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Article

In situ Derivatization of Lung Cancer Biomarker Aldehydes by Parallel-DPX-Cork and Quantification by HPLC-DAD

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Lung cancer is one of the main causes of death for thousands of people yearly around the world. Biomarker aldehydes, such as hexanal and heptanal, are compounds related to the development of lung cancer, which can be detected in the early stages of this disease. A methodology was proposed to determine these aldehydes in urine, with a new configuration associated with the sample preparation step. A novel strategy with a parallel-disposable pipette extraction (DPX)-cork device was used, offering a fast and affordable extraction methodology with analysis performed by high performance liquid chromatography with diode array detector. In optimization steps, multivariate and univariate designs were applied, providing the following conditions: urine sample centrifuged at 3500 rpm for 15 min, 30 µL and 6 min of dinitrophenylhydrazine impregnation, 10× urine diluted in ultrapure water, pH adjusted to 4.8, 7 extraction cycles with 1.5 min each, 30 mg of cork, 2 desorption cycles and solvent acetonitrile with 300 µL. Limits of detection were 0.13 ng mL^{-1} for both analytes and limits of quantification were 0.40 and 0.41 ng mL^{-1} for hexanal and heptanal, respectively. Intraday and interday precisions ranged from 4 to 21%. Relative recoveries ranged from 86 to 107%, assessed at three concentrations. Urine samples were analyzed, but the presence of aldehydes was not detected.

Keywords: lung cancer, biomarker aldehydes, parallel-DPX, cork, urine

Introduction

Lung cancer is classified as one of the main causes of death around the world, with about 2.21 million cases and 1.80 million deaths in 2020. Many causes are associated with the development of this disease in humans, such as physical (exposure to radiation), chemical (smoking, toxic compounds in human products) and biological (predisposition between generations, or infections by external agents).[1] Symptoms usually appear in middle to advanced stages of development, such as coughing, shortness of breath and coughing up blood. At this point, early-stage diagnosis needs to be performed and biomarkers that are present since the onset of lung cancer can be used.[2]

Some aldehydes are naturally present in the human body, such as formaldehyde, benzaldehyde, acetaldehyde, hexanal and heptanal. These are related to oxidative stress events in the metabolism of cells through a biological disorder, and the last two compounds are directly associated with the development of lung cancer.[3,4] Hexanal and heptanal are compounds of low molecular weight, with high volatility and reactivity. Their identification/detection can be achieved in several matrices, especially urine. Urine is easily available, accessible, and the collection is non-invasive to patients, facilitating the process of analysis.[4-7]

Urine is a complex matrix of heterogeneous composition that can also affect instrumental analysis.[8,9] Therefore, urine analysis generally requires a sample preparation step prior to injection. This step allows for the separation and concentration of the target compounds to solvents of materials compatible with the analytical instruments. Some sample preparation techniques described for the analysis of these compounds, including heptanal and hexanal, in human urine are solid-phase extraction (SPE),[4] solid-phase microextraction (SPME),[3,5,10,11] hollow fiber-liquid phase microextraction (HF-LPME)[9,12] and bar adsorptive

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The authors dedicate this work to Professor Carol H. Collins
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microextraction (BAμE).[6] In addition, a technique that has not yet been explored in these studies consists of disposable pipette extraction (DPX).

DPX is a sorbent-based sample preparation technique that has been gaining attention, described as a simple, fast, versatile, and effective technique. The device consists of a tip, containing two filters, one fixed (bottom) and one removable (top), and a free extractor material inside, allowing the extraction process based on promoting a dynamic mixture between matrix and sorbent material. With the DPX configuration, it is possible to use different materials inside, promoting versatility for the extraction of different compounds and application in matrices. Green materials have been gaining space within green analytical chemistry due to their numerous benefits, such as good sorption capacity of organic compounds, ease of handling, simple disposal, and low cost of production.[13-15] Among materials, lignocellulosic sorbents can be mentioned, which are basically comprised of lignin and cellulose. They exhibit different functional groups in their chemical structures, allowing different interactions with a wide range of organic compounds.[16]

