







The chemical memory of smoking tobacco

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The concentration in urine of *N*-acetyl-hydroxy-propyl-cysteine (3HPMA), an acrolein metabolite, has been employed as a marker of the risk of illness of smokers and the relative concentration of creatinine has been evaluated to verify the effect of moving from the practice of burning tobacco to nicotine vaping. From the results concerning the urine samples of 38 subjects, collected from 2021 to 2023 and analyzed by LC-MS/MS, corresponding to 5 active smokers, 13 previously heavy smokers who replaced traditional tobacco by vaping, and 20 non-smokers, a dramatic reduction was found in 3HPMA/creatinine in urine. 3HPMA varied from values of 2150–3100 $\mu\text{g g}_{\text{creatinine}}^{-1}$ to levels of 225–625 $\mu\text{g g}_{\text{creatinine}}^{-1}$ found for non-smokers, with the time decay described by the equation $y = 0.3661x^2 - 94.359x + 6246.4$ ($R^2: 0.757$), providing a time of approximately 10 years for tobacco memory after the cessation of the consumption of burned tobacco.

pathophysiology of these aforementioned diseases.^{3,4} Since 2009, several national and international agencies have been involved in the monitoring and control of tobacco products.⁵ In 2017, the United States Food and Drugs Administration (FDA) published a list of 20 harmful and potentially harmful constituents (HPHCs) of tobacco and tobacco smoke, including nicotine and other alkaloids, carbon monoxide, tobacco-specific nitrosamines (TSNAs), polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs), carcinogenic aromatic amines, and mineral ions, and the list is still current today.⁶ The biomarkers related to the aforementioned processes, derived from precursors included in the FDA HPHC list, are very useful for monitoring the diseases related to tobacco smoking, particularly in their first stages.⁷ Table 1 indicates some of the most common HPHCs present in tobacco smoke and their metabolites in the human body together with diseases related to tobacco smoking and the usual matrices in which they are monitored.⁸

Several studies have been published concerning the concentration of HPHC metabolites in biological fluids from smokers, former smokers who had substituted traditional practices by harm-reduction alternatives, and smokers who had ceased their smoking activities completely. In this sense, Gale *et al.* in 2021,⁹ monitored the concentration in urine of total-NNAL, total-NNN, 3HPMA, HMPMA, MHBMA, HEMA, 4-ABP or CEMA. A comparison between two different population sets, current smokers and people who had substituted traditional tobacco by alternative products, showed that while the concentrations of biomarkers in the urine of current smokers remained practically constant throughout the whole study time, in the case of people using alternative products, the studied biomarkers decreased from the initial time until the end and finally remained practically constant. The same authors performed a one-year study by comparing HPHC metabolites in four different population groups: (i) current smokers, (ii) people who has substituted traditional tobacco by alternative products, (iii) people who had completely ceased traditional tobacco consumption, and (iv) non-smokers.¹⁰ From this study, it could

Introduction

The practice of smoking damages every organ in the body, making tobacco smoking one of the most important causes of premature death around the world.¹ According to the World Health Organization (WHO), tobacco consumption causes the deaths of millions of people annually, with a higher death rate than other diseases such as human immunodeficiency virus (HIV), tuberculosis and malaria combined.²

Tobacco smoking is mainly related to several health problems, such as cancer, and cardiovascular and respiratory diseases. In this sense, smoking practices are associated with processes of inflammation and oxidative stress, related to the

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Table 1 HPHCs, metabolites, pathologies and the usual matrix employed in the monitoring of diseases related to tobacco consumption⁸

