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Effect of chemotherapy on urinary volatile biomarkers for lung cancer by HS-SPME-GC-MS and chemometrics

Ricardo Rubio-Sánchez[1] | **Rocío Ríos-Reina**[2] | **Cristina Ubeda**[2]

[1]Servicio de Análisis Clínicos, Hospital Universitario Virgen de Valme, Seville, Spain

[2]Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal, Facultad de Farmacia, Universidad de Sevilla, Seville, Spain

Correspondence
Rocío Ríos-Reina, Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal, Facultad de Farmacia, C/ Profesor García González, 2, 41012, Seville, Spain.
Email: rrios5@us.es

Abstract

Background: Volatile organic compounds (VOCs) have been studied as possible biomarkers in several diseases, including lung cancer. Early detection of cancer can improve long-term survival rates and the quality of life, so the study of VOCs in exhaled breath and urine has been increasing in recent years. This study aimed to assess the urinary VOCs that are modified after chemotherapy to identify those with the potential to be lung cancer biomarkers that can be monitored during treatment.

Methods: Three urine samples from 10 men with stage IV lung adenocarcinoma were collected, as well as urine samples from 14 men with other types of cancer (control group). All samples were analyzed by headspace-solid phase microextraction gas chromatography coupled with mass spectrometry.

Results: A total of 21 urinary VOCs were found with different levels after the administration of chemotherapy, with 2-pentanone being one of those that significantly decreased. Furthermore, 2-pentanone and 3-hydroxy-2,4,4-trimethylpentyl-2-methylpropanoate showed statistically significant differences with the control group.

Conclusions: Chemotherapy administered to patients with advanced lung adenocarcinoma modified the volatile profile of urine. 2-Pentanone, a final product of the increased rate of fatty acid oxidation and protein hypermetabolism, significantly decreased after chemotherapy. Therefore, monitoring its urinary excretion could be very useful since its decrease over time could indicate an adequate response to chemotherapy and arrest of cancer development. Another VOC that could be a potential lung cancer biomarker is 3 hydroxy-2,4,4-trimethylpentyl-2-methylpropanoate, whose origin may be due to inhibition of the propanoic acid metabolic pathway or increased aldehyde dehydrogenase activity.

KEYWORDS
2-pentanone, adenocarcinoma, biomarker, chemotherapy, lung cancer

INTRODUCTION

Lung cancer is a malignant neoplasm with the highest incidence and mortality worldwide. It causes 1.8 million deaths annually.[1] This cancer is divided into two main histological types: small cell lung cancer (SCLC) (15% of cases) and non-small cell lung cancer (NSCLC) (85% of cases), the latter being subdivided into adenocarcinoma (40%), squamous cell carcinoma (30%), and large cell carcinoma (15%).[2,3] Therefore, adenocarcinoma is the most frequent type of lung cancer, originating mainly from cells that secrete surfactant components and the most frequently mutated genes being *TP53* and *KRAS*.[4]

Smoking is the principal risk factor. It is estimated that 80% of patients with lung cancer are smokers. Although nicotine is not carcinogenic, 55 other substances in tobacco smoke are. In contrast, approximately 20% of patients have never smoked, with the development of lung cancer being associated with environmental exposures and inherited genetic susceptibility.[4,5] The greatest reduction in mortality rates, in addition to the discontinuation of smoking, is related to early diagnosis. That is

why a better understanding of the biological characteristics of this type of cancer is required.[5]

Serum biomarkers are potentially useful in the differential diagnosis of lung cancer. In addition, some provide prognostic information, and their serial measurements correlate with response to treatment. Among the most studied serum biomarkers in lung cancer, although none are specific, are neuronal specific enolase (NSE), progastrin-releasing peptide (proGRP), carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCCA), and CYFRA 21-1.[3]

Volatile organic compounds (VOCs) have been studied for years as possible biomarkers in lung cancer.[6,7] The formation of VOCs is mainly related to oxidative stress, inflammation, and changes in cellular metabolism. Consequently, these can provide information on the alteration of biochemical processes in cancer cells. Once formed, VOCs enter the bloodstream and are transported to the lungs, which is why exhaled breath is the most studied sample type.[8–10] In addition, VOCs are metabolized in the liver and excreted in urine, which is why this type of sample is being increasingly studied, also due to its ease of collection and the greater volume obtained.[8,11,12]

