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Solid-phase microextraction–gas chromatography–mass spectrometry method validation for the determination of endogenous substances: Urinary hexanal and heptanal as lung tumor biomarkers

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ABSTRACT

Hexanal and heptanal are endogenous aldehydes coming from membrane lipid oxidation, found in lung cancer patients' blood, and suggested as lung tumor biomarkers. Here the urinary matrix was investigated instead of blood and the difficulties related to the determination of endogenous substances in biological matrices were faced by developing an external calibration HS-SPME/GC/MS method. The methodology was validated according to international validation procedures and it was verified analyzing unknown biological samples from cancer patients and healthy subjects. Percentage accuracy and precision, ranging from −11.25 to 10.85% and from 0.45 to 4.46%, respectively, were obtained, together with limits of detection (LODs) and lower limits of quantification (LLOQs) of 0.11 and 0.23 pg μL^{-1} for hexanal and of 0.10 and 0.21 pg μL^{-1} for heptanal. Analytes percentage recoveries (66.3%, hexanal and 70.5%, heptanal) and stability were evaluated. No analytes degradation was found at room temperature, while the remarkable analytes loss found after 1 month storage suggests analyzing biological samples within a week from storage. Results coming from the analysis of unknown biological samples showed no evident differences of heptanal urinary excretion between lung cancer patients and healthy subjects (0.22–0.95 and 0.21–0.69 pg μL^{-1}, respectively), while hexanal urinary concentrations in cancer patients (0.24–4.36 pg μL^{-1}) were slightly higher than those found in control group ones (0.23–1.26 pg μL^{-1}). The obtained results highly suggest to do further investigations in order to collect statistically significant biological data to discriminate between the pathological state of lung cancer patients and physiological conditions of healthy subjects, using the simple, rapid and cheap method here reported for the quantification of urinary aldehydes.

1. Introduction

Membrane lipids oxidation by hydroxyl and superoxide radicals leads to the formation of saturated and unsaturated aldehydes, whose increment denotes greater oxidative stress [1,2]. Human tumor cells produce large amounts of radicals due to oxidative stress persisting during cancer [3,4], hence, the presence of aldehydes in biological fluids was considered as evidence that free-radical-mediated reactions took place [3,5] and aldehydes are considered as cancer biomarkers. High aldehyde levels were found in blood taken from cancer patients [6–8], and, in particular, concentration values of hexanal and heptanal (among all organic aldehydes) were higher in cancer patients than in healthy subjects [9]. Remarkable levels of hexanal and heptanal were also determined in exhaled air [10–14] and blood [15–18] from "lung" cancer patients. Since lung cancer is one of the most common causes of cancer death, biomarkers allowing early diagnosis are of particular interest, and several analytical methods for the determination of hexanal and heptanal in biological fluids have been proposed.

Gas chromatography (GC), gas chromatography–mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC) were used for the determination of various carbonyl compounds (among which hexanal and heptanal) in exhaled air, plasma and blood [19–22].

Deng et al. described a method for the determination of hexanal and heptanal in blood, based on solid phase microextraction (SPME) coupled with GC/MS, after aldehydes transformation into the corresponding oximes by on-fiber derivatization with *O*-2,3,4,5,6-(pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) [17].

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The derivatization step is included into the greatest part of the literature reports, where PFBHA and 2,4-dinitrophenyl hydrazine (DNPH) are mainly proposed as derivatization agents [21,23–28], and where it is possible to find the description of different and complex derivatization techniques such as headspace single-drop microextraction (HS-SDME) and, recently, dispersive liquid–liquid microextraction based on solidification of floating organic droplet (DLLME-SFO) [29].

The determination of hexanal and heptanal in blood matrix, apart from complex analytical procedures, obviously requires a more invasive sampling technique compared to the collection of urine.

Some literature data show detectable amounts of urinary aldehydes in subjects with non-pathological conditions [9,26,30,31], nevertheless, the reported methods for the determination of urinary aldehydes involved long and complex extraction and purification procedures (always including the derivatization step of analytes) and, since aldehydes are endogenous substances, in some cases the preparation of synthetic urine was necessary for analytes quantification [31]. Actually, scientific literature reports few analytical methods for the determination of urinary aldehydes by SPME/GC/MS [32–35], nevertheless, in the case of endogenous substances, the adopted quantification methods often involve high costs and long analytical times due to the necessity both to analyze several samples for each determination (as for "the addition method") and to have blank matrix based samples (or synthetic urine), involving further analytical steps aimed to bring the investigated analytes out from the biological matrix ("internal calibration method"). Moreover, although international agencies, such as the Food and Drug Administration (FDA), underline the importance of following validation protocols while developing bioanalytical quantification methods [36], most quantification methods reported in literature are not based on validation experiments.