There are different classes of lignocellulosic biosorbents that exhibit different amounts of these two biopolymers. Cork is a material belonging to this class and, in addition to the biopolymers mentioned, it exhibits suberin in its structure, which is a hydrophobic polymeric network mostly comprised of carbon and hydrogen (40%), with proportions of lignin (20%), polysaccharides (10%) and other substances. Its chemical and physical structure allow different interactions with analytes (hydrogen bonds, π-μ, van der Waals), stability at different pHs and sample compositions, in addition to good porosity. Cork has already been used as sorbent material in some applications involving different sample preparation techniques.[17-19]

In addition to sample preparation techniques, the separation/identification of heptanal and hexanal is generally performed through chromatographic techniques, especially liquid chromatography. The use of detectors with specific wavelengths is one of the simplest formats for this type of compound; however, it is generally necessary to perform a derivatization step because these spectrophotometric detectors do not provide intense response for these analytes. Thus, a derivatization reaction with 2,4-dinitrophenylhydrazine (DNPH) is an alternative, since this procedure has already been reported in the literature as possessing an efficient and simple workflow.[20-22]

In this context, a novel application of cork is proposed in this study for the determination of hexanal and heptanal in human urine, using cork powder as sorbent for a DPX procedure followed by high-performance liquid chromatography with diode with array detection (HPLC-DAD). Additionally, a device allowing for parallel *in situ* derivatization steps, and which increased the sample throughput, was proposed. Optimizations were conducted through univariate and multivariate approaches, and the analytical figures of merit were successfully determined using this affordable and eco-friendly methodology.

Experimental

Reagents and materials

Analytical standards of hexanal and heptanal were obtained from Sigma-Aldrich (St. Louis, MO, USA) and solutions were prepared at 100 μg mL^{-1} of each analyte, in HPLC grade methanol (MeOH) (J.T. Baker, Mallinckrodt, NJ, USA). The 2,4-dinitrophenylhydrazine (DNPH) derivatizer was purchased from Sigma-Aldrich, using a solution of 2.8 g L^{-1} for *in situ* derivatization step. Acetonitrile (ACN) and ethyl acetate (EtAc) were used, both commercialized by Merck (Rio de Janeiro, RJ, Brazil) in optimization of desorption solvent; solutions of citric acid and dibasic sodium phosphate were obtained from Vetec (Duque de Caxias, RJ, Brazil) to prepare the buffer solution and adjust the pH in extraction optimization, and ultrapure water (UP) was obtained from a purification system with a resistivity of 18.3 MΩ cm (Mega Purity, Billerica, USA). The tips used were DPX-Blank of 5 mL, purchased by DPX Labs (Columbia, SC, USA). Cork powder with granulometry of 75 μm was prepared using sandpaper and a sieve.

Instrumentation and chromatographic conditions

A high-performance liquid chromatographer, model LC-20AT (Shimadzu, Japan), equipped with a diode array detector, model SPD-M20A, was used. A manual injector, model Rheodyne 7725i (Rohnert Park, USA) and 20 μL injection loop were used. Mobile phase of methanol and UP water were applied using isocratic mode in a ratio of 88:12 (v/v), respectively, with a total time of 11 min and a flow rate of 1.5 mL min^{-1}. The wavelength monitored was 360 nm.[6] The chromatographic separation was carried out in a Zorbax Eclipse XDB-C18 column (Agilent, 250 mm × 4.6 mm and particle size of 5 μm). A centrifuge 12 tubes/15 mL (Centrilab, Brazil) model 80-2b was used.

Preparation of cork powder for DPX

A device for parallel-DPX with 5 coupled tips of 5 mL each was used to perform the technique.[23] Cork powder used in the DPX tip was previously prepared using

discarded stoppers, according to Dias *et al.*[24] These stoppers underwent a cleaning process with UP water and ultrasonic bath, for 2 h, with repeated washing processes until the water came out clear. For drying, they were inserted in an oven at 110 °C for 12 h and removed for sanding with wood sandpaper. The powder formed was then sieved to obtain particles with sizes ≤ 75 μm. DPX-Blank tips containing cork powder were inserted in the parallel-DPX device. For conditioning, 300 μL of ACN and MeOH 1:1 (v/v) were used and 10 cycles of 1 mL of UP water were performed.

Optimization of the parallel-DPX-cork procedure

Urine obtained from a male volunteer was centrifuged to sediment possible proteins and organic material, with 15 min at 3500 rpm; this procedure may alleviate problems with foaming and clogging of the filters. Then, urine was spiked with 300 ng mL^{-1} of the analytes, pH adjusted with citric acid/dibasic sodium phosphate buffer to 4.8, diluted 10 times with UP water, and 2 mL of sample was used for each extraction cycle. For desorption, 300 μL of EtAc/MeOH (1:1, v/v) volume were established.