These two sample types have already been studied in lung cancer detection by dogs. These animals have a very sensitive olfactory system with low perception thresholds. Canine can differentiate odors up to 100 000 times better than humans. Dogs detected cancer in urine samples in 84.5% of patients, compared with the 56.1% sensitivity of bronchoscopy, demonstrating that olfactory detection of lung cancer is possible.[13] Although the diagnostic gold standard is computed tomography, with a sensitivity of 100%, its application in a large population is not appropriate due to the side effects related to the radiation used.[13,14]

Surgery is the main form of treatment for patients diagnosed with early-stage (stage I/II) NSCLC, while radiation therapy in combination with platinum-based chemotherapy (CTX) may be used for those with advanced disease not amenable to surgery.[3] However, most patients have advanced cancer (stage III/IV) at diagnosis, and survival remains low.[2] Consequently, early detection can improve long-term survival rates and the quality of life. This requires a rapid, reliable, noninvasive, accessible, and inexpensive screening method.[14,15] The use of VOCs can be useful for this purpose, as well as for disease monitoring.

In this context, the electronic nose (e-nose) is a fast, efficient, and cheap device that detects VOCs in different biological matrices and has demonstrated its potential utility in noninvasive diagnosis and early detection of various diseases, including lung cancer.[14,16] Knowledge of tumor-specific VOCs is necessary to configure these devices. The combination of different volatile markers is more useful for detecting lung cancer patients in clinical settings than a single compound.[8,13] However, to our knowledge, no follow-up study of volatile disease biomarkers before and after CTX has been performed. The identification of biomarkers that are present in cancer and disappear during treatment could shed some light on the complex understanding of the evolution of lung cancer. Therefore, this study aimed to assess the urinary VOCs that are modified after CTX in order to identify those with the potential to be lung cancer biomarkers that can be monitored during treatment.

METHODS

Samples and patients

Urine samples from 10 men with stage IV lung adenocarcinoma were included in this study. Three samples were obtained from each patient: one before and two after starting CTX (45 days after starting the treatment and 60 days after the previous sample). The mean age of the patients was 66 years (range: 52–83). All the patients had smoked at some point in their lives, three of them being smokers at the time of the study. Most patients (9 of 10) received carboplatin + pemetrexed as CTX, while the least used treatment was docetaxel (2 of 10). Detailed information about the samples is shown in Table 1.

The control group included urine samples from 14 men with other types of cancer (seven with colorectal cancer and another seven with bladder cancer) who had not yet received CTX, to identify potential lung cancer-specific biomarkers. The mean age of the patients was 68 years (range: 48–81). Of these patients, four were smokers, seven were ex-smokers, and three had never smoked.

All urine samples were collected in a BD Vacutainer tube without additives (Becton Dickinson). Then, 2 mL of each sample was placed in a 20 mL headspace vial and frozen at −80°C until the VOCs were analyzed. This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Clinical Research Ethics Committee of the Virgen de Valme University Hospital (code: 0170-N-20).

Reagents and solvents

As internal standard, a solution of a commercial standard of 4-methyl-2-pentanol, supplied by Merck and stored at −20°C, was used. The linear retention index (LRI) was calculated by injecting a series of C10 to C40 straight-chain n-alkanes (50 mg/L in *n*-hexane), purchased from Fluka. Moreover, the available VOCs standards used for identification were bought from different commercial sources (Merck and Sigma-Aldrich).

Analysis of volatile compounds

VOCs were analyzed by gas chromatography coupled to mass spectrometry (GC–MS) after headspace-solid phase microextraction (HS-SPME), using a 2 cm 50/30 µm Carboxen/DVB/PDMS SPME fiber (Supelco), as described in Rubio-Sánchez et al.[17] Analyses were performed on an

TABLE 1 Data of interest of the patients with lung adenocarcinoma included in the study.