Under these circumstances, the research study reported here was based on four aspects: (I) Since blood collection is an invasive technique, here the urinary matrix was examined. (II) A rapid, low cost and easy-to-use HS-SPME/GC/MS method was developed for urinary hexanal and heptanal determination, avoiding the derivatization step and adopting the external calibration quantification methodology. (III) The method was validated according to FDA guidelines. Selectivity, linearity, detection and quantification limits, precision and accuracy of the analytical procedure were evaluated and the recovery percentage of analytes from biological matrix, together with the sample stability during handling and storage were also determined. (IV) Unknown biological samples from lung cancer patients and healthy subjects (control group) were analyzed with the aim both to verify the actual applicability of the proposed methodology for the analysis of real samples, and to study if hexanal and heptanal were excreted in urine in detectable amounts, able to discriminate between the pathological state of lung cancer patients and the physiological conditions of healthy subjects.

2. Materials and methods

2.1. Chemicals and SPME fibers

Hexanal (purity ≥97%) and HPLC grade methanol were purchased from Sigma–Aldrich (St Louis, MO, USA) and Carlo Erba (Milan, Italy), respectively. Benzaldehyde and heptanal (purity ≥97%) were purchased from Merck (Whitehouse Station, NJ, USA), solid-phase microextraction manual holder and 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers were obtained from Supelco (Bellafonte, PA, USA). Sodium chloride, 10 mL vials and "superior standard" silicone/Teflon lined (0.1 mm thick coating) septa were purchased from Carlo Erba (Milan, Italy).

2.2. Preparation of methanolic, aqueous and urinary solutions

Aldehydes stock solutions were prepared in methanol with concentration levels of 500 ng μL^{-1} for hexanal and heptanal and of 1 μg μL^{-1} for benzaldehyde (used as internal standard, IS), and were stored at −20 °C. The IS stock solution was diluted up to 1 ng μL^{-1} (IS working solution). Hexanal and heptanal stock solutions were diluted to obtain a 10 ng μL^{-1} hexanal and heptanal methanolic solution. The latter was further diluted to have a set of six *methanolic working solutions* (3.00, 1.50, 0.75, 0.37, 0.19, and 0.09 ng μL^{-1}), used for the preparation of *matrix-based calibration standards* and of *standard aqueous samples*. Two other methanolic solutions (10 and 1 ng μL^{-1}), which were obtained by dilution from analytes stock solutions, were used in *working solutions stability* experiments. Analogously, a methanolic solution of hexanal, heptanal and internal standard (1 ng μL^{-1}) was prepared for the optimization of analytical conditions.

Matrix-based calibration standards and *aqueous standards* were prepared by adding 50 μL of analytes *working solutions* to 10 mL sealed vials (previously added with 1 g NaCl) containing 3 mL of urine and 3 mL of water, respectively, in order to obtain analytes concentrations of 50.00, 25.00, 12.50, 6.25, 3.12, and 1.56 pg μL^{-1}. Finally, 30 μL of the 1 ng μL^{-1} IS solution were added to each sample, obtaining a final IS concentration of 10 pg μL^{-}1. Analogously, *matrix-based blank samples* were prepared by adding only 30 μL of 1 ng μL^{-1} IS solution (without hexanal and heptanal) to 3 mL urine samples. *Urinary quality control samples* (QCi) were prepared six times, as above reported for matrix-based calibration standards, using fresh and independent methanolic solutions at 1.80, 0.48 and 0.15 ng μL^{-1} hexanal and heptanal concentrations, obtaining urinary analytes concentrations of 29.20 pg μL^{-1} (QC1), 7.80 pg μL^{-1} (QC2) and 2.40 pg μL^{-1} (QC3).

Matrix-based blank samples were analyzed: (I) for the evaluation of the detection technique selectivity, (II) together with *matrix-based calibration standards*, for the evaluation of the analytical response linearity, and (III) together with QCi, for the evaluation of accuracy and precision. *Matrix-based standards* were used for the optimization of the analytical procedure and during long term stability experiments, too. S*tandard aqueous solutions* were adopted for the quantification of unknown samples and were used in working solutions stability experiments and in the evaluation of detection and quantification limits. Both *matrix-based* and *aqueous standards* were used in the calculation of the analytes percentage recovery from the biological matrix.

2.3. SPME/GC/MS analysis

Hexanal and heptanal were extracted from aqueous or urinary samples by SPME, as follows: 10 mL sealed vials containing 1 g NaCl, 3 mL of sample and 30 μL of the 1 ng μL^{-1} IS solution were kept in an ultrasonic bath at 60 °C. PDMS/DVB fibers were directly exposed on the head space above the sample for 20 min. At the end of the sampling time, the fiber was immediately inserted into the GC injector (injector temperature, 200 °C) allowing the thermally desorption of analytes.

Analyses were performed on a gas chromatograph GC 8000 series (Fisons Instruments, Milan, Italy) interfaced with a single quadrupole mass spectrometer Voyager (Thermo Finnigan, Waltham, MA, USA), operated using Xcalibur software version 1.2.

Compounds were separated using a 25 m length × 0.25 mm ID × 3 μm film thickness CP-PoraBOND Q capillary column (Varian, Palo Alto, CA, USA). The carrier gas was helium with flow rate of 1 mL min^{-1}. Splitless mode was used with a 0.75 mm ID inlet liner for SPME (Supelco, Bellafonte, PA, USA). The temperature program used was the following: 160–270 °C at 15 °C min^{-1}, 270–300 °C at 5 °C min^{-1}.