Parent	Metabolite	Usual matrix	Developed pathology
1,3-Butadiene	Monohydroxybutenyl mercapturic acid (MHBMA)	Urine	Cancer Respiratory disease Reproductive toxicant
2-Aminonaphthalene	2-Aminonaphthalene (2-AN)		Cancer
4-Aminobiphenyl	4-Aminobiphenyl (4-ABP)		Cancer
<i>o</i> -Toluidine	<i>o</i> -Toluidine (<i>o</i> -T)		Cancer
Acrolein	3-Hydroxypropyl mercapturic acid (3-HPMA)		Respiratory disease Cardiovascular disease
Benzene	<i>S</i> -phenyl mercapturic acid (SPMA)		Cancer Cardiovascular disease Reproductive toxicant
Ethylene oxide	2-Hydroxyethyl mercapturic acid (HEMA)		Respiratory disease Reproductive toxicant Cancer
Hydrogen cyanide	Thiocyanate (SCN)		Respiratory disease Cardiovascular disease
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol and related glucuronides (total NNAL)		Cancer
Carbon monoxide	Carboxyhemoglobin (COHb)	Blood	Reproductive toxicant Cardiovascular disease

be concluded that the concentration in current smokers remained constant throughout the time, while the presence of biomarkers in urine decreased substantially when traditional tobacco consumption was substituted or completely avoided.

In a previous study,¹¹ we evaluated exposure to tobacco and nicotine vaping through the urine control of six metabolites of acrylonitrile, acrolein and crotonaldehyde in urine samples of active traditional smokers, past strong smokers who moved to vaping, and non-smokers, which confirmed that *N*-acetyl-*S*-(2-cyanoethyl)-*L*-cysteine (CEMA) is present only in active smokers and that levels of 2*R*-*N*-acetyl-*S*-(4-hydroxybutan-2-yl)-*L*-cysteine (HMPMA) and *N*-acetyl-*S*-[1-(hydroxymethyl)-2-propen-1-yl]-*L*-cysteine (MHBMA) in vapers' urine are at the same level of magnitude as smokers and non-smokers. The level of *N*-acetyl-*S*-(3,4-dihydroxybutyl)-*L*-cysteine (DHBMA) in the urine of previous smokers reached similar values to those determined in non-smokers and it was also observed that levels of *N*-acetyl-hydroxy-propyl-cysteine (3HPMA) in urine showed a strong reduction on moving from smoking to vaping. *N*-acetyl-*S*-(3-carboxy-2-propyl)-*L*-cysteine (CMEMA) in the urine of vapers can reach concentrations higher than those found in the cases of non-smokers and smokers.

In this study, the relationship was evaluated between the 3HPMA in the urine of previous smokers who moved to nicotine vaping as their single nicotine consumption practice, in order to undertake a preliminary determination based on molecular markers of the time required after consumption of burnt tobacco ceased to reduce the risk of illness.

From a medical point of view, several studies have shown the biological effect of cessation of smoking on disease risks. Jeong *et al.*¹² found a 20% decrease in cardiovascular disease risk on cessation of smoking after one to three years. In the same way,

Polosa *et al.*¹³ evidenced the harm reduction in chronic obstructive pulmonary disease (COPD) twenty-four months after cessation of traditional smoking practices and substitution by alternative practices, while Huang *et al.*¹⁴ and Fares *et al.*¹⁵ established a relationship between negative pulmonary diagnosis and smoking cessation from one to five years after stopping smoking. So, it can be concluded that there is evidence of a certain memory of tobacco smoking which must be clarified. On the other hand, specialized sources argue that heavy smokers can reduce the risk of health diseases in a period of five to ten years after cessation in spite of the risks of pulmonary diseases that are difficult to solve.¹⁶

Acrolein is considered one of cigarette smoke's most toxic and harmful components.^{16,17} It is involved in the development of several diseases, including multiple sclerosis, neurodegenerative diseases such as Alzheimer's disease, cardiovascular and respiratory diseases, diabetes mellitus and even the development of cancer due to its high reactivity, cytotoxicity and genotoxicity.^{18,19} Smokers are particularly exposed to the harmful effects of acrolein due the high concentration found in tobacco smoke. Chronic exposure to acrolein has been linked to the development of asthma, acute lung injury, chronic obstructive pulmonary disease and even respiratory cancers.²⁰ Considering the importance of acrolein, its exposition monitoring seems to be very important for making a diagnosis, assessing disease progression, and validating treatments that specifically target acrolein. In recent years, the relevance of 3HPMA, a specific and stable metabolite of acrolein, has been demonstrated as a reliable biomarker of acrolein in urine, being very useful for bio-monitoring purposes and to assess the evolution and the memory, in terms of possible disease exposition, of tobacco consumption and its cessation.^{21–23}