Patient	Cancer	Age	Tobacco	Chemotherapy
1	Lung ADC	60	Smoker	Carboplatin + pemetrexed
2	Lung ADC	65	Smoker	Carboplatin + pemetrexed
3	Lung ADC	71	Smoker	Carboplatin + pemetrexed
4	Lung ADC	52	Ex-smoker	Carboplatin + pemetrexed
5	Lung ADC	65	Ex-smoker	Carboplatin + pemetrexed
6	Lung ADC	66	Ex-smoker	Carboplatin + pemetrexed
7	Lung ADC	78	Ex-smoker	Carboplatin + pemetrexed
8	Lung ADC	83	Ex-smoker	Carboplatin + pemetrexed
9	Lung ADC	61	Ex-smoker	Carboplatin + pemetrexed, docetaxel
10	Lung ADC	59	Ex-smoker	Docetaxel
11	Colorectal	48	Ex-smoker	-
12	Colorectal	63	Ex-smoker	-
13	Colorectal	69	Smoker	-
14	Colorectal	70	-	-
15	Colorectal	77	-	-
16	Colorectal	78	Ex-smoker	-
17	Colorectal	81	Ex-smoker	-
18	Bladder	62	Smoker	-
19	Bladder	65	Smoker	-
20	Bladder	66	Smoker	-
21	Bladder	66	Ex-smoker	-
22	Bladder	68	Ex-smoker	-
23	Bladder	69	Ex-smoker	-
24	Bladder	70	-	-

Abbreviation: ADC, adenocarcinoma.

Agilent 8890 gas chromatograph coupled to an Agilent 5977B Inert Plus quadrupole mass spectrometer with a Gerstel autosampler (Müllheim an der Ruhr).

Data treatment and chemometrics

Chromatographic data were exported to AIA format using MSD ChemStation software (Agilent Technologies) and processed using PARADISe. This software enables peak deconvolution using PARAllel FACtor analysis2 (PARAFAC2) modeling and simultaneously extracts the pure mass spectra of the coeluting compounds used for peak identification.[18] Modeling options were set to a maximum of seven components per interval, applying non-negativity constraint, and carefully optimizing fit and core consistency to select the correct number of components for each model. Finally, a report with the areas of the resolved peaks and a tentative identification of each one, made by comparing the resolved mass spectra with the database of the NIST MS library, was generated. Then, identification was assessed after a series of alkanes injections and the calculation of the LRIs of each compound, which were then compared with the NIST spectrum library (version 2.0), the LRI of reference standards, when available, and LRIs reported in the literature. In addition, compounds were semi-quantified by means of relative areas concerning the internal standard.

The relative areas obtained were subjected to principal component analysis (PCA) to explore the clustering and differentiation of the samples. In addition, partial least squares-discriminant analysis (PLS-DA) was applied not for a pure classification approach, but to reduce the data and select the variables with importance in the projection (VIPs). PLS-DA models were cross-validated (CV) by venetian blinds, and the appropriate number of latent variables were selected according to the minimum CV classification error average. According to the literature,[19] all predictors having a VIP value ≥1 are considered relevant, and the higher the VIP score, the higher their contribution to the classification. PCA and PLS-DA models were performed using PLS Toolbox 7.9.5 (Eigenvector Research Inc.), working in a MATLAB 2016a environment (Mathworks).

In addition, an analysis of variance (ANOVA), followed by a post-hoc comparison test (Tukey's test), was performed using the INFOSTAT software 2016 (FCA, National University of Cordoba, Argentina) to study the significant differences between samples of different groups.