Initially, the study of urine dilution was performed using undiluted urine, and diluted at 10, 20 and 40× in UP water. Afterward, *in situ* derivatization was optimized using the variables impregnation time of DPNH (0 to 6 min) and volume of derivatizing agent (30 to 170 μL) through a central composite design. Then, extraction time (17 to 102 s) and number of cycles (1 to 7 cycles) were also optimized using a central composite design. Regarding cork mass, this variable was evaluated from 10 to 30 mg using univariate planning. Moreover, a Simplex Lattice design was performed to optimize the desorption solvent using ACN, MeOH, and EtAc, and desorption cycles were also examined from 1 to 5 cycles, with univariate mode. All the multivariate and univariate designs were treated using Statsoft Statistica 10 (Statsoft, USA)[25] and Microsoft Excel 2010 software.[26] The DNPH was handled with care for the safety of the analyst and the laboratory.

Urine samples

For the development of the methodology, analyte-free urine samples were obtained from a male volunteer, then stored in polytetrafluoroethylene (PTFE) bottles and kept under refrigeration at 4 °C, until the analysis. The application of the proposed method was carried out in four samples from anonymous volunteers between 20 and 30 years old, both men and women. The Ethics Committee from the Federal University of Santa Catarina approved this study with the number 11300913718.3.0000.0121.

Analytical parameters of merit

To obtain the analytical parameters of merit, calibration curves were constructed for the two analytes using extractions performed in a urine sample obtained from a male volunteer, free from the compounds studied. The following parameters were obtained: limit of detection (LOD), limit of quantification (LOQ), coefficient of determination (R^2) and linear working range. LOD was calculated using 3 times the standard deviation of the first concentration of the calibration curve divided by the slope obtained in the linear equation, and LOQ was calculated as 3.3 times the LOD. To assess accuracy (relative recovery) and precision (intraday and interday), urine obtained from a female volunteer's urine was spiked at 3 concentrations.[27,28] In this study, four urine samples obtained from male and female volunteers (20 to 30 years old) were collected and analyzed.

Results and Discussion

Urine dilution study

Urine dilution can directly affect the pre-concentration of the analytes in the sorbent phase. In some cases, diluting complex matrices that contain a heterogeneous composition allows for mass transfer to be favored, thus improving the analytical response. In this way, pH of the matrix was previously adjusted to 4.8, and a derivatization was performed with 50 μL of DNPH in 5 min of agitation. This optimization was carried out with the matrix undiluted and diluted 10, 20 and 40 times in UP water. In Figure S1 (Supplementary Information (SI) section) a bar graph of these results is presented with the chromatographic peak data normalized in relation to the highest response. As can be seen, the best response was for urine diluted 10 times.

Chromatographic separations using the aforementioned condition are shown in Figure 1, consisting of: (a) extraction of a blank urine with DNPH derivatization; (b) extraction of urine spiked with 1 μg mL^{-1} of the aldehydes; and (c) direct injection of 1 μg mL^{-1} of the aldehydes derivatized with DNPH. There were no interfering peaks observed in those chromatograms. The retention times were 8.0 min for hexanal, 10.25 min for heptanal, and 4.4 min for DNPH.

Desorption solvent optimization

In this step, a Simplex Lattice design with 10 experiments including a triplicate in central point using ACN, MeOH, and EtAc was evaluated. These solvents were selected