Materials and methods

Reagents and standards

N-acetyl-*S*-(3-hydroxypropyl)-*L*-cysteine sodium salt (3HPMA, CAS: 14369-42-7), with a purity higher than 98%, was obtained from TLC Pharmaceutical Standards (New Market, ON, Canada). Terfenadine and Val-Tyr-Val from Sigma Life Sciences (St. Louis, MO, USA), triallyl phosphite from Alfa Aesar Thermo Fischer (Kandel, Germany) and sulfaguanidine, sulfadimethoxine, reserpine and acetaminophen obtained from Sigma-Aldrich (St. Louis, MO, USA) were employed as internal standards for LC-MS/MS in 3HPMA determination.

Methanol (LC-MS grade), acetonitrile (LC-MS quality) and buffer constituents, acetic acid and ammonium formate, were provided by VWR Chemicals (Radnor, PA, USA) and Scharlau (Barcelona, Spain). Ultrapure water with a maximum resistivity of $18.2 \text{ M}\Omega \text{ cm}^{-1}$ from an Adrona B-30 Bio system (Adrona, Riga, Latvia) was used.

Sample population

The first morning urine samples of 38 subjects who did not suffer from renal affections, including 5 smokers, 20 non-smokers and 13 declared vapers, were collected using sterile flasks. Men and women volunteers were selected, taking into account their former smoking practice and present vaping habits. All the participants were informed of the objective of the study and a consent document including a confidentiality agreement between the University and the participants was provided and signed. The original samples and aliquots of 10 mL from the freshly received samples were frozen until their analysis.

Creatinine analysis

Creatinine was used to normalize the data of the target analyte in order to minimize the effect of the characteristics and habits of the volunteers. Creatinine determination was performed using a Linear Kroma autoanalyzer (Holliston, MA, USA). The analysis methodology is based on the colorimetric determination of creatinine using 20 μL of urine by the formation of the Janovsky complex through reaction with alkaline picrate based on the Jaffé reaction.²⁴ The employed methodology allowed the determination of creatinine in urine samples down to concentrations of 0.01 g L^{-1} .

Determination of 3HPMA by LC-MS/MS

The determination of 3HPMA was carried out using a Vanquish UHPLC chromatograph from Thermo Scientific (Waltham, MA, USA) with a triple quadrupole mass spectrometer TSQAltis, operated in multiple reaction mode, from Thermo Scientific (Waltham, MA, USA). A Hypersil GOLD C_{18} column, $1.9 \mu\text{m}$ ($150 \times 2.1 \text{ mm}$), provided by Thermo Scientific (Waltham, MA, USA), was employed using a gradient of two mobile phases, ammonium formate (5 mM) in 0.1% acetic acid in ultrapure water (A) and acetonitrile of HPLC-MS quality (B). The column temperature was $40 \text{ }^\circ\text{C}$. To perform the chromatographic separation, the

applied mobile phase gradient was: 99.5% A from the beginning to 0.5 minutes, then decreasing to 70% A until minute 2, maintaining 70% A until minute 7, followed to decreasing to 0% A at minute 8 and maintaining only phase B until minute 12. Finally, increasing the proportion of mobile phase A to 99.5% at minute 12.1 and maintaining this proportion until the end of the program (minute 18).

The analysis of 3HPMA was carried out using negative ionization (ESI⁻) by applying a voltage of 3300 V. The injections were made in cycles of 0.8 seconds and the injection volume was 10 μL . The determination of the analyte was performed using the 220m/z precursor ion, 90m/z for the quantification ion and 89m/z for the confirmation ion, with collision energies of 13.72 V and 21.51 V applied for quantification and confirmation ions, respectively.

A concentrated standard solution in water was prepared from the commercial standard. The calibration solutions were prepared in the concentration range between 9 and 750 ng mL^{-1} , and were made by mixing 100 μL of non-smokers' urine (urine blank), 20 μL of $8 \mu\text{g mL}^{-1}$ internal standard solution, and the appropriate volume of 3HPMA standard solution to reach the required concentration. Ultrapure water was added to a final volume of 1 mL. Then, 1 mL of properly diluted urine samples were spiked with 20 μL of multi-internal standard solution of an adequate concentration, and analyzed.