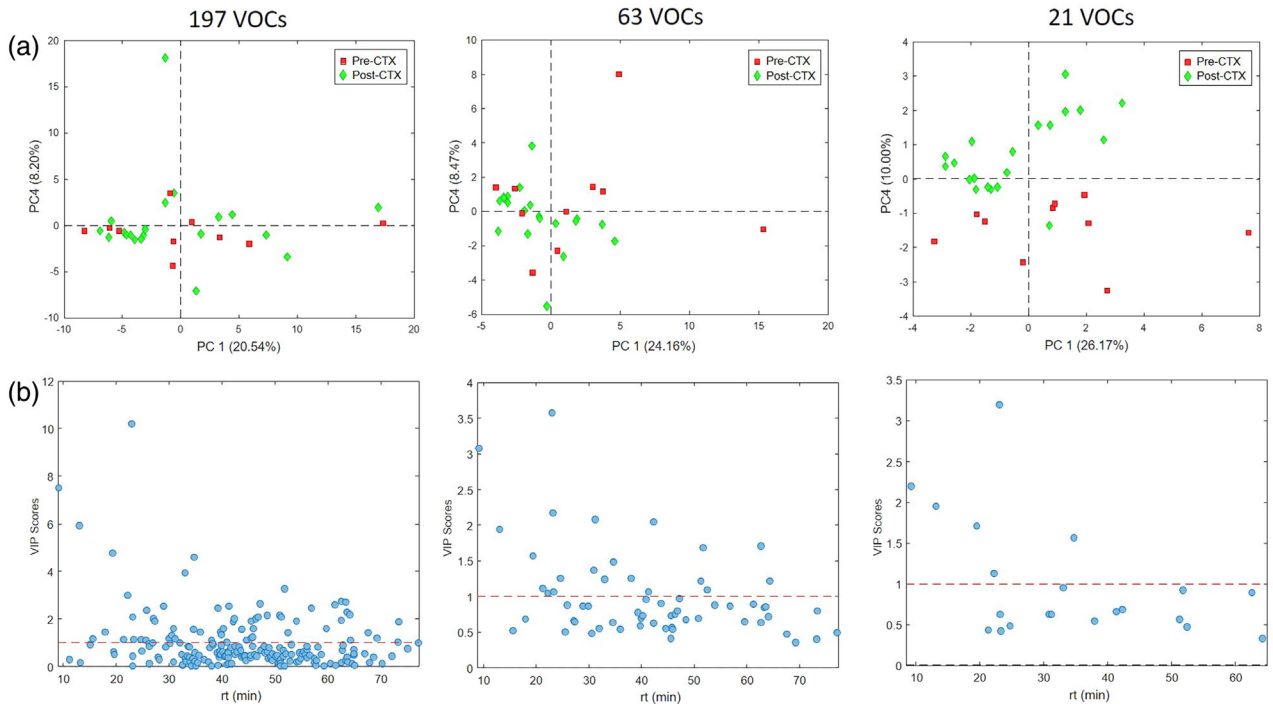

FIGURE 1 Variables with importance in the projection (VIP) evaluation process and feature reduction carried out for the prechemotherapy (CTX) and post-CTX samples classification. (a) Principal component analysis (PCA) score plots with different datasets. (b) VIP score plots of partial least squares-discriminant analysis (PLS-DA) models.

RESULTS

Influence of chemotherapy on the urine volatile profile of lung cancer patients

PARADISe software was used to process the global GC–MS dataset consisting of 30 urine samples (10 pre-CTX and 20 post-CTX) from lung cancer patients. A total of 197 VOCs were deconvoluted obtaining their peak areas and were tentatively identified. Subsequently, the relative areas were calculated with respect to the internal standard. The dataset with the relative areas of the 197 VOCs was subjected to PCA, with no evident results in terms of the grouping of samples. Therefore, a PLS-DA model was then performed to reduce the number of VOCs, through a data selection done by studying the VIPs. Those VOCs with VIP values ≥1 were selected, reducing the dataset to 63 VOCs. A second PCA was performed, but the separation between the two groups remained unclear, selecting those VIPs ≥1 and obtaining a dataset with only 21 VOCs. When a third PCA model was performed, in this case, the separation of the two groups, the ones without CTX and samples after CTX, was clearly observed. Figure 1a shows the score plots of the three PCA models, showing how reducing the number of variables (i.e., VOCs selected with VIP values ≥1 shown in Figure 1b) improves the separation of the two groups. This improvement was also observed in the correct classification percentages in CV as the VOC reduction continued (Table 2). Figure 2 shows the PCA score (A) and loading (B) plots obtained with the 21 selected VOCs where the grouping of the samples can be observed depending on whether or not CTX was administered.

In addition, the mean and standard deviation of the relative area of these 21 selected VOCs, together with their LRI, identification, and chemical families, are shown in Table 3. Most VOC families identified were ketones (5), and those with a cyclohexane ring (5), two of them being benzene derivatives. The compound with the highest relative area identified in these samples was 2-pentanone, followed by 2-ethyl-1-hexanol, both found before and after CTX, although higher in pre-CTX samples. Moreover, among these 21 VOCs, 11 displayed a decrease in relative area after CTX treatment, while the relative area of the other 10 compounds increased.