Mass spectrometric data were obtained under the following conditions: electron ionization, 70 eV; source temperature, 250 °C; transfer line temperature, 230 °C; emission current, 200 μA.

Mass spectra were first obtained in full scan mode (range of acquisition, 30–300 *m*/*z*) in order to define analytes retention times. Following analyses were performed in selected ion monitoring (SIM) by dividing the chromatographic run into three different segments, one for each analyte. In the first segment (from 6.5 to 7.3 min) the ions at *m*/*z* 56.1, 72.1 and 82.1 (hexanal characteristic ions) were acquired, in the second segment and the third one (7.8–8.3 min and 8.3–9.5 min, respectively) the ions at *m*/*z* 55.0, 70.1, and 86.1 (heptanal) and at *m*/*z* 51.0, 77.0, and 106.0 (benzaldehyde) were registered.

2.4. Quantitative analysis of urinary hexanal and heptanal

Spot urine specimens were collected (in 100 mL sterile containers) form 25 healthy subjects (control group) and from 10 lung cancer patients. Then samples were stored at −20 °C and analyzed within a week. After thawing, 3 mL urine was transferred in sealed vials previously added with 1 g NaCl, then 30 μL of the 1 ng μL^{-1} IS solution were added and the sample was analyzed by HS-SPME/GC/MS.

Hexanal and heptanal amounts in unknown samples were quantified using calibration curves equations from the analyses of standard aqueous solutions, and then the concentrations obtained with this method were normalized according to the measured percentage recovery of each analyte from the biological matrix (see Sections 3.2.2, 3.2.4 and 3.3).

2.5. Statistical analysis

Statistical analyses were carried out using the SPSS software, version 17.0 for windows (SPSS ITALIA s.r.l., Bologna, Italy). Heptanal and hexanal urinary concentrations data were divided into four sub-distributions, defined by the health state of the urinary sample donor (i.e. heptanal and hexanal concentrations measured in controls – healthy subjects – and in lung cancer patients). Then, the normality of the frequency sub-distributions of the collected data was evaluated by Shapiro–Wilk test (the normality test adopted for sample size lower than 30 data). Since only two sub-distributions resulted normal, parametric tests were not applied, and the statistical analysis was performed using Kruskal–Wallis and Mann–Whitney non parametric tests.

3. Results and discussion

3.1. Optimization of SPME analysis

The complete chromatographic separation among the investigated analytes (hexanal, hexa; heptanal, hepta, internal standard, IS) was verified (Fig. 1) by analyzing 1 ng μL^{-1} methanolic solution. Then the HS-SPME extraction efficacy was evaluated.

HS-SPME is an extraction procedure based on analytes partition between liquid and vapor phases in equilibrium conditions and on the analytes affinity with the HS-SPME fiber coating. The efficacy of HS-SPME technique increases when the vapor phase concentration of the interested analyte increases. Various parameters can be modified in order to facilitate the transition of volatile substances contained into the liquid matrix to the gaseous phase above the sample (head space), and to allow the absorption of analytes on the fiber coating. Here, the salt effect was evaluated through the addition of sodium chloride to urinary samples in order to increase the ionic strength of the liquid phase, facilitating the aldehydes transition into the head space. Five identical urinary samples were prepared with same analytes concentrations (1 ng μL^{-1}), but various NaCl amounts (0, 0.5, 1.0, 1.5, and 2.0). As expected, the HS-SPME/GC/MS-SIM analyses related to the NaCl added samples showed higher signal to noise ($S\,N^{-1}$) values of all the analytes' chromatographic peaks, and the obtained $S\,N^{-1}$ plateau (Fig. 2) suggested the addition of 1 g NaCl for a better extraction recovery of analytes from the urinary matrix.

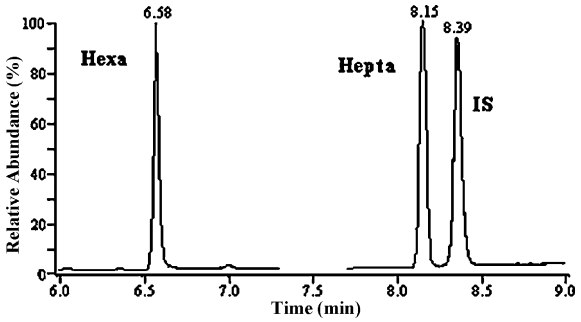

Fig. 1. Hexanal, heptanal and internal standard chromatographic separation. GC/MS-SIM analysis of 1 ng μL^{-1} methanolic solution.