The precision and accuracy of analyte determination were established from three independent replicates of non-smoker urine blanks spiked at concentrations between 9 and 750 ng mL^{-1} and analyzed as unknown samples.

Results and discussion

Characteristics of studied population

A sampling process following the University of Valencia ethics committee guidelines, verification code X0H21EQATBAG6TVE, was performed. Table 2 indicates the characteristics of the 38 subjects included in this study, 13 male and 25 female, spanning the age range from 20 to 79 years for men and from 16 to 76 years for women. Urine samples were obtained from 13 vapers (5 male and 8 female), 5 smokers (2 male and 3 female) and 20 non-smokers (6 male and 14 female) as a control group. Vapers were former smokers who had smoked for 8–30 years and who had used vaping systems for from 84 to 132 months. The urinary biomarker levels were normalized using the concentration of creatinine in the samples. The Jaffé reaction was employed to determine the creatinine concentration in the samples. The data found for creatinine varied from 0.31 to $2.83 \text{ g L}_{\text{urine}}^{-1}$, in concordance with levels found in the literature.²⁵

Evaluation of 3HPMA

Analytical features of the 3HPMA. The analytical procedure employed has been validated in terms of linearity, sensitivity accuracy and precision. A 0.9974 determination coefficient value (R^2) was obtained for the linear calibration range from 9 ng mL^{-1} to 750 ng mL^{-1} . The limits of detection (LOD) and quantification (LOQ) were calculated as 3 and 10 times,



Table 2 Sample population included in the study together with their gender, type of nicotine consumption system used, smoking time, use of an alternative practice to traditional tobacco and creatinine concentration in urine

Sample	Gender	Age	Weight	Practice	Smoking time (years)	Last year averaged cig per day	Alternative practice (months)	Nicotine liquid refill concentration (mg mL ⁻¹)	Consumed volume (mL per day)	Vaping solution type free base/salts	Living with smokers	Creatinine (g L _{urine} ⁻¹)
Sample 01	Man	78	80	Smoker	61	20	—	—	—	—	No	0.68
Sample 02	Woman	64	59	Smoker	40	13	—	—	—	—	—	0.32
Sample 03	Woman	59	59	Smoker	40	10	—	—	—	—	Yes	0.776
Sample 04	Woman	60	55	Smoker	40	10	—	—	—	—	Yes	1.92
Sample 05	Man	60	78	Smoker	40	20	—	—	—	—	Yes	1.38
Sample 06	Man	53	73	Vaper	26	30	132	3	20	Free base	No	2.48
Sample 07	Woman	47	70	Vaper	18	15	108	12	2	Free base	No	1.98
Sample 08	Woman	48	60	Vaper	24	10	84	0	4	—	No	0.64
Sample 09	Woman	39	72	Vaper	16	30	99	3/	10	Free base	No	0.96
Sample 10	Woman	40	61	Vaper	28	10	84	5	10	Salts	Yes	1.23
Sample 11	Man	54	107	Vaper	30	40	108	6	10	Free base	Yes	0.98
Sample 12	Woman	39	90	Vaper	18	40	84	20	4	Salts	No	1.59
Sample 13	Woman	40	70	Vaper	18	40	89	20	4	Salts	No	0.56
Sample 14	Man	56	90	Vaper	30	40	90	3	40	Free base	Yes	0.40
Sample 15	Woman	46	74	Vaper	24	30	84	18	1	Free base	No	0.85
Sample 16	Woman	41	61	Vaper	28	10	84	5	10	Salts	Yes	0.59
Sample 17	Woman	29	52	Non-smoker	—	—	—	—	—	—	—	1.25
Sample 18	Woman	34	67	Non-smoker	—	—	—	—	—	—	Yes	1.63
Sample 19	Woman	74	70	Non-smoker	—	—	—	—	—	—	Yes	1.76
Sample 20	Man	20	63	Non-smoker	—	—	—	—	—	—	No	0.87
Sample 21	Woman	75	76	Non-smoker	—	—	—	—	—	—	Yes	2.09
Sample 22	Woman	29	52	Non-smoker	—	—	—	—	—	—	—	2.60
Sample 23	Man	44	77	Non-smoker	—	—	—	—	—	—	—	2.05
Sample 24	Man	21	60	Non-smoker	—	—	—	—	—	—	No	1.18
Sample 25	Man	52	110	Vaper	23	6	86	0	2	Free base	Yes	1.34
Sample 26	Woman	33	67	Non-smoker	—	—	—	—	—	—	Yes	0.68
Sample 27	Woman	34	65	Non-smoker	—	—	—	—	—	—	Yes	0.59
Sample 28	Man	44	77	Non-smoker	—	—	—	—	—	—	—	0.58
Sample 29	Man	68	81	Non-smoker	—	—	—	—	—	—	Yes	0.87
Sample 30	Man	69	74	Non-smoker	—	—	—	—	—	—	Yes	1.88
Sample 31	Woman	21	50	Non-smoker	—	—	—	—	—	—	Yes	0.35
Sample 32	Man	52	110	Vaper	23	6	86	0	2	Free base	Yes	0.77
Sample 33	Woman	28	52	Non-smoker	—	—	—	—	—	—	No	1.51
Sample 34	Woman	21	50	Non-smoker	—	—	—	—	—	—	Yes	0.85
Sample 35	Woman	75	70	Non-smoker	—	—	—	—	—	—	Yes	0.48
Sample 36	Woman	76	68	Non-smoker	—	—	—	—	—	—	Yes	0.66
Sample 37	Woman	28	52	Non-smoker	—	—	—	—	—	—	No	0.63
Sample 38	Woman	16	42	Non-smoker	—	—	—	—	—	—	No	0.67