According to the VOCs with statistically significant differences after CTX, the results showed that the VOCs 2-pentanone ($p = 0.0059$), 5-methyl-3-hexanone ($p = 0.0096$), 3-methyl-2-heptanone ($p = 0.0180$), mesitylene ($p = 0.0005$), and 2-ethyl-1-hexanol ($p = 0.0213$) were the ones with significant differences between pre-CTX and post-CTX. Thus, these five VOCs showed a significant decrease in relative area after the treatment.

Identification of lung cancer biomarkers

The chromatographic peaks corresponding to 21 VOCs selected in lung cancer samples (VIPs ≥1) were integrated by

TABLE 2 Classification results (confusion matrices and classification performance) obtained by the PLS-DA models developed with the total number of VOCs (197) and the two selections of VIPs (63 and 21 VOCs).

		Cal		CV	
		Real classification		Real classification	
PLS-DA models		Pre-CTX (set of 197/63/21 VOCs)	Post-CTX (set of 197/63/21 VOCs)	Pre-CTX (set of 197/63/21 VOCs)	Post-CTX (set of 197/63/21 VOCs)
Model prediction	Pre-CTX (set 197/63/21 VOCs)	**10/10/10**	0/0/0	1/4/**8**	6/3/**0**
	Post-CTX (set 197/63/21 VOCs)	0/0/0	**20/20/20**	9/6/**2**	14/17/**20**
	Total number of samples	10	20	10	20
Classification performance	Correct category classification (%)	**100**	**100**	10/40/**80**	70/85/**100**

Note: The best results are highlighted in bold.
Abbreviations: Cal, calibration; CTX, chemotherapy; CV, cross-validation; PLS-DA, partial least squares-discriminant analysis; VIPs, variables with importance in the projection; VOCs, volatile organic compounds.

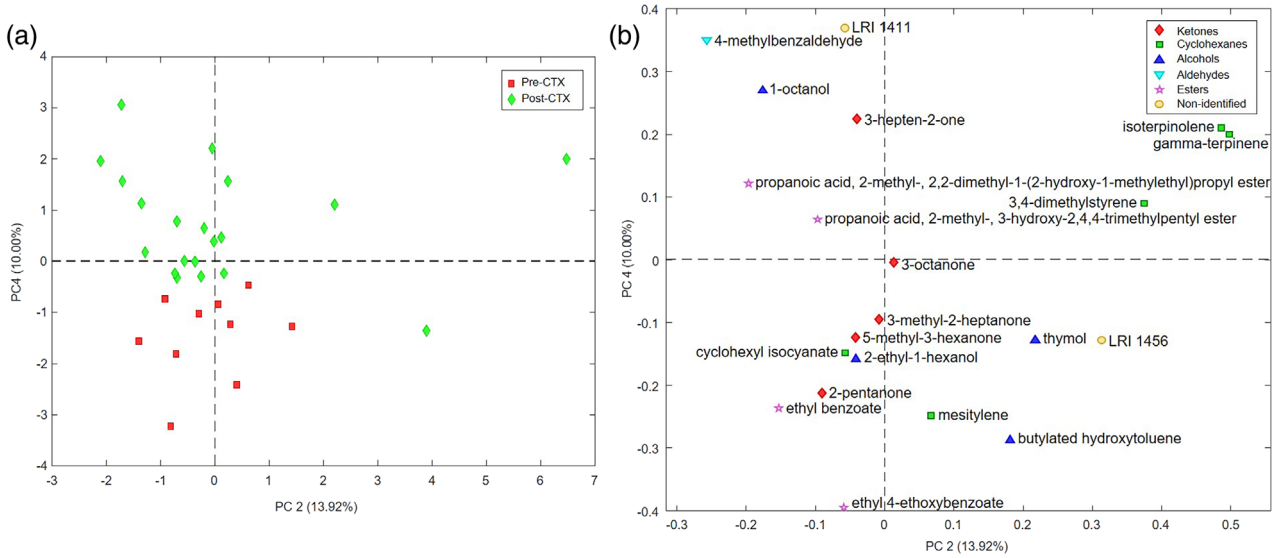

FIGURE 2 Score (a) and loading (b) plots of the principal component analysis (PCA) model carried out with 21 volatile organic compounds (VOCs) selected as variables with importance in the projection (VIPs) and the prechemotherapy (CTX) and post-CTX samples.