The HS-SPME technique optimization was also performed by comparing various extraction times (times of contact between the HS-SPME fiber and analytes) and residence times of the liquid matrix in the ultrasonic bath. Two urinary samples, containing 1 ng μL^{-1} hexa, hepta and IS, were immediately treated with PDMS/DVB microfibers for 30 min and for 20 min at 60 °C. A third sample, with same concentration of analytes, was first kept in the ultrasonic bath at 60 °C for 15 min (in order to allow to reach the partition equilibrium), and then analytes were extracted by the PDMS/DVB fiber, with a contact time of 15 min more. After the extraction procedures, samples were analyzed by GC/MS-SIM, and the ratios between analytes and IS chromatographic peaks areas were compared, showing that an immediate contact time of 20 min guaranteed a good compromise between high analytes recoveries and reduced analysis times (data not shown).

3.2. Analytical method validation

3.2.1. Selectivity

According to FDA Guidelines, *selectivity is the ability of an analytical method to differentiate (. . .) the analyte in the presence of other*

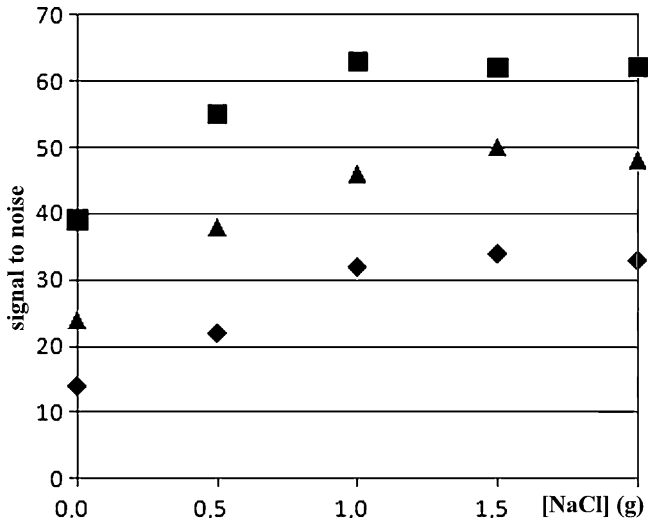

Fig. 2. Signal to noise values of HS-SPME/GC/MS-SIM analyses of hexanal, heptanal, internal standard 1 ng μL^{-1} urinary samples, added with different amounts of NaCl. The addition of at least 1 g NaCl improves the analytes extraction from the urinary matrix (salt effect). ♦, hexa; ■, hepta; ▲, IS.

Table 1
Recovery.

	Analytical response[a]						Slope coefficient (α)	
	$A_{S,U\text{-}i}$ (25.00 pg μL^{-1})		$B_{S,U\text{-}i}$ (6.25 pg μL^{-1})		$C_{S,U\text{-}i}$ (1.56 pg μL^{-1})			
	Hexa	Hepta	Hexa	Hepta	Hexa	Hepta	Hexa	Hepta
U								
I	0.401	1.741	0.122	0.426	0.037	0.113	0.0153	0.0697
II	0.412	1.721	0.120	0.413	0.038	0.114	0.0158	0.0689
III	0.415	1.694	0.142	0.465	0.041	0.124	0.0174	0.0666
S	0.608	2.397	0.144	0.536	0.039	0.146	0.0244	0.0970
Recovery (Rec% ± SD)							66.3 ± 4.5	70.5 ± 1.7

[a] Analytical response: areas ratio between chromatographic peaks of the investigated analyte and the internal standard, measured by HS-SPME/GC/MS-SIM analyses of samples at three different concentration levels (*A–C*); U, urinary samples; S, standard aqueous solutions; i, I-III: repeated experiments (triplicate).

components in the sample, by analyzing blank samples of the appropriate biological matrix (. . .) from at least six sources [36]. In the specific case here reported, this concept could mean that the GC/MS-SIM analysis of matrix-based blank samples (added only with IS) should show the absence of interfering compounds eluting at the same retention time of analytes and giving signals at the same *m*/*z* values. When the investigated analytes are endogenous substances, even if interfering compounds are absent, the analysis of blank samples necessarily shows chromatographic peaks at analytes retention times, which are related to the analyte amount actually present in the biological matrix, and the evaluation of selectivity is often neglected [31,37].

Here six *matrix-based blank samples* from different sources (healthy volunteers) were analyzed by adopting two acquisition modes, full scan and SIM mode. Full scan analyses showed the absence of interfering compounds at analytes retention time, nevertheless, due to the lower sensitivity of full scan mode compared with SIM one, these analyses cannot guarantee the analytical method's selectivity by themselves. Hence, SIM analyses were evaluated in order to verify the correspondence (within a 5% tolerance) of selected ions' relative heights between SIM mass spectra coming from samples and hexanal and heptanal reference mass spectra reported in literature (NIST libraries, Xcalibur software, version 1.2). Relative heights of characteristic ions were analogous, suggesting the absence of interfering substances. An example is reported in Fig. 3.

3.2.2. Linearity of urinary and aqueous calibrators

Analytical response linearity was evaluated by calculating the coefficients of the determination of calibration curves based on the analysis of urinary samples (*matrix-based calibration standards* and *matrix-based blank samples*) coming from subjects with no occupational exposure to aldehydes and no respiratory pathologies.