Table 3 Recovery values obtained for 3HPMA determination in spiked urine samples

	$C_{\text{added}} \text{ (ng mL}^{-1} \pm s \text{ (RSD\%, } n = 3))$					
	9	50	100	250	500	750
Recovery (%)	93 ± 2 (2)	91 ± 4 (4)	98 ± 5 (5)	97 ± 1 (1)	96 ± 2 (2)	103 ± 4 (4)

Table 4 3HPMA concentrations, expressed as $\mu\text{g g}_{\text{creatinine}}^{-1}$, in analyzed urine samples

3HPMA concentration in urine ($\mu\text{g g}_{\text{creatinine}}^{-1}$)					
Sample 01	1924.33	Sample 14	737.27	Sample 27	335.82
Sample 02	2543.66	Sample 15	1064.38	Sample 28	356.51
Sample 03	2675.10	Sample 16	757.32	Sample 29	374.31
Sample 04	2860.00	Sample 17	207.43	Sample 30	401.02
Sample 05	3105.97	Sample 18	228.45	Sample 31	482.65
Sample 06	172.12	Sample 19	235.26	Sample 32	529.81
Sample 07	236.12	Sample 20	251.53	Sample 33	533.74
Sample 08	979.97	Sample 21	254.87	Sample 34	620.97
Sample 09	284.26	Sample 22	271.37	Sample 35	643.37
Sample 10	819.04	Sample 23	286.11	Sample 36	709.05
Sample 11	475.71	Sample 24	301.95	Sample 37	768.61
Sample 12	726.54	Sample 25	324.52	Sample 38	927.24
Sample 13	1048.26	Sample 26	325.60		

respectively, the standard deviation of the intercept of the calibration line divided by the calibration slope. Values of 3 and 9 ng mL^{-1} were obtained for the instrumental LOD and LOQ, respectively, of 3HPMA in urine samples. These data were confirmed with the 3 and 10 ratio signal/noise in the chromatogram. On the other hand, the accuracy of the method was established from the recovery of spiked urine samples ($n = 3$). Table 3 indicates the recovery values found. It can be seen that

the values varied from 93% around the LOQ to 103% at the highest spiked concentration level. The precision of the measurements, evaluated as the relative standard deviation (RSD%), ranged from 1 to 5%.