PARADISe in the set of control samples to find out which ones had the potential to be lung cancer biomarkers, in order to find those responsible for the differentiation between lung cancer patients and those with other types of cancer. A new PLS-DA model was performed with the relative area of these VOCs including the two groups of patients, but only those who had not yet received CTX (pre-CTX lung cancer vs. the control group). As the scores plot of the first two latent variables shows (Figure 3a), a separation between lung cancer patients and patients with other types of cancer was again observed through these selected VOCs detected in urine samples. Once again, VOCs whose VIP values were ≥1 were highlighted to search for volatile biomarkers of this cancer. Thus, in the loading plot (Figure 3b), the VIPs ≥1 identified in the new PLS-DA model have been marked in purple, with the highest VIP score obtained for 3-hydroxy-2,4,4-trimethylpentyl-2-methylpropanoate (VIP score = 5.412) being more related to control samples, and 2-pentanone (VIP score = 3.912) more present in the lung cancer samples.

Moreover, there were statistically significant differences between patients with lung cancer (pre-CTX) and patients with other types of cancer (control group) in some VOCs. In this context, 2-pentanone, which decreased after CTX ($p = 0.0014$), and 3-hydroxy-2,4,4-trimethylpentyl-2-methylpropanoate, which increased after treatment ($p = 0.0001$), stand out. These compounds matched with the VOCs with the highest VIP scores identified in the previous PLS-DA model.

Thus, 2-pentanone was the only VOC that appeared to be an advanced lung adenocarcinoma biomarker whose relative area decreased significantly after CTX.

DISCUSSION

According to the results, CTX administered to patients with advanced lung adenocarcinoma modifies the volatile profile of urine (Table 3). Among the 21 VOCs that best allowed differentiating pre-CTX samples from post-CTX ones, the chemical

TABLE 3 Mean relative areas (multiplied by 100) and statistical results of the 21 VIPs that allowed the differentiation of the pre-CTX samples from the post-CTX ones.

			Pre-CTX		Post-CTX		Control	
VOCs	**LRI**	**Id**	**Mean**	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**
Ketones								
2-pentanone*#	963	A	20.9	12.9	8.92	8.98	6.63	5.94
5-methyl-3-hexanone*	1058	A	2.93	2.63	1.15	0.87	1.37	1.02
3-methyl-2-heptanone*	1190	B	1.73	1.47	0.70	0.79	2.76	2.13
3-octanone	1244	A	1.19	1.18	0.55	0.64	0.64	0.31
3-hepten-2-one	1291	A	0.32	0.30	0.52	1.03	0.31	0.52
Cyclohexanes								
Gamma-terpinene	1228	A	0.12	0.19	0.59	1.29	2.88	5.97
Mesitylene*	1260	A	0.36	0.29	0.09	0.08	0.37	0.21
Cyclohexyl isocyanate	1262	B	1.77	5.59	0.45	1.46	0.35	0.87
Isoterpinolene	1265	A	0.01	0.02	0.09	0.23	0.34	0.94
3,4-dimethylstyrene	1418	B	1.62	0.61	1.93	1.05	2.04	2.28
Alcohols								
2-ethyl-1-hexanol*	1492	A	10.5	5.21	7.32	2.02	11.5	21.0
1-octanol	1562	A	0.41	0.16	0.46	0.22	0.49	0.42
Butylated hydroxytoluene	1902	A	0.10	0.18	0.04	0.11	0.11	0.12
Thymol	2212	A	0.03	0.05	0.06	0.13	0.11	0.23
Aldehydes								
4-methylbenzaldehyde	1637	A	0.17	0.14	0.30	0.31	0.26	0.29
Esters								
Ethyl benzoate	1656	A	0.21	0.14	0.16	0.07	0.18	0.11
3-hydroxy-2,4,4-trimethylpentyl-2-methylpropanoate#	1871	B	0.83	0.27	0.94	0.28	0.32	0.26
2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl-2-methylpropanoate	1886	B	0.65	0.24	0.80	0.27	0.79	0.18
Ethyl 4-ethoxybenzoate	2172	C	0.85	0.77	0.54	0.23	0.97	0.49
Nonidentified compounds								
LRI 1411 (43, 113, 175)	1411	-	0.04	0.06	0.07	0.10	0.07	0.11
LRI 1456 (85, 112, 69)	1456	-	0.05	0.05	0.02	0.03	0.07	0.07