The ratios between chromatographic peaks areas of hexa and hepta compared to the IS chromatographic peak area (analytical response) were measured, and areas ratios (from 0.043 to 0.745 for hexa and from 0.123 to 3.667 for hepta) coming from the GC/MS-SIM analysis of *matrix-based calibration standards* were normalized (subtracted) with areas ratios (0.006 for hexa and 0.010 for hepta) from *matrix-based blank samples* analysis. This subtraction is indispensable because of hexa and hepta endogeneity, leading to the urinary excretion of analytes not only in lung cancer patients but also in non-pathological conditions.

The obtained determination coefficients (hexa, $R^2 = 0.9909$; hepta, $R^2 = 0.9969$) demonstrated the analytical response linearity in the investigated concentration range (50–1.56 pg μL^{-1}). Nevertheless, the percentage deviations of the calculated concentrations, for each matrix-based calibrator, compared to the nominal ones did not respect validation protocols' requirements, i.e. percentage deviations of calibrators should have been lower than 15% for each calibrator and lower than 20% for the lower limit of quantification [36]. Since for "exogenous" substances this kind of problem does not occur, the obtained high percentage deviations could be attributable to the endogenous nature of the investigated analytes, which lead to remarkable errors, especially at low analytes concentration levels, influencing the quantification process. As a consequence, matrix-based calibration curves could not be used for the quantification of aldehydes in unknown samples, therefore calibration curves based on aqueous samples, rather than urinary ones, were constructed. Determination coefficients of 0.9971 (hexa) and 0.9949 (hepta) were obtained, with percentage deviations of calibrators being in the range 1.04–14.7%, that is to say within the accepted limits suggested by international validation protocols.

3.2.3. Recovery

Extraction recovery is defined as the analytical response *obtained from an amount of the analyte added to and extracted from the biological matrix, compared with the analytical response obtained for the true concentration of the pure standard. Recovery experiments should be performed by comparing the analytical results from extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery* [36]. Here, three standard aqueous solutions (S) and nine urinary samples (U) at different analytes concentrations, low (samples C_S and $C_{U\text{-}I}$, $C_{U\text{-}II}$, $C_{U\text{-}III}$, 1.56 pg μL^{-1}), medium (samples B_S and $B_{U\text{-}I}$, $B_{U\text{-}II}$, $B_{U\text{-}III}$, 6.25 pg μL^{-1}) and high (samples A_S and $A_{U\text{-}I}$, $A_{U\text{-}II}$, $A_{U\text{-}III}$, 25.00 pg μL^{-1}) where prepared and four calibration curves were calculated. The first one was based on the analyses of standard aqueous solutions and the others were obtained by the GC/MS-SIM analyses of urinary samples. Table 1 reports the analytical responses measured by GC/MS-SIM analyses, together with the slope coefficients (α) of each curve. Percentage recovery from the urinary matrix (Rec%$_i$, where i = I, II, III) was calculated as the percentage ratio between the slope coefficients of urinary curves ($\alpha_{U\text{-}i}$) and the standard one (α_S): Rec%$_i$ = $\alpha_{U\text{-}i}/\alpha_S \times 100$ [38,39]. Then the mean recovery values with the relative standard deviation (Rec% ± SD) were calculated both for hexa and hepta (Table 1).

It is noteworthy that, due to the endogeneity of hexa and hepta, the analytical responses obtained from the analyses of urine samples added with analytes' known amounts should be subtracted with those from the analysis of matrix-based blank samples (Sections 3.2.2 and 3.2.5). But, in this case, the subtraction (normalization) is not necessary, because the slopes of calibration curves from "subtracted" and "unsubtracted" analyses would be the same.

3.2.4. Limits of detection and quantification

As suggested by international validation protocols agencies, the limit of detection (LOD) and the lower limit of quantification (LLOQ) can be obtained by analyzing six matrix-based blank samples and they can be *defined by various conventions to be the analyte*