Analysis of urine samples. Table 4 indicates the concentration of the acrolein metabolite 3HPMA found in urine samples normalized to their respective creatinine concentrations. It should be highlighted that the highest 3HPMA concentrations were obtained for smokers, with a smoking history equal to or more than forty years. The 3HPMA values found were in concordance with those reported in the literature for smokers' urine.²⁶ On the other hand, low 3HPMA concentrations were found for non-smokers, spanning 207 to 927 $\mu\text{g g}_{\text{creatinine}}^{-1}$, in good agreement with those found by other authors.^{27–30} For vapers, the 3HPMA concentration found in urine ranged from 172 to 1064 $\mu\text{g g}_{\text{creatinine}}^{-1}$, showing a situation between that of smokers and non-smokers and similar to that found by Bjurlin *et al.*³¹

Can be vaping a kind of cessation? Fig. 1 shows the average of the 3HPMA values for smokers and non-smokers, together with their respective standard deviations. An averaged 880 ± 150 $\mu\text{g g}_{\text{creatinine}}^{-1}$ 3HPMA concentration was found for heavy smokers who had replaced the smoking of tobacco by nicotine vaping systems for 80–100 months, with the 3HPMA concentration in urine found to have fallen to an average 330 ± 130 $\mu\text{g g}_{\text{creatinine}}^{-1}$

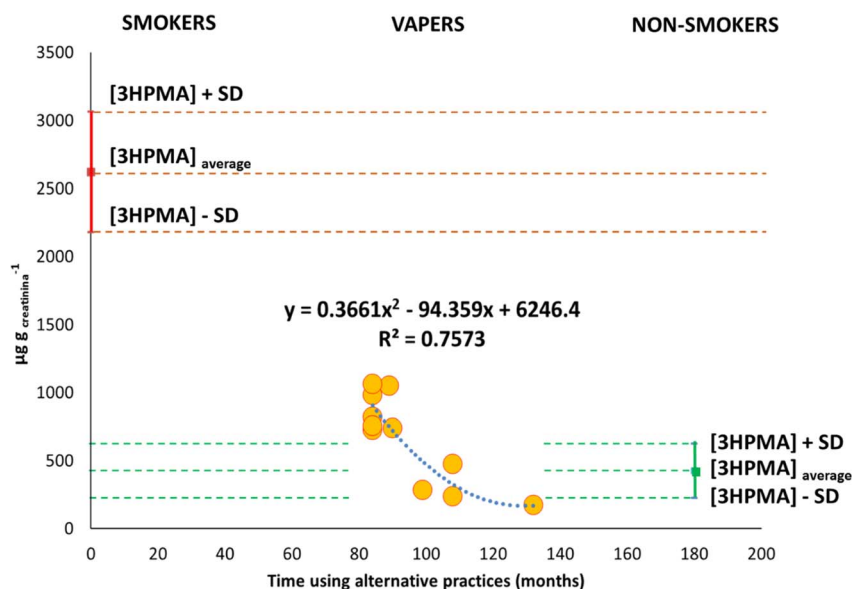


Fig. 1 Evolution with time using alternative practices for 3HPMA concentration, expressed as $\mu\text{g g}_{\text{creatinine}}^{-1}$, found in the urine of former smokers who have substituted smoking by vaping compared with $[3\text{HPMA}]_{\text{averaged}} \pm \text{SD}$ found in smokers' (■) and non-smokers' (■) urine.



$g_{\text{creatinine}}^{-1}$ concentration for those who had replaced traditional smoking practices for a duration of 100–120 months. In short, a quadratic relationship can be established between the 3HPMA/creatinine levels and the duration of replacement of traditional smoking by vaping, providing a value for the memory tobacco time of approximately 10 years.

Conclusions

An evaluation of the 3HPMA/creatinine relationship in the urine samples of smokers who actively burn tobacco, and those who have replaced smoking by nicotine vaping seems to indicate a relationship between the persistence of 3HPMA and the duration of using an alternative practice of nicotine consumption. This relationship provides an approximate value of 10 years, or 120 months, for the tobacco memory effect of the acrolein metabolite.

Ethical statement

The presented project was approved by Ethics Committee of the University of Valencia (Valencia, Spain), verification code X0H21EQATBAG6TVF. Volunteers enrolled in the study have provided their written informed consent for the research study protocol.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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