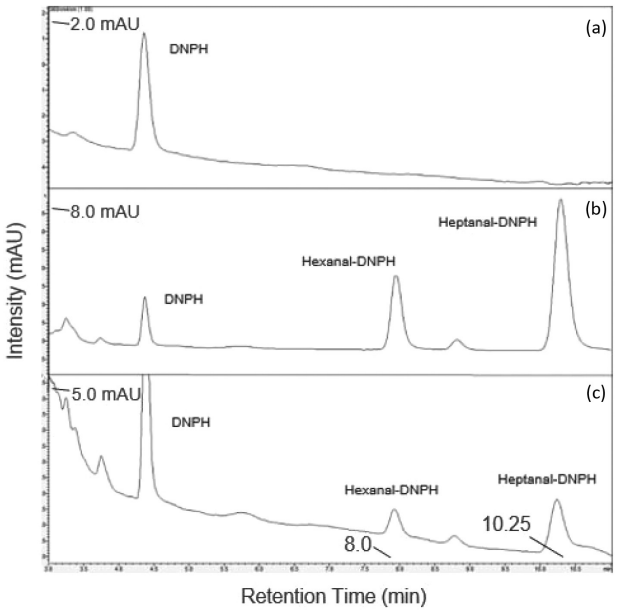

Figure 1. Comparative chromatogram, at 360 nm, for: (a) urine blank extraction with DNPH derivatization; (b) extraction in 1 μg mL^{-1} of aldehydes in urine samples; and (c) direct injection of 1 μg mL^{-1} of DNPH-derivatized aldehydes. Conditions: derivatization with 50 μL of DNPH and 5 min of impregnation, 10× urine diluted in UP water, 15 mg of cork, pH adjusted to 4.8, 3 extraction cycles, 2 desorption cycles, acetonitrile desorption solvent with 300 μL.

based on physico-chemical properties and interactions with the analytes, as well as compatibility with the analytical instrumentation. A triangular surface obtained in this optimization is shown in Figure 2.

The surface obtained (R^2 = 0.9968), using a special cubic model, presents a maximum response around 50%

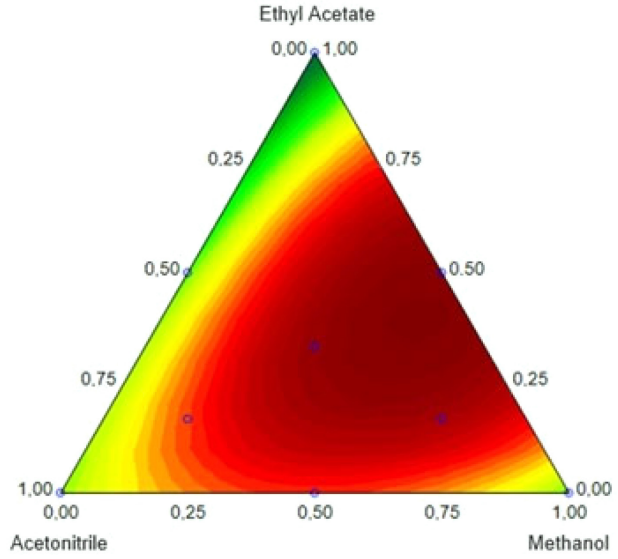

Figure 2. Ternary surface generated from the Simplex Lattice design to evaluate the desorption solvent. Conditions: derivatization with 50 μL of DNPH and 5 min of impregnation, 300 ng mL^{-1} in fortification, 10× urine diluted in UP water, 15 mg of cork, pH adjusted to 4.8, 3 extraction cycles, 2 desorption cycles, desorption solvent in 300 μL.

of EtAc and 50% of MeOH. This mixture allowed a good dynamic mixing with the cork, guaranteeing a high contact area for the desorption of the aldehydes, in addition to allowing the breakdown of interaction between the extracting phase and the analytes.

Optimization of desorption cycles

The number of cycles of the DPX procedure that allows for satisfactory interaction of cork with the solvent mixture is crucial for desorbing the analytes properly, reducing errors and avoiding possible carryover effect. In Figure S2 (SI section) a bar graph for the normalized chromatographic peak area is shown in relation to the highest response for 1, 3 and 5 desorption cycles with EtAc/MeOH (1:1) as desorption solvent.

As can be seen, using 3 cycles allowed for the best response. In 1 cycle, not all analytes are desorbed from cork, and in 5 cycles, a process of re-extraction can occur in the matrix, explaining the lower result comparing with 3 cycles. Therefore, 3 cycles were established as the optimal condition.

Optimization of impregnation time *vs.* DNPH volume for *in situ* derivatization

In situ derivatization study was carried out by aspiration of the DNPH solution through the DPX-cork tip. In this process, the time required for the cork to interact with the derivatizing agent was verified, while the volume of DNPH added is sufficient for the reaction to be effective with the analytes. Thus, a central composite multivariate design was applied, where times of 0, 1, 3, 5 and 6 min were evaluated, and volumes of 30, 50, 100, 150 and 170 μL were also examined. Figure 3 shows the response surface obtained for the chromatographic responses of the two analytes.

