method are collected in Table 1.

The linearity was studied by measuring standard solutions prepared in synthetic saliva at different concentrations, all of them containing 50 ng mL^{-1} of surrogate. Calibration curves were plotted using the ratio of the peak area of each target analyte to the surrogate (A_i/A_s) versus the analyte concentration. The results indicated that the linearity reached at least 50 ng mL^{-1} for the target analytes. However, the working range was set from 1 to 10 ng mL^{-1} due to the low expected values. The determination coefficients, residues graphs and ANOVA p-values reveal good linearity (see Supplementary Material).

The EFs were estimated as the ratio of the signal obtained after the extraction of a standard solution prepared in synthetic saliva containing 10 ng mL^{-1} of the analytes and the signal of a standard solution of the same concentration prepared in acetonitrile without extraction. An aliquot of surrogate was added to act as internal standard to both extracted and non-extracted solutions. EFs of 12 ± 2 and 15 ± 2 were achieved for hexanal and heptanal, respectively. The EEs were calculated considering a maximum EF of 20. In this sense, the EE values were 60 ± 9 and 74 ± 8% for hexanal and heptanal, respectively.

The LODs and LOQs were calculated as 3 times and 10 times, respectively, the signal-to-noise ratio of a standard solution prepared in synthetic saliva after applying the proposed approach. The LOD and LOQ values were 0.22 and 0.75 ng mL^{-1}, respectively, for hexanal and

Table 1
Analytical figures of merit of the proposed method.

Analyte	EF[a]	EE[b] (%)	LOD[c] (ng mL^{-1})	LOQ[c] (ng mL^{-1})	Precision (RSD, %)[d]					
					Intra-day			Inter-day		
					1 ng mL^{-1}	5 ng mL^{-1}	10 ng mL^{-1}	1 ng mL^{-1}	5 ng mL^{-1}	10 ng mL^{-1}
Hexanal	12 ± 2	60 ± 9	0.22	0.75	10.4	6.5	9.8	12.0	8.6	4.7
Heptanal	15 ± 2	74 ± 8	0.26	0.85	5.6	7.0	4.0	8.7	6.7	8.9

[a] EF: enrichment factor; mean of three replicates ± standard deviation.
[b] EE: extraction efficiency; mean of three replicates ± standard deviation.
[c] LOD: limit of detection; LOQ: limit of quantification; calculated as 3·SN and 10·SN criteria, respectively, where SN is the signal-to-noise ratio of a standard solution.
[d] RSD: relative standard deviation (n = 5).

0.26 and 0.85 ng mL^{-1}, respectively, for heptanal.

The precision of the method was evaluated applying the proposed method to five replicates of standard solutions prepared in synthetic saliva containing the target analytes at three levels of concentration (*i.e.*, 1, 5 and 10 ng mL^{-1}). The results, expressed as the relative standard deviation (RSD, %) revealed that good precision was achieved for the target analytes.

3.5. Analysis of human saliva samples

Saliva samples from five healthy volunteers (three male and two female) and from five lung cancer patients at Stage IV (three male and two female) were collected as described in Section 2.3. The volumes of samples from lung cancer patients were variable and below 1 mL (0.2–0.85 mL), therefore, they were diluted up to 1 mL with synthetic saliva. Then, they were treated by the proposed M–HS–AME approach and measured by GC-MS. Results, besides other clinical parameters, are shown in Table 2.

Analytes from all healthy volunteers were below the LOD or LOQ, whereas lung cancer patients showed higher values of the analytes. These results serve as a starting point for clinical research of this disease and show the diagnostic potential of the proposed method, which is the unique study where these biomarkers have been determined in saliva. These results will be extended by analyzing samples from a larger cohort of volunteers both healthy and unhealthy in different stages of the disease.

Three randomly selected saliva samples from healthy volunteers were spiked at two concentration levels (*i.e.*, 1, and 5 ng mL^{-1}) in order to evaluate the matrix effects by means of the relative recovery (% RR) values (eq. (1)), calculated as follows:

$$\%\ \mathrm{RR} = \frac{\mathrm{Concentration}_{\text{spiked sample}} - \mathrm{Concentration}_{\text{sample}}}{\text{Spiked amount}} \cdot 100 \quad (1)$$

The results presented in Table 3 proved matrix effects were low and external calibration was suitable for quantification of hexanal and heptanal in saliva samples by the proposed method.

3.6. Comparison of the proposed method with previously reported approaches

The proposed approach has been compared with the previous M–HS–AME methodologies reported in terms of extraction conditions and greenness.

As evidenced in Table 4, the proposed approach requires a smaller amount of sample and considerably lower sorbent amount compared with previous approaches. It is true, however, that they are methods for

Table 3
Relative recoveries obtained from real samples at different spiked amounts.