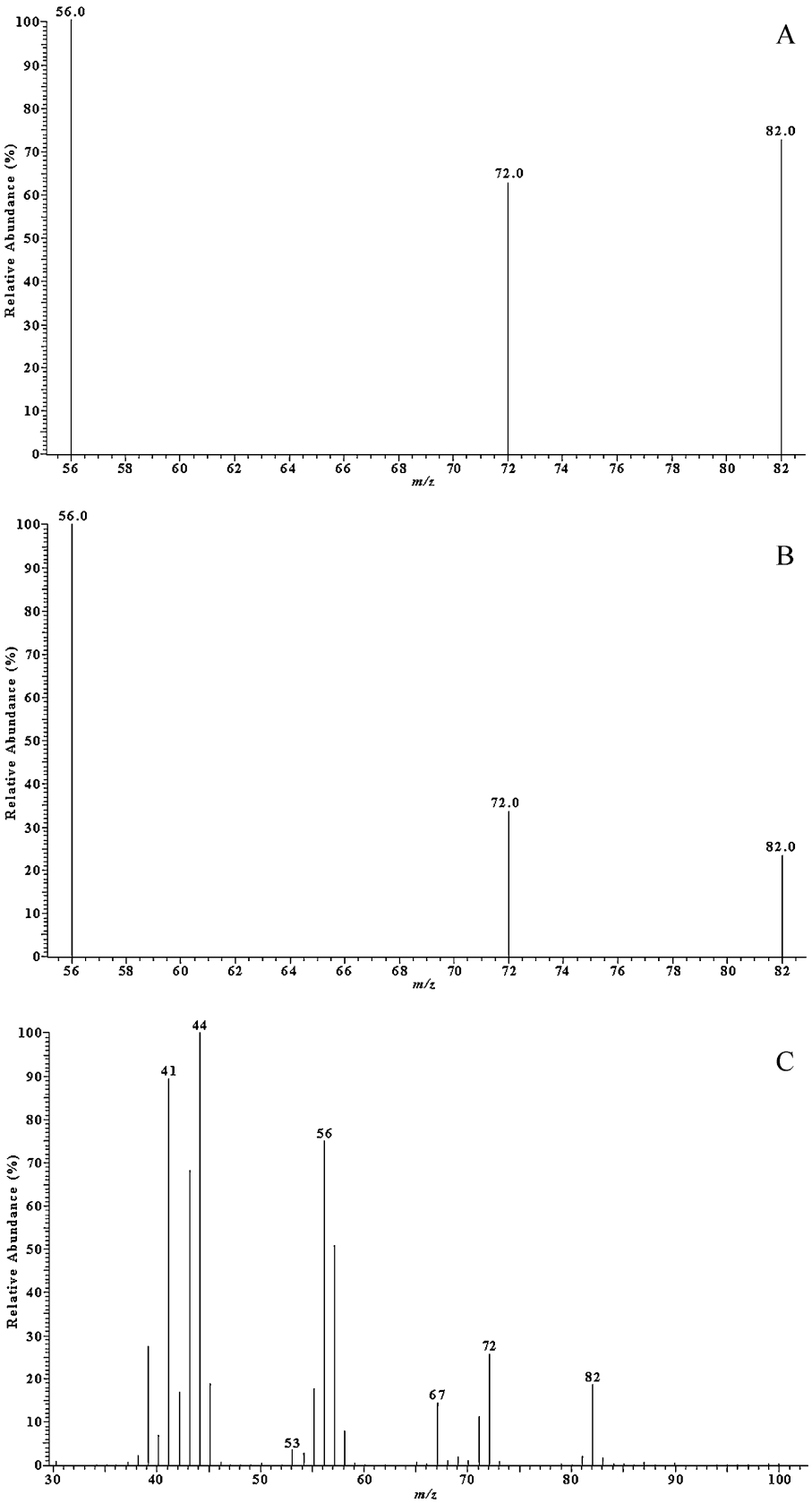

Fig. 3. GC/MS-SIM analysis of a matrix-based blank sample: mass spectra from chromatographic peaks chosen into the background (A) and at hexanal retention time (B). The correspondence, within a 5% tolerance, between the relative intensities of signals with respect to those from the full scan hexanal mass spectrum of NIST library (C) allows hexanal identification: A and B mass spectra, respectively, correspond to an interference and to the analyte under examination.

Table 2
Detection and quantification limits.

	Limits of detection and quantification	
	LOD (pg μL^{-1})	LLOQ (pg μL^{-1})
Hexanal	0.11	0.23
Heptanal	0.10	0.21

Table 3
Quality control samples: accuracy and precision.

	QC1, 29.20 pg μL^{-1}		QC2, 7.80 pg μL^{-1}		QC3, 2.40 pg μL^{-1}	
	Acc%	CV%	Acc%	CV%	Acc%	CV%
Hexanal	−11.25	1.98	−3.41	1.21	7.80	4.46
Heptanal	−6.86	0.45	10.85	2.82	6.59	4.02

concentration corresponding to the sample blank value plus 3 (LOD) and *plus 5, 6 or 10* (LLOQ) *standard deviations of the blank mean* [40]. Alternatively, some authors define LODs and LLOQs as the analyte concentration giving a signal to noise ratio of 3 and 10, respectively [41,42]. Here, due to the endogenous nature of hexa and hepta, *standard aqueous solutions* containing decreasing analytes concentrations were used instead of matrix-based blank samples and LOD and LLOQ were evaluated by measuring the signal to noise ratios of chromatographic peaks obtained from the HS-SPME/GC/MS analyses of these aqueous standards. Then the aldehydes concentrations giving the lowest signal to noise ratio were normalized by taking into account the percentage recovery of each analyte from the biological matrix (hexa recovery = 66.3% and hepta recovery = 70.5%).

The analyses of *standard aqueous solutions* containing hexa and hepta at 0.07 and 0.15 pg μL^{-1}, gave chromatographic peaks with a signal to noise ratio of 4:1 and 11:1 for hexa and 5:1 and 12:1 for hepta, respectively. The obtained LODs and LLOQs, calculated by dividing these concentration values by 0.663 (for hexa) and 0.705 (for hepta) are reported in Table 2.