According to the response surface (R^2 = 0.98628), there is a trend as the time increases and smaller volumes of DNPH are added. In this way, a volume of 30 μL of DNPH was maintained (in relation to 300 ng mL^{-1} of added analytes during the methodology development, with the volume adjusted according to the molar ratio of the analytes) and 6 min of cork impregnation as optimized conditions.

Optimization of extraction cycles (*in situ* reaction) *vs.* derivatization time reaction

In addition to the steps involving the impregnation and volume of derivatizing agent, the number of cycles

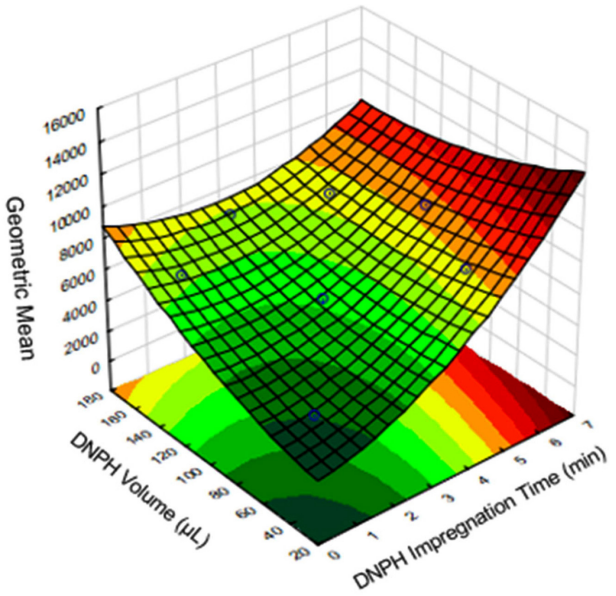

Figure 3. Response surface for the central composite between impregnation time and DNPH volume. Conditions: fortification of 300 ng mL^{-1} of analytes, 10× urine diluted in UP water, 15 mg of cork, pH adjusted to 4.8, 3 cycles of extraction, 2 cycles of desorption, desorption solvent acetonitrile with 300 µL.

and the time required for the reaction/extraction to occur simultaneously were also studied. Thus, another central composite design was applied with 1, 2, 4, 6 and 7 cycles and 17, 30, 60, 90 and 102 s. Figure 4 shows the response surface obtained for this optimization.

This response surface (R^2 = 0.89785) allowed us to observe a trend when both the number of cycles and time

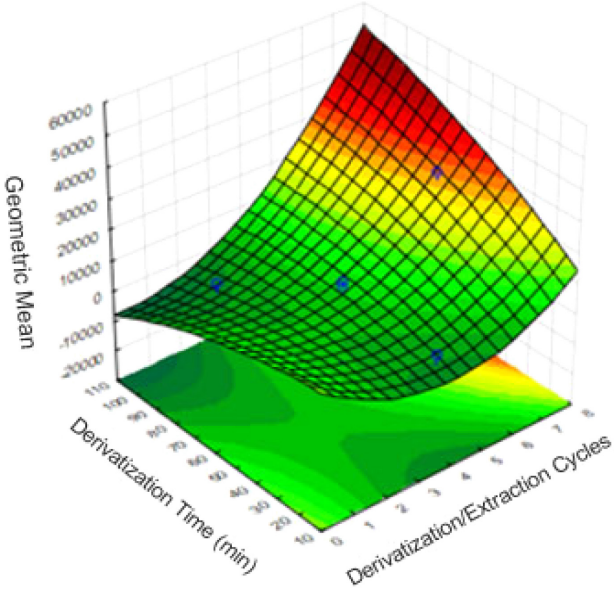

Figure 4. Response surface for optimizing extraction cycles (*in situ* reaction) by reaction time. Conditions: 30 µL and 6 min of DNPH impregnation, 300 ng mL^{-1} of analyte fortification, 10× urine diluted in UP water, 15 mg of cork, pH adjusted to 4.8, 2 desorption cycles, acetonitrile desorption solvent with 300 µL.

for the reaction/extraction increased. Longer reaction/extraction time allowed more satisfactory interactions with the analytes, which made the reaction yield higher. However, to ensure a suitable sample throughput while maintaining satisfactory responses, 7 extraction cycles were established, keeping 90 s for the derivatization of the aldehydes occurring directly on the cork surface.