Sample[a]	Spiked amount (ng mL^{-1})	Relative recovery (%)[b]	
		Hexanal	Heptanal
A	1	135 ± 5	133 ± 4
	5	94 ± 7	105 ± 2
B	1	131 ± 6	125 ± 4
	5	101 ± 6	96 ± 6
D	1	75 ± 9	82 ± 9
	5	101 ± 8	123 ± 7

[a] Sample A and B: male; Sample D: female.
[b] Mean of three replicates ± standard deviation.

Table 2
Relevant data from saliva samples and concentration of the analytes.

Group	Sample	Sex[a]	Smoker[b]	Cancer type	Concentration (ng mL^{-1})[c]	
					Hexanal	Heptanal
Healthy volunteer	A	m	n	–	<0.22[d]	<0.85[e]
	B	m	n	–	<0.22[d]	<0.26[d]
	C	m	a	–	<0.75[e]	<0.85[e]
	D	f	n	–	<0.75[e]	<0.85[e]
	E	f	n	–	<0.75[e]	<0.85[e]
Lung cancer patient	F	m	e	small-cell	3.3 ± 0.2	<1.0[e]
	G	m	e	adenocarcinoma	1.1 ± 0.1	<1.1[e]
	H	m	e	small-cell	6.6 ± 1.3	2.1 ± 0.3
	I	f	e	adenocarcinoma	9.5 ± 0.2	<1.3[d]
	J	f	n	adenocarcinoma	10.8 ± 0.3	2.0 ± 0.1

[a] f: female; m: male.
[b] a: active smoker; e: ex-smoker; n: non-smoker.
[c] Mean of two replicates ± standard deviation.
[d] Estimated limit of detection (LOD), according to sample dilution, if any.
[e] Estimated limit of quantification (LOQ), according to sample dilution, if any.

Table 4
Comparison of the proposed M–HS–AME approach with the previous configurations.

Matrix	Analytes	Sample amount	Sorbent amount (mg)	Extraction time (min)	Extraction temperature (°C)	Desorption conditions	EF[c]	AGREEprep Score	Reference
Traditional Chinese Medicine	VOCs[a]	1.0 g	10	15	90	500 μL ethyl acetate, 5 min	n.r.[d]	0.61	[39]
Environmental water	Chlorobenzenes	20 mL	10	30	RT[b]	Thermal desorption, 240 °C, 5 min	n.r.	0.56	[40]
Baby food	Volatile phenols	0.50 g	10	10	90	100 μL 0.01 M NaOH, 10 min	n.r.	0.57	[41]
Saliva	Linear aldehydes	1 mL	1	40	30	50 μL acetonitrile, 30 s	12–15	0.62	This work

[a] VOCs: volatile organic compounds.
[b] RT: room temperature.
[c] EF: enrichment factor.
[d] n.r.: not reported.

different analytical applications and the availability of samples differs (for example, 20 mL of environmental water are more affordable than saliva).

On the other hand, extraction time is slightly higher in the proposed methodology, but it must be noted that the extraction time, and also the temperature, to carry out the headspace extraction depends on the nature of compounds under study, which are different in the compared methods.

In terms of analytical performance, none of the previously reported methods stated the enrichment factors, therefore we were not able to compare the preconcentration capability of the techniques. However, it is expected that methods with high donor-acceptor phase ratio (even more avoiding liquid desorption such as Vidal et al. [40]) are able to achieve higher EFs and therefore lower LODs.

The greenness of these methods was evaluated by estimating their AGREEprep score [51], a new metric tool for the greenness assessment of analytical sample preparation techniques. AGREEprep grades from 0 to 1 each one of the ten principles of Green Sample Preparation [37], considering environmental and health impact factors, such as the use of reagents, the energy consumption, sample size and throughput, and generated waste. As can be seen in Table 4, all of the presented approaches were comparable in terms of greenness. Slight differences are due to higher sample size and/or higher energy consumption and lower sample throughput in Refs. [40,41].

4. Conclusions

In this work, a method based on M–HS–AME followed by GC-MS for the determination of hexanal and heptanal as lung cancer biomarkers in human saliva samples is proposed. For this purpose, an adapted extraction device for M–HS–AME has been described for the first time for the analysis of biological samples, whose volume is usually limited. The presented work contributes to the development of simple methods for bioanalysis, being useful not only for the Analytical Chemistry field but also for medical science.

Good analytical features were obtained and it was successfully applied to saliva samples from healthy and ailing volunteers, showing notably differences between them. Further research should be done in order to assist whether it is possible or not to detect the disease at earlier stages by means of this simple saliva analysis. This would allow the proposed methodology to be a promising tool for clinical research, focused on achieving earlier and easier cancer diagnosis through target screening of high-risk people.

CRediT authorship contribution statement

Cristian Azorín: Methodology, Validation, Investigation, Data curation, Writing – original draft. **Andreu L. López-Juan:** Methodology, Validation, Investigation, Data curation. **Francisco Aparisi:** Writing – review & editing. **Juan L. Benedé:** Methodology, Writing – review & editing, Supervision. **Alberto Chisvert:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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