3.2.5. Accuracy and precision

Accuracy (Acc%) and precision (expressed as percentage variation coefficient, CV%) of the proposed quantification method were calculated by analyzing *quality control samples* (QCi), prepared in urine at three different concentrations QC1, QC2, QC3.

Commonly, when exogenous substances are quantified by matrix-based calibration curves, the analysis of QCi directly gives Acc% and CV% values [38,39]. Here, together with QCi, other urinary blank samples were prepared using the same urine adopted for QCi preparation and then analytical responses obtained from QCi analyses (from 0.057 to 0.684 for hexa; from 0.186 to 2.427 for hepta) were subtracted to blank samples ones (0.005 for hexa and 0.010 for hepta). Calibration curves based on *standard aqueous solutions* were used for analytes quantification, and the so obtained analytes concentrations were normalized according to the average percentage recovery of each analyte from the biological matrix (66.3 and 70.5% for hexa and hepta, respectively, as above reported). For all the three examined concentration levels, the obtained Acc% and CV% values are lower than 15% (Table 3), and satisfy FDA requirements.

3.2.6. Stability

Hexa and hepta stability in working solutions was evaluated at room temperature for 6 and 24 h (which are the analytical conditions adopted during analytes handling and analysis), using methanolic solutions at two aldehydes concentration levels, *A* (10 ng μL^{-1}) and *B* (1 ng μL^{-1}). These concentration levels were chosen for different reasons. The *A* concentration was tested because all the other experiments were performed using samples (both aqueous and urinary samples) freshly prepared by diluting the 10 ng μL^{-1} solution. The *B* level was chosen within the concentration range of working solutions.

A and *B* solutions were both divided into three aliquots, then the IS was added to the first one and it was immediately analyzed. The other two aliquots were analyzed after storage at room temperature for 6 and 24 h. The experiment was repeated in triplicate.

Long term analytes' stability was evaluated reproducing the analytical conditions encountered during biological samples' storage, as regarding storage times and temperature. 3 sets of *matrix-based samples* at three analytes concentration levels within the calibration range were prepared: 9 *A* samples (25.00 pg μL^{-1}), 9 *B* (6.25 pg μL^{-1}) and 9 *C* (1.56 pg μL^{-1}). The first samples set (constituted by 3 *A* samples, 3 *B* and 3 *C*) was immediately analyzed, the others were stored at −20 °C (storage temperature) and analyzed after 1 week and 1 month (storage times). In all cases the IS was added immediately before analysis.

Working solutions stability and long term stability were, respectively, evaluated comparing the analytical response of methanolic samples kept at room temperature for 6 and 24 h ($X_{6\,h}$, $X_{24\,h}$, where: *X* = analytical responses corresponding to *A* and *B* concentration levels) and of matrix-based frozen samples ($X_{1\,w}$, $X_{1\,m}$, where: *X* = analytical responses corresponding to *A*–*C* concentration levels; 1 w = 1 week, 1 m = 1 month) to the analogous samples immediately analyzed (X_0, where *X* = analytical responses corresponding to *A*–*C* concentration levels). The following formula was used: Analytes Percentage Stability = $X_j/X_0 \times 100$, where *X* = analytical responses corresponding to *A*–*C* concentration levels; *j* = 6 h, 24 h, 1 week, and 1 month.

In both cases, GC/MS analyses were necessarily carried out on different times (hours, weeks and months), during which instrumental conditions could vary, leading to uncomparable results. The addition of IS immediately before analysis and the measurement of analytical response as the ratio between analytes areas and the IS one assure that different analytical responses were actually due to analytes' degradation rather than instrumental variations.

The obtained results, expressed as percentage, are reported in Table 4. Analytes' working solutions stability is guaranteed at room temperature for 24 h, while the remarkable analytes loss after 1 month storage (especially at low concentrations) suggests that biological samples should be analyzed within a week after storage at −20 °C.

Table 4
Working solution and long-term stability.

	Working solution stability ± SD (%)				Long-term stability ± SD (%)					
	A (10 ng μL^{-1})		*B* (1 ng μL^{-1})		*A* (25.00 pg μL^{-1})		*B* (6.25 pg μL^{-1})		*C* (1.56 pg μL^{-1})	
	6 h[a]	24 h[a]	6 h[a]	24 h[a]	1 week[b]	1 month[b]	1 week[b]	1 month[b]	1 week[b]	1 month[b]
Hexanal	95.3 ± 10.9	94.7 ± 0.7	96.2 ± 6.8	102.6 ± 26.6	102.1 ± 7.0	85.1 ± 3.4	97.3 ± 7.1	77.1 ± 2.3	94.6 ± 8.2	73.1 ± 8.4
Heptanal	93.2 ± 4.2	98.0 ± 4.3	92.3 ± 7.5	98.2 ± 19.5	98.4 ± 6.5	87.4 ± 7.3	95.5 ± 1.7	80.1 ± 2.3	96.4 ± 6.5	74.1 ± 7.5

[a] Room temperature.
[b] Storage at −20 °C.