Optimization of sorbent phase mass

The amount of sorbent phase is a crucial factor to ensure satisfactory extraction of the analytes. Particularly for alternative materials, this parameter must be evaluated considering the nature of the material, physico-chemical properties, and interactions with the analytes. Cork exhibits sites of interaction with aldehydes; however, its hydrophobicity affects the dynamic mixing with urine. Therefore, masses of 15, 20, 25 and 30 mg of cork added to the DPX tip were evaluated. Results are shown in a bar graph of Figure S3 (SI section), considering the normalized areas of the chromatographic peaks.

It is possible to observe that 25 and 30 mg provided similar responses. However, for 30 mg, the response was slightly better than 25 mg. Larger masses were not tested because a dynamic mixture with urine was difficult, and satisfactory responses were not obtained in this condition. Therefore, 30 mg was established for cork mass.

Analytical parameters of merit and urine analysis

In this step, calibration curves obtained with extractions directly in the spiked urine were constructed considering *in situ* derivatized aldehydes, with 7 concentrations (in triplicate). The figures of merit obtained for hexanal and heptanal are presented in Table 1.

For the linear working range, two formats are presented, the first from 50 to 800 ng mL^{-1} for both analytes, and 0.49-7.99 ng mL^{-1} and 0.44-7.00 ng mL^{-1} for hexanal and heptanal, respectively. The second range format is used in the literature to compare the range of 0.12-0.97 nmol L^{-1} of hexanal and 0.09-1.30 nmol L^{-1} for heptanal in healthy patients. For patients with lung cancer, the ranges are from 0.99 to 6.70 nmol mL^{-1}, and 2.50 and 6.40 nmol mL^{-1} for hexanal and heptanal, respectively. For the coefficients of determination, the values were 0.9900 for hexanal and 0.9911 for heptanal. LODs were 0.13 nmol mL^{-1} for both analytes and LOQs were 0.41 nmol mL^{-1} for hexanal and 0.40 nmol mL^{-1} for heptanal. From this format, it is possible to identify patients affected by lung cancer, as well as achieving concentrations that indicate that the individual is healthy.[6,29] Relative recovery and intraday

Table 1. Analytical parameters of merit obtained for hexanal and heptanal by the proposed method

Analyte	LOD / (nmol mL^{-1})	LOQ / (nmol mL^{-1})	R^2	Linear working range / (ng mL^{-1})	Linear working range / (nmol mL^{-1})	Linear equation
Hexanal	0.13	0.41	0.9900	50-800	0.49-7.99	$y = 3636.2x + 5609.8$
Heptanal	0.13	0.40	0.9911	50-800	0.44-7.00	$y = 1444.9x + 6661.6$

LOD: limit of detection; LOQ: limit of quantification; R^2: coefficient of determination.

(n = 3) and interday (n = 9) precisions were assessed (at three concentrations) in triplicate. The results obtained are shown in Table 2.

Regarding relative recoveries, concentrations evaluated were 50, 400 and 800 ng mL^{-1}, with recoveries ranging from 86 to 103% for hexanal and 103 to 107% for heptanal, and intraday precisions were ≤ 17% for hexanal and ≤ 15% for heptanal, and for interday were ≤ 21% and ≤ 12%, respectively. Data presented small variations, which were justified because of the complexity of the urine, since it is heterogeneous and varies from day to day. However, these results are acceptable and satisfactory for validation guidelines.[27]

The application of the proposed method was carried out in four urine samples of male and female volunteers, aged between 20 and 30 years. Chromatograms of the analyses are shown in Figure S4 (SI section), where it is possible to observe that no peaks of hexanal and heptanal were detected.

Comparison of the method developed with the literature

Other works are reported in the literature for the analysis of aldehydes as cancer biomarkers for pre-diagnosis of lung cancer. Table 3 presents the studies reported in the literature for the determination of hexanal and heptanal in human urine.