Note: Relative areas are multiplied by 100. A, mass spectrum agreed with the mass spectral data base, and LRI agreed with the literature data. B, Mass spectrum agreed with mass spectral data base, but there is no LRI in a polar column reported in the literature. C, Mass spectrum agreed with mass spectral data base R.Match >800 but not with LRI in the literature.

Abbreviations: CTX, chemotherapy; Id, identification reliability; LRI, linear retention index; SD, standard deviation; VIPs, variables with importance in the projection; VOCs, volatile organic compounds.

*VOCs with statistically significant difference between pre-CTX and post-CTX.

#VOCs with statistically significant difference between pre-CTX and control.

group of ketones was predominant, such as 2-pentanone, 5-methyl-3-hexanone, and 3-methyl-2-heptanone (Figure 2). These VOCs may be the final product of fatty acid oxidation or protein metabolism, as these events increase during cancer development.[10] In addition, significantly increased activity of the alcohol dehydrogenase enzyme, which oxidizes secondary alcohols and contributes to ketone production, has been observed in many cancerous tissues.[8] This higher activity was observed, for example, by Orywal et al.[20] in liver cancer tissue.

One VOC that decreased significantly after CTX was 2-ethyl-1-hexanol, which is a compound already identified in other studies as a potential lung cancer biomarker.[7,21] In contrast, according to our results, 2-ethyl-1-hexanol could not be a lung cancer biomarker because its relative area in patients with lung cancer was smaller than in other cancer patients (Table 3). It should be noted that Hanai et al.[7] and Pérez et al.[21] used healthy people as a control group, so although this alcohol could be a cancer biomarker, it is probably not lung-specific. As can be seen in our results, it also increased in colorectal and bladder cancer. Another potential lung cancer biomarker, according to Santos et al.,[22] is 3-octanone. However, although it decreased after CTX administration (Table 3), there were no significant differences between patients with lung cancer and other cancer patients, according to our results.

The second VOC whose relative area decreased significantly after CTX administration was 2-pentanone (Table 3), which is a ketone already identified as a potential urinary