Table 5
Hexanal and heptanal urinary concentrations of unknown samples.

	Hexanal (pg μL^{-1})	Heptanal (pg μL^{-1})
Control group		
1	0.24	0.22
2	1.26	0.29
3	0.23	0.22
4	0.30	0.23
5	0.24	0.27
6	0.24	0.21
7	0.69	0.32
8	0.79	0.21
9	0.25	0.23
10	0.71	0.37
11	0.25	0.42
12	0.57	0.30
13	0.26	0.21
14	0.28	0.38
15	0.28	0.23
16	0.25	0.22
17	0.29	0.21
18	0.23	0.22
19	0.28	0.22
20	0.24	0.21
21	0.25	0.22
22	0.52	0.22
23	0.27	0.29
24	0.86	0.69
25	0.42	0.21
Patients		
1	0.81	0.30
2	3.13	0.40
3	4.36	0.95
4	0.24	0.38
5	1.26	0.42
6	2.28	0.41
7	0.78	0.22
8	0.47	0.50
9	1.39	0.23
10	0.65	0.63

3.3. Quantitative analysis of hexanal and heptanal in normal urine and lung cancer urine

Unknown samples coming from health subjects (control group) and lung cancer patients were collected, stored at −20 °C and analyzed within a week. Aldehydes' concentrations in biological samples were worked out by normalizing results coming from aqueous calibration curves equations (hexa: $y = 0.0252x + 0.0138$; hepta: $y = 0.0996x + 0.0029$) compared to the average percentage recovery of each analyte from the biological matrix (66.3% and 70.5% for hexa and hepta, respectively).

The choice of using calibration curves based on the analysis of aqueous samples rather than urinary ones was due to the endogenous nature of the examined analytes which makes difficult to have "blank" urine, i.e. urine completely without the investigated analytes. Some authors overcame this problem by preparing synthetic urine in laboratory [31], with obvious increments of analytical costs and times. Besides, although the notable advantage of this approach, urine chemical composition may differentiate from subject to subject and synthetic urine could not reflect individual variations of urinary excretion. In contrast, the analytical procedure here reported is independent from the biological matrix. The normalization of analytes concentration values compared to the average percentage recovery of each analyte from various urine samples takes into account both any different chemical compositions of urine and the differences in the analytes behavior when extracted from water rather than from urine.

Unknown samples analysis (Table 5) showed urinary aldehydes concentrations much lower than expected values of about 15 pg μL^{-1}, reported in literature [31]. In particular, heptanal and hexanal concentrations were within ranges of 0.22–0.95 and 0.21–0.69 pg μL^{-1} (heptanal urinary excretion in patients and control group, respectively), and of 0.24–4.36 and 0.23–1.26 pg μL^{-1} (hexanal urinary levels in patients and control group).

The measured concentration levels, being beyond the calibration range, can only be considered as indicative values, nevertheless, the obtained results showed that higher analytes urinary concentrations could be expected in patients suffering from lung cancer. In fact, even at first glance, while in the case of heptanal, no evident differences of urinary excretion were revealed between lung cancer patients and healthy subjects, on the contrary, higher hexanal urinary concentrations were already evident in patients with respect to controls. In order to support these findings, after having performed normality tests, non-parametric tests were applied to the collected biological data, aimed to verify the analytes' different urinary excretions between patients and healthy subjects. Both for heptanal and hexanal, p-values of 0.002 were obtained, indicating a significant difference of median values of analytes urinary concentrations measured in patients with respect to controls ones (hepta, 0.41 and 0.22 pg μL^{-1}, hexa, 1.04 and 0.28 pg μL^{-1}, in biological data from patients and controls, respectively).

4. Conclusions

Urinary levels of hexanal and heptanal can be measured by HS-SPME/GC/MS-SIM, avoiding the analytes derivatization step, often reported in literature. After the optimization of the analytical technique, the quantification method here reported was tested by following international validation protocols, in order to guarantee analytical data quality and reproducibility. The proposed methodology, being based on the external calibration method, presents many advantages in terms of analytical rapidity and simplicity, avoiding any costs increment and long analysis times related to the use of other quantitative methods, such as the addition one, adopted for the determination of endogenous substances in biological matrix. Analytical costs and times are also reduced in comparison to the commonly used internal calibration method, whose application needs either synthetic urine or matrix-based blank samples involving further analytical steps aimed to bring the investigated analytes out from the biological matrix.

The proposed methodology was applied to the analysis of real unknown samples from lung cancer patients and healthy subjects, and the obtained results suggest two considerations. The determination of aldehydes levels in urine should be based on lower calibration ranges compared to concentration data reported in literature. The possibility of using hexanal and heptanal urinary levels as lung tumor biomarkers, able to discriminate the pathological state of lung cancer patients from physiological conditions of healthy subjects, is not excluded. Given the importance of having urinary biomarkers allowing lung cancer diagnosis, further investigations aimed to collect a large number of biological data are needed and highly recommended.

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