Only one work[6] had been previously reported using a biosorbent (cork), which was developed and applied by our group. However, this proposed method was the first to use cork powder associated with DPX, and it was therefore unprecedented methodology in this application. LODs were satisfactory, considering a relative comparison due to different forms of calculation. LC-MS (liquid chromatography-mass spectrometry and GC-MS (gas chromatography-mass spectrometry) are very important instruments in current applications due to the possibility of peak confirmation through integrated libraries, ensuring the identity of different compounds in complex matrices, however, an HPLC-DAD instrument was used, which also has the possibility of identifying different compounds with the use of other tools, which was a simpler, cheaper, and easier to maintain instrument compared to the others mentioned, The linear ranges studied were satisfactory for use with patients who present lung cancer. Derivatization took place in a simple way, with only aspiration, without discarding the solution because the volume is very small, in addition to the reaction occurring *in situ* through the sample aspiration in the different cycles. The preparation time took approximately 25 min, and considering that 5 extractions can be performed simultaneously, the analysis throughput is 5 min *per* sample, consisting of an advantage compared to methods that require centrifugation/derivatization/extraction/desorption, generally with analysis time up to 70 min. The methodology developed was unprecedented, with the use of a renewable material in combination with DPX, employed for the first time in the application of heptanal and hexanal with a high-throughput analytical device.

Conclusions

The present method was proposed as a new tool with high sample throughput, simplicity, green aspects and as

Table 2. Recovery and precision assays for analytes at different concentrations in human urines

Analyte	Relative recovery / %		Intraday precision (n = 3)		Interday precision (n = 9)	
	Fortified / (ng mL^{-1})	Recovery / %	Fortified / (ng mL^{-1})	RSD / %	Fortified / (ng mL^{-1})	RSD / %
Hexanal	50	86	50	4	50	9
	400	92	400	17	400	17
	800	103	800	9	800	21
Heptanal	50	107	50	15	50	9
	400	103	400	9	400	8
	800	106	800	11	800	12

RSD: relative standard deviation.

Table 3. Comparative table between different methodologies described in the literature for determination of hexanal and heptanal in human urine

Technique	Extractive phase	Derivatization	Sampling time / min	LOD / (nmol mL^{-1})		Linear working range / (nmol mL^{-1})		Instrumentation	Reference
				Hexanal	Heptanal	Hexanal	Heptanal		
DPX	cork	*in situ*-DNPH	ca. 5	0.13		0.49-7.99	0.44-7.00	HPLC-DAD	this study
BAμE	cork	*in situ*-DNPH	ca. 70	1.00	0.73	3.00-8.00	2.19-7.00	HPLC-DAD	6
SPME	poli(MAA-*co*-EDMA)	HAHC	ca. 35	15.00	9.00	50-500	30-500	LC-MS	7
SPME	poli(MAA-*co*-EDMA)	DNPH	ca. 15	0.81	0.76	0.02-5.00		HPLC-UV	10
SPE	(Fe_3O_4/SiO_2/P(MAA-*co*-EDMA)	*in situ*-DNPH	ca. 9	1.7	2.5	6-5000	9-5000	HPLC-UV	4
SPME	graphene oxide	–	ca. 10	0.004	0.0026	0.01-0.50	0.08-0.43	GC-MS	11
SPME	PDMS/DVB	–	ca. 20	0.11	0.10	1.56-50		GC-MS	5
SPME	Fe_3O_4/SiO_2/polipirrol	*in situ*-DNPH	ca. 10	0.10	0.50	10-15000	10-15000	GC-FID	3
HF-LPME	1-octanol	2-thiobarbituric acid	ca. 70	2.7	0.97	4.00-100	3.5-88	CZE-AD	12

DPX: disposable pipette extraction; BAμE: bar adsorptive microextraction; SPME: solid phase micrextraction; SPE: solid phase extraction; HF-LPME: hollow fiber-liquid phase microextraction; poli(MAA-*co*-EDMA): methacrylic acid-*co*-ethylene glycol dimethacrylate; PDMS/DVB: polydimethylsiloxane/divinylbenzene; DNPH: 2,4-dinitrophenylhydrazine; HAHC: hydroxylamine hydrochloride; HPLC-DAD: high-performance liquid chromatography with diode array detection; LC-MS: liquid chromatography-mass spectrometry; GC-MS: gas chromatography-mass spectrometry; GC-FID: gas chromatography-flame ionization detection; CZE-AD: capillary zone electrophoresis-amperometric detection.

a pre-diagnosis for patients with lung cancer, through the determination and identification of hexanal and heptanal as biomarkers of this type of disease. The device in parallel allowed the use of up to 5 tips simultaneously, making it viable for routine laboratories. Some green aspects can be highlighted, such as miniaturization of the methodology, using a natural material for the extraction, reducing the use of energy to perform the procedure, and generating less waste due to the low usage of samples and solvents.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgments

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