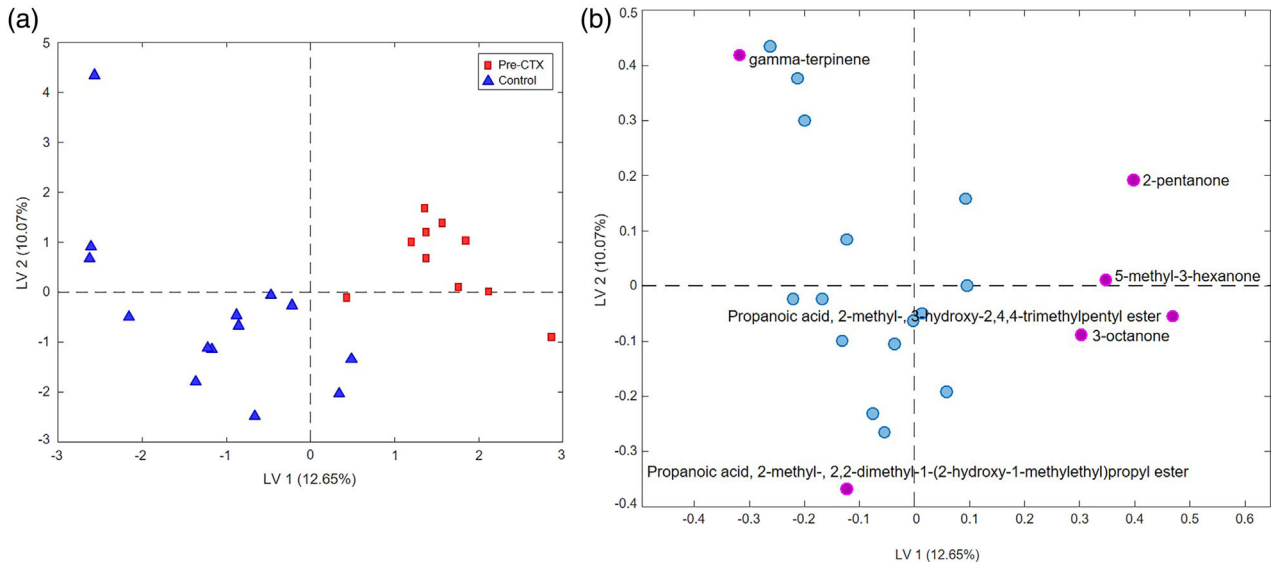

FIGURE 3 Score (a) and loading (b) plots of the partial least squares-discriminant analysis (PLS-DA) model carried out with the 21 volatile organic compounds (VOCs) selected as variables with importance in the projection (VIPs) between prechemotherapy (CTX) lung cancer and the control group samples. VOCs with VIP ≥1 obtained from the PLS-DA model are highlighted in purple in the loadings plot.

lung cancer biomarker in several studies.[7,10,21] According to our results, 2-pentanone could also be a lung cancer biomarker because there were significant differences in the relative areas between these patients and those with colorectal or bladder cancer (Table 3; Figure 3). To our knowledge, this is the first time that this potential biomarker has been identified by comparing lung cancer patients with other cancer patients. In most previous studies, lung cancer patients were compared with healthy people.[6,7,10–12,14,21,22] This ketone, as previously discussed, could be a final product of the increased rate of fatty acid oxidation and protein hypermetabolism that occurs specifically during cancer development.[10] In addition, Hanai et al.[7] and Gasparri et al.[10] included patients with stage I, II, and III cancer, while our study only included stage IV patients, confirming that 2-pentanone could also be a biomarker of lung cancer in advanced stages. Although only patients with adenocarcinoma were included in our study, Hanai et al.[7] also included patients with squamous cell carcinoma, the second most common type of lung cancer, demonstrating that 2-pentanone could serve to differentiate these two types of cancer because patients with adenocarcinoma tend to have higher concentrations.

The other VOC identified in this study as a potential lung cancer biomarker was 3-hydroxy-2,4,4-trimethylpentyl-2-methylpropanoate (Figure 3). Wang et al.[23] identified this ester as a potential lung cancer biomarker in exhaled breath. Its increase in urine, as these authors argue, may be due to inhibition of the propanoic acid metabolic pathway or increased aldehyde dehydrogenase activity in lung cancer cells.[23]

In addition to its noninvasive nature and availability in large volumes, the advantage of analyzing VOCs in urine is that the kidneys concentrate the analytes before excreting them, so their detection can be greater than in other biological fluids, such as exhaled breath and blood.[10,16] Therefore, the analysis of the volatile urine profile could be a feasible detection method in many parts of the world, being also much cheaper than computed tomography and without exposing the patient to the risks associated with the radiation used.[12,13]

In conclusion, after chemotherapy administration in patients with advanced lung adenocarcinoma, the urinary excretion of five VOCs decreased significantly, one of them being 2-pentanone. Our results are in line with previous studies that identify this ketone as a potential lung cancer biomarker, useful both in early and advanced stages. Monitoring urinary 2-pentanone excretion during treatment in these patients could be very meaningful, since its decrease over time could indicate an adequate response to chemotherapy and the arrest of cancer development.

AUTHOR CONTRIBUTIONS

Ricardo Rubio-Sánchez: Conceptualization, investigation, writing–original draft. Rocío Ríos-Reina: Data curation, methodology, writing–review and editing. Cristina Ubeda: Conceptualization, methodology, supervision, writing–review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

ORCID

Ricardo Rubio-Sánchez https://orcid.org/0000-0002-6313-4191
Rocío Ríos-Reina https://orcid.org/0000-0002-9703-5853
Cristina Ubeda https://orcid.org/0000-0002-1320-4438

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